Functional Development and Embryology ©2007 Global Science Books

Fetal Spleen Development, the Ride toward Multiple Functions

Guillaume E. Desanti[1] • Julien Y. Bertrand[2] • Rachel Golub[1*]

[1] Unité du Développement des Lymphocytes, INSERM U668, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France
[2] University of California, San Diego, Natural Sciences Building, #6105, 9500 Gilman Drive La Jolla, CA 92093-0380 USA

Corresponding author: * **rgolub@pasteur.fr**

ABSTRACT

The aim of this review is to clarify the different steps of fetal and neonatal spleen development that lead to the formation of a functional adult immune organ. The adult spleen harbors a highly organized architecture directly correlated with its roles in the innate and adaptive immune system. Before achieving these immune functions, the spleen undergoes two important steps: the acquisition of the hematopoietic capacities during the fetal period followed by the implant of distinct organ areas during the neonatal phase. It is first a site of hematopoiesis, producing cells that will then be segregated into different areas. The spleen primordium appears around embryonic day E11.5 and is colonized early by several hematopoietic progenitors, via blood circulation. The interactions between the stromal microenvironment and the hematopoietic progenitors constitute a key step to understand spleen organogenesis. We discuss herein all the data concerning fetal spleen hematopoiesis characteristics that will provide the main cell subsets involved in its organogenesis. We actually highlight the relationship between these different hematopoietic compartments, the acquisition of architecture complexity and the gain of organ functions that take place at the neonatal period. We also report several models of aberrant murine spleen development, which constitute important tools to study the specificities of the fetal spleen microenvironment.

Keywords: embryogenesis, hematopoiesis, left/right asymmetry, lymphoid tissue, macrophage, rodent, stroma
Abbreviations: DC, dendritic cells; **MΦ,** macrophages; **MZ,** marginal zone; **NALT**, nasal-associated lymphoid tissues; **LN,** lymph nodes; **LTi,** lymphoid tissue inducer cells; **PP,** Peyer's patch

CONTENTS

INTRODUCTION

The spleen is an asymmetric organ present only in vertebrates that filters the blood and participates to the immune response to pathogens. It is situated on the left side of the abdomen, on top of the stomach. Due to its functions, the spleen is largely vascularized and is protected by a capsule composed of connective tissue. Compared to other secondary immune organs, the spleen possesses a complex architecture with the establishment of a special area, the MZ that surrounds the white pulp and separates it from the red pulp. In this review, we are reporting the last findings on its embryonic origin and we establish the basis to understand the parallel between its organogenesis and the establishment of its hematopoietic capacities. This review focuses on spleen development in mice. To understand the events that drive this complex organogenesis, a large description of the adult spleen composition and architecture is required.

THE ADULT SPLEEN: COMPOSITION AND ARCHITECTURE

The mammalian adult spleen is compartmentalized into red and white pulp, two regions that are markedly different by their constitution and function (reviewed in Mebius and Kraal 2005). Between these two areas, the marginal zone (MZ) is important and its anatomical difference constitutes a place where cells circulate from the bloodstream to the white pulp area. The establishment of this zone and its integrity largely depends on the interactions of the different

Received: 27 February, 2007. Accepted: 22 March, 2007.

Invited Review

Table 1 Characterization of splenic macrophage subsets.

	Mac1	F4/80	CD163	SIGNR1	MARCO	Siglec1	MOMA	References
MZ Metallophilic Macrophages	+	-	-	-	-	+	+	reviewed in
MZ Macrophages	+	-	-	+	+	-	+	Mebius and Kraal 2005;
Red Pulp Macrophages	-	+	+	-	-	-	-	Taylor *et al.* 2005
Fetal Spleen Macrophages (E15)	+	+	N.D	N.D	N.D	N.D	N.D	Bertrand *et al.* 2006
HSC-derived macrophages cultures on FSS	+	+	N.D	N.D	N.D	N.D	N.D	Bertrand *et al.* 2006

N.D: not determined.

Table 2 Knock-out mice presenting defects in adult spleen organization.

Genes deleted	Cells affected	MZ MΦ	MM MΦ	MZ B	MS	T/B	FDC	GC	Reference
Id2	hema	N.D	+	↓	N.D	+	N.D	+	Yokota *et al.* 1999; Berker-Herman *et al.* 2002
Rorγ	hema	N.D	N.D	+	N.D	+	N.D	+	Zhang *et al.* 2003
Ltα	hema	-	-	-	-	- / ↓	-	↓	de Togni *et al.* 1994; Matsumoto *et al.* 1996; Banks *et al.* 1997; Koni *et al.* 1997; Alexopoulos *et al.* 1998; Liepinsh *et al.* 2006
Ltβ	hema	-	-	-	-	↓	↓	-/↓	Alimzhanov *et al.* 1997; Koni *et al.* 1997; Alexopoulos *et al.* 1998; Kuprash *et al.* 1999
Tnf	hema	N.D	N.D	+	-	↓	↓	-	Körner *et al.* 1997
Light	hema	+	+	+	+	+	+	+	Scheu *et al.* 2002
B cells *Ltβ*-/-	hema	↓	↓	↓	↓	↓	↓	↓	Tumanov *et al.* 2002
T cells *Ltβ*-/-	hema	+	+	+	+	+	+	+	
T/B cells *Ltβ*-/-	hema	↓	↓	↓	↓	↓	-	-	
Ltb / Tnf	hema	-	-	-	-	↓	-	-	Kuprash *et al.* 1999
Ltα+/- / *Ltβ*+/-	hema			↓		+		+	Koni *et al.* 1998
Tnf / Ltα / Ltβ	hema	-	-	-	-	-	-	-	Kuprash *et al.* 2002
Light / Ltβ	hema	-	-	-	-	↓	↓	↓	Scheu *et al.* 2002
TRANCE-R	hema	N.D	+	↓	N.D	+	+	↓	Dougall *et al.* 1999
Cxcr5	hema	N.D	N.D	N.D	+	↓	N.D	N.D	Ansel *et al.* 2000; Ohl *et al.* 2003
Ccr7	hema	N.D	N.D	N.D	+	↓	N.D	N.D	Förster *et al.* 1999; Ohl *et al.* 2003
Cxcr5 / Ccr7	hema	N.D	N.D	N.D	+	-	N.D	N.D	Ohl *et al.* 2003
Trance	hema/stroma	↓	N.D	N.D	N.D	+	N.D	N.D	Kong *et al.*1999; Kim *et al.* 2000
Tnfr1	stroma	N.D	-	↑	-	↓	-	↓	Neumann *et al.* 1996; Alcamo *et al.* 2001
Tnfr1 / Rela	stroma	N.D	-	↓	N.D	↓	-	-	Alcamo *et al.* 2001
Aly / Aly	stroma	-	-	-	-	- / ↓	-	-	Miyawaki *et al.* 1994; Sinkura *et al.* 1999
Ltβr	stroma	-	-	-	-	-	-	↓	Fütterer *et al.* 1998
Nfkb1	stroma	+	+	-	+	+	+	+	Weih *et al.* 2003
Nfkb2	stroma	+	-	N.D	-	↓	-	-	
Relb	stroma	-	-	-	-	-	-	-	
Plt / Plt	stroma	↓	+	+	N.D	↓	N.D	N.D	Nakano *et al.* 1998; Ato *et al.* 2004
Cxcl13	stroma	N.D	N.D	N.D	↑	↓	-	+	Ansel *et al.* 2000

+: similar to wild-type; -: absent; ↓: organization partially impaired or smaller in size and/or number; ↑: larger in size; N.D: not determined;
hema: hematopoietic; MZM: marginal zone macrophages; MMM: marginal zone metallophilic macrophages; MZB: marginal zone B cells; MS: MadCAM1+ sinus lining cells; T/B: T and B follicle segregation; FDC: follicular dendritic cells: GC: germinal centers.

MZ cell types. The marginal sinus consists of the blood conduit localized between the follicular zone of the white pulp and the MZ. New arriving leukocytes or molecules enter into the follicular and/or MZ thanks to numerous small arterial branches that terminate in the marginal sinus. The latter is coated with MadCAM1^{+} sinus lining cells that allow leukocyte adhesion and diapedesis.

The red pulp is mainly composed of erythrocytes, macrophages (MΦ) and plasma cells and is largely alimented by the red pulp venous system (reviewed in Mebius and Kraal 2005). Red pulp MΦ play an essential role in iron metabolism (**Table 1**). Indeed, they can phagocyte senescent erythrocytes retained in the red pulp. Those erythrocytes are then hydrolyzed, and non-toxic iron can be released in circulation. Also, red pulp MΦ can directly bind circulating hemoglobin, released when erythrocytes are damaged, through the CD163 receptor (Kristiansen *et al*. 2001). This subset is also involved in pathogens removal from the blood.

The adult spleen contains resident myeloid cells, consisting of diverse MΦ and dendritic cells (DC) subsets. Amongst splenic MΦ, two other major populations have been described, harboring different phenotype (**Table 1**) and playing different roles (reviewed in Mebius and Kraal 2005). The MZ Metallophilic MΦ are located in the white pulp, lining the MZ. Their functions have not yet been totally elucidated. However, it was shown that they are the main producers of interferon-α and -β after a viral infection by herpes simplex strain (Eloranta and Alm 1999). The MZ MΦ are located in the MZ, and are specialized in the clearance of pathogens, through a set of membrane receptors (SIGNR1, MARCO). However, these MΦ lack the expression of MHC Class II, necessary for the activation of MZ B cells (van Rooijen 1990). It has been suggested that MZB cells are activated via the complement and the opsonization of pathogen (van Rooijen 1990). The mechanisms underlying this process remain unknown. The MZ also enclosed reticular cells, DC, and MZ B cells (MZB). Splenic mature B cells can differentiate either into MZB cells or follicular B cells depending on BCR signal strength (reviewed in Pillai *et al*. 2005). MZB cell generation is dependent on Notch2 signaling, while follicular B cells express high levels of MINT (a Notch signaling inhibitor) (Tanigaki *et al*. 2002; Kuroda *et al*. 2003; Saito *et al*. 2003). The respective levels of Id and E proteins are also crucial in this lineage commitment. Indeed MZB cells only express low levels of E2A and Id3 blocks the differentiation into follicular B (Quong *et al*. 2004).

The white pulp is mainly lymphoid by its composition and organization, and operates similarly to other secondary lymphoid tissues such as lymph nodes (LN), nasal-associated lymphoid tissues (NALT) or gut-associated lymphoid tissues (GALT). In all these tissues, B and T cells are organized into follicles. After a pathogenic challenge DC, B and T cells interact to build the adaptative immune response. In the adult spleen, three main subsets of DC have been iso-

lated on their phenotype: namely, $CD11c^{hi}CD4^{-}CD8^{-}$, $CD11c^{hi}CD4^{+}CD8^{-}$, and $CD11c^{hi}CD4^{-}CD8^{+}$ cells (Shortman and Liu 2002). Another subset of $CD11c^{lo}B220^{+}$ plasmacytoid-DC can be isolated (Shortman and Liu 2002). These DC function as antigen-presenting cells, and can be found predominantly in the MZ and in T-cell area of the white pulp (reviewed in Mebius and Kraal 2005).

Secondary lymphoid tissue organization results from dynamic processes maintained throughout life. Many of these molecules belong to the tumor-necrosis factor (TNF) family, such as the secreted lymphotoxin-α3 (LTα3), the membrane-bound LIGHT, the surface LTα1β2 complex and their receptors TNFRI (p55), TNFRII (p75) and LTβR (reviewed in Chaplin and Fu 1998; Fu and Chaplin 1999; Chaplin 2002). The interaction between the LTα1β2 complex and its specific receptor LTβR triggers the classical and alternative nuclear factor-κB (NF-κB) pathways. The classical pathway activates the inhibitor of the κB kinase complex. Its downstream components, RelA and NF-κB1, enhance the expression of the vascular-cell adhesion molecule 1 (VCAM1) on stromal cells (Dejardin *et al*. 2002; Yilmaz *et al*. 2003). The alternative pathway, through the NF-κb inducing kinase (NIK) and its downstream components (RelB and NF-κB2), leads to the expression of the CXCL12 (SDF-1), CXCL13 (BLC), CCL19 (ELC) and CCL21 (SLC) chemokines by stromal cells (Yin *et al*. 2001). The localized expression of CCL12 in the red pulp, CXCL13 in the B cell follicles and both CCL19 and CCL21 in the T cell follicles consitutes the mechanism of splenic lymphocyte segregation (reviewed in Fu and Chaplin 1999; Chaplin 2002; Cyster 2005). Indeed, the mature follicular B cells express CXCR5 until they recognize an antigen and undergo the affinity selection process by interaction with follicular dendritic cells in the germinal centers. Once activated, B cells differentiate into plasma cells, down-regulate CXCR5 and up-regulate CXCR4 to migrate toward the red pulp, following the CCL12 gradient (Hargreaves *et al*. 2001). $CCR7^{+}$ T cells can reach the follicular T cell zone of secondary lymphoid tissues, following to the CCL19/CCL21 gradient (Willimann *et al*. 1998). For an efficient immune response, activated DC subsets up-regulate CCR7 to be able to migrate toward the T cell area (Sanchez-Sanchez *et al*. 2006). Thus, the spleen organization seems highly dependent on chemokine expression. Several knock-out mice for chemokines or their receptors present alterations of their spleen architecture as well as specific immune response deficiencies (**Table 2**).

ORIGIN OF THE EMBRYONIC SPLEEN

The embryonic origin of the spleen is not yet clear (reviewed in Brendolan *et al*. 2007). The spleen organogenesis was first described after histological studies as a mesenchymal condensation in the dorsal mesogastrium (Thiel and Downey 1921). Its early position has been difficult to evaluate since no early specific marker was known. The spleen is an asymmetric organ situated in the posterior part of the stomach, in close contact with the omentum and the pan-

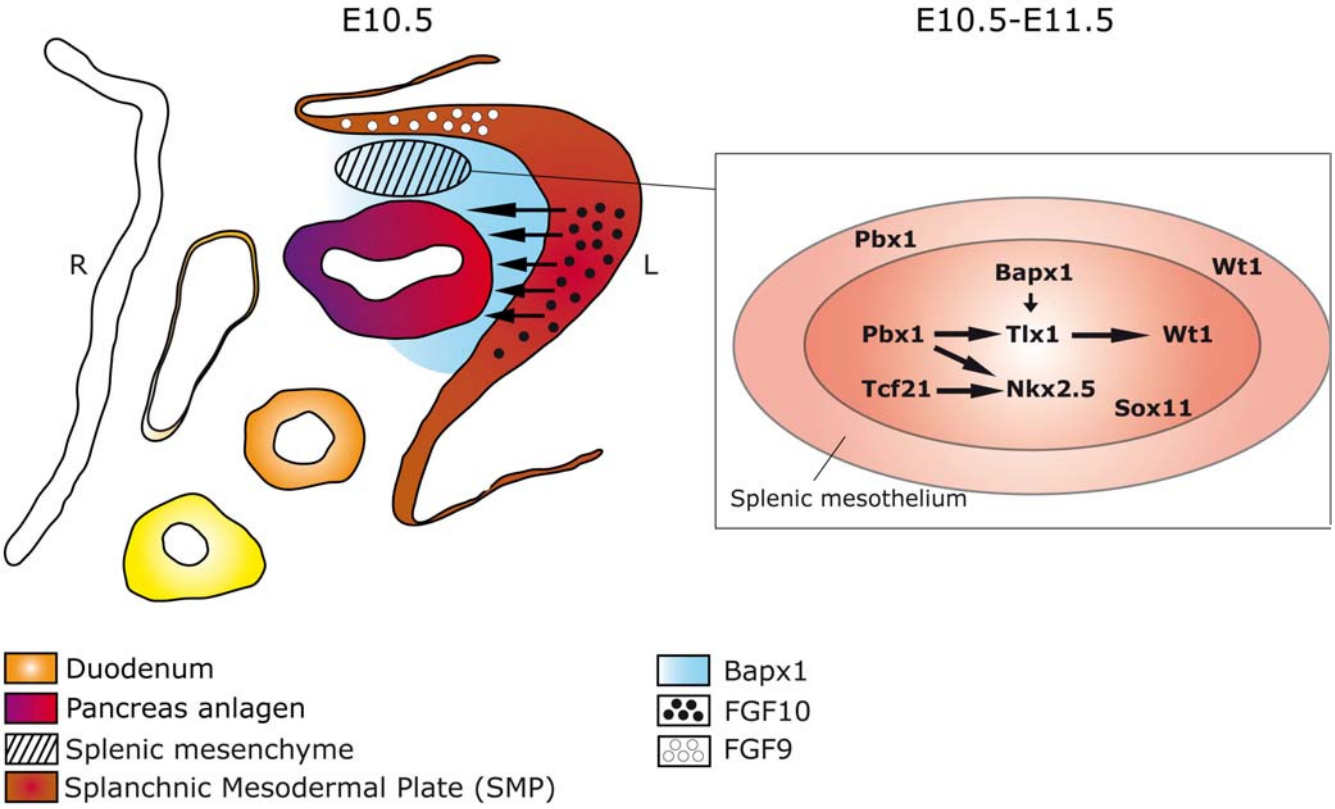

Fig. 1 The embryonic origin of the spleen. At E10.5, the SMP is asymetric and its lateral left growth is under the control of the left/right pathway (Hecksher-Sorensen *et al*. 2004). The SMP is a source of numerous growth factors. It expresses Fgf 9, 10, 11 and 13. Fgf 9 and 10 display a gradual expression, respectively localized in the dorsal and ventral SMP. Fgf9 is thought to promote the outgrowth of the SMP in association with FGFR3 (Hecksher-Sorensen *et al*. 2004). The mesenchymal region situated underneath the SMP is called spleno-pancreatic mesenchyme and expands along with the left SMP. The dorsal mesenchyme outgrowth results in spleen formation but the inductive signals are still unknown. In the SMP ventral region, the Fgf10 gradient maintains the pancreatic proliferation and induces its leftward growth (Hecksher-Sorensen *et al*. 2004). The expression of Bapx1 is found in the SMP, spleno-pancreatic and surrounding mesenchyme (Hecksher-Sorensen *et al*. 2004). It was shown that Bapx1 is responsable for the Fgf10 gradient and the separation of the spleen and pancreas around E11.5 (Hecksher-Sorensen *et al*. 2004; Asayesh *et al*. 2006). On top of Bapx1 expression, the genes (Tlx1, Tcf21, Pbx1, Wt1, Sox11 and Nkx2.5) involved in the expansion and/or survival of the specific spleen precursors were detected at E10.5-E11.5. Their hierarchical interactions were deduced from studies (Lettice *et al*. 1999; Tribioli and Lufkin 1999; Patterson *et al*. 2000; Koehler *et al*. 2000; Hecksher-Sorensen *et al*. 2004; Brendolan *et al*. 2005) using the respective deficient mice: Tlx1 (Dear *et al*. 1995; Kanzler and Dear 2001), Tcf21 (Lu *et al*. 2000), Pbx1 (DiMartino *et al*. 2001), Wt1 (Herzer *et al*. 1999), and Sox11 (Sock *et al*. 2004). Later in development, the E15 spleen mesenchyme was shown to express Nkx2.3, an another specific marker (Pabst *et al*. 1999). At E11.5, the mesothelial layer of the spleen is formed and will form the adult protective capsule. This mesothelium express Pbx1 and Wt1 but Tlx1 suggesting an independent expression of these two genes in this tissue (Brendolan *et al*. 2005).

creas. While the pancreas clearly originates from a mesodermic induction of the endoderm (Edlund 2002), the spleen is considered as exclusively mesoderm derived.

The left/right axis in mouse and human is involved in spleen formation. It has been stated by the laterality defects like polyspenia and asplenia, respectively observed in left or right isomerism as well as reversed axes (e.g. *situs inversis* mutation in mice) (Yokoyama *et al*. 1993; Bartram *et al*. 2005). Spleen abnormalities are frequently associated with other irregular organ disposition that relies on left/right asymetry (Ivemark 1955) even if rare cases of unique asplenia were reported (Ferlicot *et al*. 1997; Germing *et al*. 1999; Gilbert *et al*. 2002; Halbertsma *et al*. 2005; Hummler *et al*. 2005). Studies on the left/right axis have participated to the characterization of the spleen primordium and its specific precursors (Green 1967; Patterson *et al*. 2000; Hecksher-Sorensen *et al*. 2004). The hemimelia dominant mutant (Dh) (Green 1967) and Bapx1$^{-/-}$ mice (Lettice *et al*. 1999; Akazawa *et al*. 2000) have been used to analyze the spleen formation (Hecksher-Sorensen *et al*. 2004). These mice clearly showed that spleen and pancreas primordia are tightly linked. Furthermore, the cardiac, lung and liver development were shown to be independent of the spleno-pancreatic mesenchyme creation. These two mice have disorders in the splanchnic mesodermal plate (SMP) structure (Green 1967; Hecksher-Sorensen *et al*. 2004). The SMP derives from the lateral plate mesoderm and is constituted of epithelial-like cells. Initially bilateral, the SMP starts to specify its left side at E9.5 by the expression of Pitx2 and Barx1 genes (Hecksher-Sorensen *et al*. 2004). This first asymmetry is enhanced by the regression of the SMP right side. At E10.5, the left outgrowth of the SMP is specifically under the control of the left/right pathway. In parallel, the mesenchyme underlying the SMP increases and starts to form a splenic anlagen attached to the pancreas bud. Histological studies at E10.5 have underlined the link between the spleno-pancreatic rudiment and the left SMP (Hecksher-Sorensen *et al*. 2004; **Fig. 1**). The importance of the SMP in spleen organogenesis is clear since the disruption of its structure in Dh mutant and Bapx1$^{-/-}$ embryos lead to asplenia. The SMP represent the rigid tissue layer that guide the morphogenesis. It is still not clear if SMP signals are necessary to induce the splenic mesenchyme. On the other hand, the elevated levels of Fgf10 in the ventral SMP are known to activate the pancreatic endodermal bud from the gut and to initiate its asymmetry (Bhushan *et al*. 2001).

The spleen fate acquisition is shown by the co-expression of numerous genes: Tlx1/Hox11(Hecksher-Sorensen *et al*. 2004), Nkx2.5 (Hecksher-Sorensen *et al*. 2004), Wt1 (Hecksher-Sorensen *et al*. 2004), Tcf21/capsulin (Hecksher-Sorensen *et al*. 2004), Bapx1 (Hecksher-Sorensen *et al*. 2004), Barx1 (Hecksher-Sorensen *et al*. 2004), Sox11 (Lioubinski *et al*. 2003) and Pbx1 (Kim *et al*. 2002). A recent paper using Bapx1$^{-/-}$ embryos has highlighted the role of this gene in the separation of the two tissues (Asayesh *et al*. 2006). Indeed, the morphological events that separate the spleen rudiment from the pancreas are not sustained in Bapx1$^{-/-}$ embryos, despite the priming of the spleen mesenchyme by the expression of the specific Nkx2.5 and Tcf21 genes (Asayesh *et al*. 2006; **Fig. 1**).

The later events of spleen organogenesis were analyzed from mouse models that exhibit different anomalies of spleen development at E13.5 though they harbor normal pancreatic development. While at E10.5, Tlx1, Tcf21, Pbx1, and Wt1 genes are expressed in both splenic and pancreatic mesenchyme, only abnormalities of spleen architecture are observed in their respective deficient E13.5 embryos (Dear *et al*. 1995; Herzer *et al*. 1999; Lu *et al*. 2000; DiMartino *et al*. 2001; Kanzler and Dear 2001).

In order to build a pathway and define a hierarchy between these transcription factors, their relative expression was analyzed in the deficient mice (**Fig. 1**). Tlx1 was the first gene described as involved in the spleen organogenesis since its deletion causes an asplenia in the adult. Normal until E13.5, Tlx1$^{-/-}$ spleen mesenchyme stops its proliferation and decreases until complete spleen disappearance. This process is cell-autonomous and could not result from a deficient interaction with the hematopoietic lineage. The absence of Tlx1 expression in Bapx1 and Pbx1 deficient embryos implied that both genes are able to genetically control Tlx1 (**Fig.1**). It was confirmed that Pbx1 binds to Tlx1 promoter and activates its expression in spleen precursors (Brendolan *et al*. 2005). Wt1 was also absent from Pbx1$^{-/-}$ splenic mesenchyme suggesting a putative regulation of Wt1 by Pbx1 (Brendolan *et al*. 2005). As a consequence of splenic mesenchyme apoptosis around E13.5, Wt1$^{-/-}$ mice are asplenic (Herzer *et al*. 1999). In Wt1$^{-/-}$ mice, Tlx1 expression was detected by in situ hybridization suggesting that it is situated upstream in the pathway controlling the splenic mesenchyme formation (Herzer *et al*. 1999). However, data are conflictual concerning Tlx1 and Wt1 interaction since in Tlx1$^{-/-}$ embryos, Wt1 is absent or present according to different studies (Herzer *et al*. 1999; Koehler *et al*. 2000). More data are required to positively prove that Wt1 is directly regulated by Tlx1 in the spleen mesenchyme, and consequently by Pdx1 *via* its regulation of Tlx1. In the mesothelial layer that surrounds the spleen mesenchyme, Wt1 expression was observed in normal, Pbx1$^{-/-}$ and Tlx1$^{-/-}$ embryos (Brendolan *et al*. 2005). In this tissue, Pbx1 is normally expressed while Tlx1 is not. Hence, Wt1 expression is not dependent on Pbx1 nor Tlx1 in this tissue. It suggests that Wt1 expression may be generally independent of the Pdx1-Tlx1 axis.

Nkx2.5 is essential for heart morphogenesis, myogenesis and function (Lyons *et al*. 1995). In *Xenopus*, Nkx2.5 is a perfect marker for the presplenic tissue with an expression on both sides of the embryonic stomach (Patterson *et al*. 2000). However, in mice, its expression is uniform in E10.5 spleen mesenchyme (Hecksher-Sorensen *et al*. 2004). Its importance is not clear since Nkx2.5$^{-/-}$ embryos die around E9 due to a failure of the heart looping morphogenesis. The pattern of Nkx2.5 expression in Tlx1$^{-/-}$, Tcf21$^{-/-}$ and Pbx1$^{-/-}$ embryos suggests that Pbx1 and Tcf21 are direct regulators of Nkx2.5 although their expression is not related to Tlx1 (Brendolan *et al*. 2005).

Asplenic Tcf21$^{-/-}$ mice die at birth with specific problems of breathing (Lu *et al*. 2000). In Tcf21$^{-/-}$ embryos, the E12.5 splenic precursors display a normal expression of the splenic anlagen markers, Bapx1 and Tlx1. However, the splenic primordium of these embryos undergoes apoptotic cell death around E13.5 (Lu *et al*. 2000).

Nkx2.3 is normally expressed in gut and spleen mesenchyme of embryonic and adult mice. Nkx2.3$^{-/-}$ mice present severe morphological alterations of both organs with a varying penetrance that result in an early postnatal lethality in most homozygous mutants. Asplenic mice represent 20% of the mutants and the rest possess small spleens with severely reduced number of lymphoid cells and a lack of ordered tissue architecture (Pabst *et al*. 1999, 2000). More data are needed to understand the regulation of these transcription factors in spleen mesenchyme and to confirm the hypothetical gene cascade.

THE FETAL SPLEEN IS DEDICATED TO HEMATOPOIESIS

Embryonic development of the spleen also consists in the colonization of the splenic microenvironment by hematopoietic cells. By E12.5, pancreas and spleen are distinguishable enough to be individually isolated. Fetal spleen at E13 represents the earliest stage analyzable for its hematopoietic capacities and cell content (Mebius *et al*. 1997; Godin *et al*. 1999; Bertrand *et al*. 2006).

The fetal spleen hematopoietic progenitors

Long-term reconstitution assays have detected hematopoitic stem cells in fetal spleen as soon as E13.5 (Godin *et al*. 1999; Christensen *et al*. 2004; Bertrand *et al*. 2006). Hematopoitic stem cells from fetal liver and spleen share the same

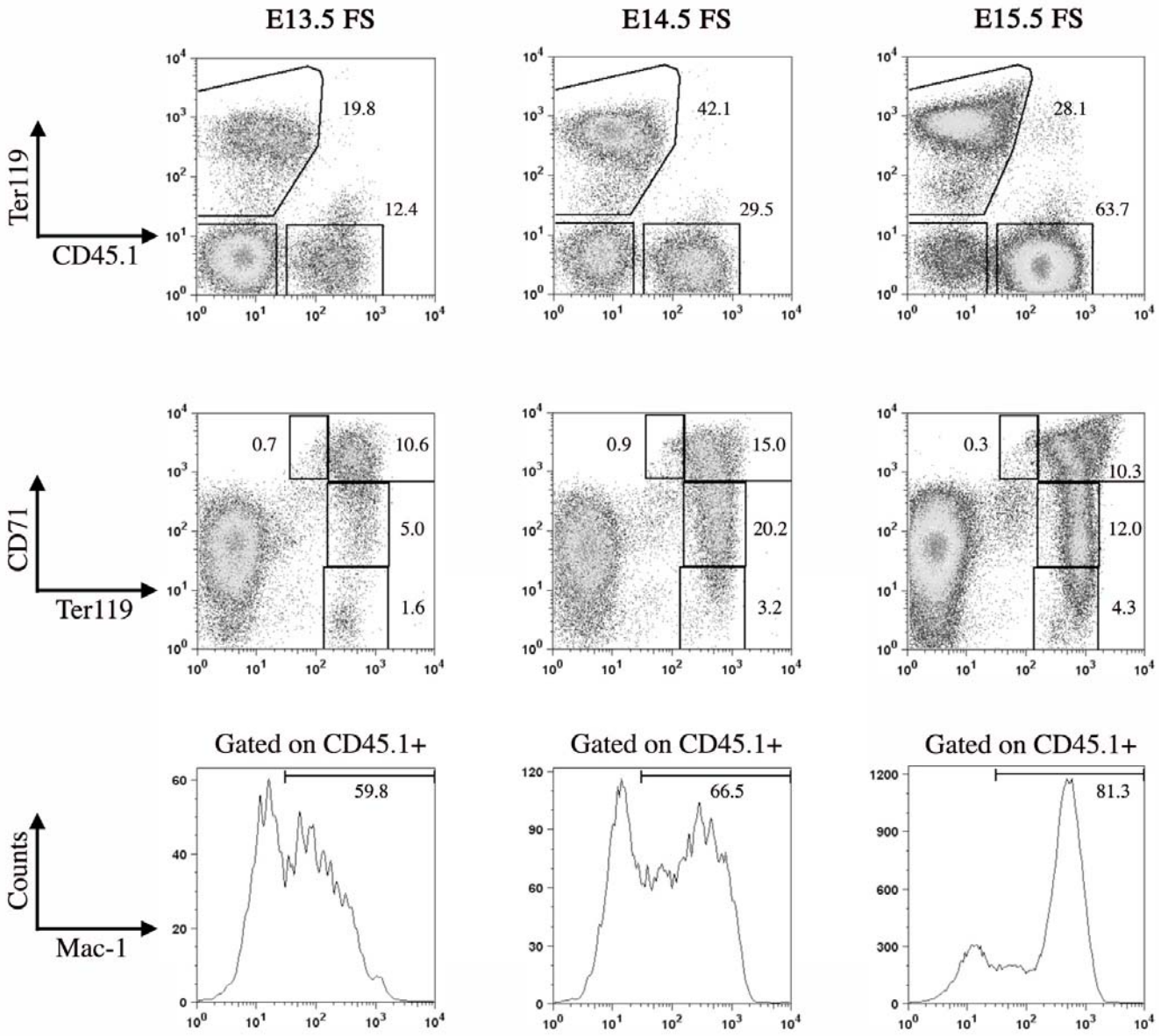

Fig. 2 Erythrocytes and leukocytes content in the fetal spleen from E13.5 to E15.5. C57BL/6 fetal spleen from E13.5 to E15.5 embryos were analyzed by FACS for erythrocytes (Ter119, CD71) and leukocytes (CD45.1, Mac-1). The CD71 marker is highly expressed by early erythroid precursors. Here, we distinguish four erythroid populations from the immature to mature: proerythroblasts ($Ter119^{int}CD71^{hi}$), basophilic erythroblasts ($Ter119^{hi}CD71^{hi}$), late basophilic and polychromatophilic erythroblasts ($Ter119^{hi}CD71^{int}$), and orthochromatophilic erythroblasts ($Ter119hiCD71^{-/lo}$). The Mac-1 marker is expressed by $CD45.1^{+}$ macrophages. Numbers indicate the percentage of cells comprise in the gate.

phenotype and potential (Christensen *et al*. 2004; Kiel *et al*. 2005; Bertrand *et al*. 2006). In contact with the fetal spleen environment, the hematopoietic stem cells loose their capacity to self-renew, and commit toward $F4/80^{+}$ macrophages (Bertrand *et al*. 2006). The fetal spleen is a hematopoietic organ that is constantly colonized by circulating HSC (Christensen *et al*. 2004; Bertrand *et al*. 2006). The first erythrocytes, myeloid, B and T/NK progenitors are observed at E13-E13.5 and increase until E16.5 (Metcalf and Mas 1971; Carlyle and Zuniga-Pflucker 1998; Bertrand *et al*. 2006; **Fig 2**). Indeed, erythrocytes and leukocytes represent around 32% of the total splenocytes at E13.5 and 92% at E15.5 (**Fig. 2**). At this time, the splenic cellularity is doubling each day.

We have isolated the splenic hematopoietic progenitors as $Lin^{-}CD4^{int}CD3^{-}$ cells (Desanti, unpublished observations). Using $RAG2^{GFP}$ mice, this population was further subdivided into $RAG2^{-}$, $RAG2^{lo}$ and $RAG2^{hi}$ subsets that respectively possess a majority of myeloid, T/NK and B cell precursors (Desanti, unpublished observations). These progenitors probably arise from circulating fetal liver cells that differentiate herein.

The lymphoid subsets are detectable at E16.5 in both fetal liver and spleen. Only few $CD19^{+}IgM^{+}B$ cells are detected in the spleen compared to the liver (Desanti, unpublished observations). The lymphoid populations increase from E17.5 to birth (Velardi and Cooper 1984), probably by both colonization and in situ differentiation. NK cells from fetal and neonatal spleen have a restricted Ly49 repertoire and half of them express the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) typical of immature stages (Koo *et al*. 1982; Takeda *et al*. 2005). These cells are probably not fully functional *in vivo* since they have reduced cytotoxic capacity and reduced cytokine secretion after *in vitro* stimulation (Koo *et al*. 1982; Takeda *et al*. 2005).

Establishment and segregation of MΦ in the spleen

At E12, MΦ can be detected by their expression of the F4/80 antigen in the spleen rudiment (Morris *et al*. 1991). These MΦ colonize the fetal spleen before it actually proves any hematopoietic potential, supporting the idea that these first MΦ are born from the yolk sac, which constitutes the

first source of mature MΦ at this stage (Bertrand *et al*. 2005). The number of F4/80$^+$ MΦ in the spleen remains relatively constant until E15 (Morris *et al*. 1991; **Fig. 2**). This increase in splenic MΦ correlates with the onset of hematopoiesis in the fetal spleen. Indeed, we have shown that the fetal spleen starts to be colonized by HSCs at E13 (Bertrand *et al*. 2006). Using fetal spleen organ cultures, and taking advantage of fetal spleen stromal cell lines (FSS) that we generated, we could show that these HSC commit only toward the MΦ lineage *in vitro* (Bertrand *et al*. 2006). We also showed that the phenotype of splenic MΦ evolves during embryogenesis and the neonatal period. Indeed, all splenic MΦ are F4/80$^+$Mac1$^+$ at E15, but lose Mac1 expression after birth, thus acquiring the phenotype of adult red pulp MΦ (**Table 1**). We thus suggest that fetal spleen hematopoiesis gives rise to the first neonatal red pulp MΦ from HSC. During fetal and neonatal life, these MΦ have been found associated to nucleated erythrocyte precursors (Bertrand *et al*. 2006), likely indicating that these first splenic MΦ play a role in erythropoiesis, such as their counterpart in the fetal liver (Sadahira and Mori 1999). From E13.5 to E15.5, the percentage of leukocytes displays a 5-fold increase and 80% of them are Mac-1$^+$ myeloid cells (**Fig. 2**).

NEONATAL SPLEEN: A HINGE BETWEEN HEMATOPOIESIS AND SECONDARY IMMUNE FUNCTIONS

As described, the fetal spleen has settled the basis of the future secondary organ. The fetal organ is highly hematopoietic and this function decrease at the neonatal stage to end with the structuration of the secondary lymphoid organ. The neonatal stage is the transition between hematopoiesis and establishment of efficient immune responses.

Splenic erythropoiesis

Erythropoiesis in mice is developmentally regulated and takes place in successive organs (rewieved in Godin and Cumano 2002). Primitive erythropoiesis starts at E7.5 in the yolk sac. From E12 to birth, the fetal liver is the main site for definitive erythropoiesis while the bone marrow produces the blood cells during the neonatal and adult life. A few studies have shown that fetal and neonatal spleens constitute additional erythropoietic sites. Most of these data have been obtained from microscopic observations showing some rare proerythroblasts as soon as E12. At this early stage, erythrocytes could be yolk sac derived. A kinetic was established as follow: a clear population of proerythroblasts is observed at E13 followed by the apparition of basophilic erythroblasts at E14 and orthochromatophilic erythroblasts expulsing their nuclei at E15 (Djaldetti *et al*. 1972; **Fig. 2**). To complete these previous observations, we analyzed by FACS the maturation of splenic erythrocytes from E13.5 to E15.5 using the erythroid lineage-specific Ter119 marker and CD71, a marker highly expressed by erythrocyte progenitors (**Fig. 2**). We observed that the proerythroblast (Ter119^{int}CD71^{hi}), the basophilic erythroblasts (Ter119^{hi} CD71^{hi}), late basophilic and polychromatophilic erythroblasts (Ter119^{hi}CD71^{int}), and the most mature orthochromatophilic erythroblasts (Ter119hiCD71$^{-/lo}$) are already present at E13.5. The early erythroid committed cells (Ter119^{int} CD71^{hi} cells and Ter119^{hi}CD71^{int} cells) are the most represented at E13.5. However, the proportion of splenic late erythroid-committed cells (Ter119^{hi}CD71^{int} cells and Ter119^{hi}CD71$^{-/lo}$ cells) starts to increase as soon as E14.5.

We have also described the presence of erythroblastic islands where MΦ are tightly associated with nucleated erythrocytes (Bertrand *et al*. 2006). From E16 to birth, most splenic red cell precursors are polychromatophilic erythroblasts, some of them already containing ferritin. The same cell types predominate in one-week newborns, although a few proerythroblasts were still detected in spleens at birth (Djaldetti *et al*. 1972). The spleen and liver contributions were also observed using colony-forming progenitor cells (CFCs), colony-forming unit erythrocytic (CFU-E) and burst forming unit erythrocytic (BFU-E) assays (Wolber *et al*. 2002). At later fetal stages (E18), FS and BM displayed a similar quantifiable contribution to hematopoiesis that appears weak compared to fetal liver.

Stromal cell lines from newborn spleen particularly sustain the proliferation and differentiation of erythroid progenitors *in vitro* (Yanai *et al*. 1989). In the neonatal spleen, two peaks of erythropoiesis were detected at 2 days and 14-15 days with numbers of erythrocytic progenitors that are comparable to those found in the BM. However, splenectomy did not alter CFC or HSC frequencies in the BM restricting the neonatal spleen to a minor alternative hematopoietic organ. Livers of splenectomized mice showed a higher CFC number, which underlines a cooperative contribution of the spleen, BM, and liver to the hematopoietic homeostasis (Wolber *et al*. 2002). A specificity of the neonatal spleen erythropoeisis concerns the requirement of the thyroid hormone T3 pathway via the thyroid receptor (TRα) for the erythrocytic terminal stage of differentiation (Angelin-Duclos *et al*. 2005). Hence, fetal and neonatal splenic erythropoietic processes are differentially regulated since fetal environment is known to be a hypothyroid situation. The action of T3 thyroid hormone pathway on erythroblast differentiation was demonstrated as a cell autonomous process only active in the spleen. The authors favor a difference of spleen and bone marrow environment action on erythrocytic progenitors rather than a difference of progenitors (Angelin-Duclos *et al*.2005). At the adult stage, few early (Lin$^-$ cKit$^+$ CD71$^-$ GATA1$^+$) and late (Lin$^-$ cKit$^+$ CD71$^+$ GATA1$^+$) erythroid progenitors are found in the spleen (Suzuki *et al*. 2003). Interestingly, after a phenylhydrazine-induced hemolytic anemia, the proportion of these progenitors increase indicating that the spleen could work as an extra-medullar erythropoietic site in hemorrage conditions (Migliaccio *et al*. 2000; Suzuki *et al*. 2003).

Splenic macrophage ontogeny

The spleen starts to be regionalized into red and white pulp at 5-7 days after birth, concomitantly to the establishment of the MZ. This coincides with the emergence of two new subsets of MΦ, known as MZ MΦ and MZ metallophilic MΦ (**Table 1**). The origin of these two subsets is still unclear in the mouse. But the divergence between red pulp and MZ MΦ could already exist during fetal life. Indeed, studies in the rat embryo have shown the existence in the fetal spleen of two different subsets of MΦ precursors, that appear sequentially at E15 and E16-17 and differentiate into red pulp and MZ MΦ, respectively (Takeya and Takahashi 1992). Some data obtained in the mouse also point to a different origin for red pulp and MZ MΦ. Indeed, after *in vivo* elimination of MΦ (after injection of liposome-entraped dichloromethylene diphosphate), red pulp MΦ, MZ metallophilic and MZ MΦ have different kinetics of splenic repopulation, recovering at 4 days, 8 days and 16 days, respectively (van Rooijen *et al*. 1989). This could suggest that different differentiation pathways are involved in the generation of these three subsets. As far as molecular events are concerned, the generation of these different MΦ subsets is critical on the transcription PU. 1 but might require different

Table 3 Deficient mice showing defects in splenic macrophages.

Knock-out mouse	MZ Metallophilic Macrophages	MZ Macrophages	Red Pulp Macrophages	References
Op / *Op* (*M-CSF*)	-	-	+	Witmer-Pack *et al.* 1993
PU.1	-	-	-	McKercher *et al.* 1996
Lymphotoxins or receptor	-	-	+	De Togni *et al.* 1994; Futterer *et al.* 1998

microenvironmental signals (McKercher *et al*. 1996; **Table 3**). Indeed, red pulp MΦ can differentiate in the absence of M-CSF, whereas MZ MΦ cannot, as observed in the op/op mouse (Witmer-Pack *et al*. 1993; **Table 3**). Thus, it was shown that administration of exogenous MCSF/CSF-1 could restore the populations of MZ and MZ metallophilic MΦ in the spleen (Cecchini *et al*. 1994). These subsets thus possess different requirements for cytokines during their ontogeny, suggesting different origins. However, it was recently shown that the $CX3CR1^+$ $cKit^+$ Lin^- bone marrow progenitor could differentiate *in vivo* into all types of splenic MΦ (Fogg *et al*. 2006).

Splenic DC ontogeny

DC are major players in the establishment of the immune function of the spleen, since they initiate T-cell dependant immunity. So far, splenic DC development has not been investigated during fetal life. Two groups have described the establishment of splenic DC starting at day 1 after birth (Sun *et al*. 2003; Dakic *et al*. 2004). These two groups describe the same sequence of events: after birth, the major DC subset comprises $CD4^-CD8^-CD11c^+$ cells, a subset very low represented in the adult spleen. During the second week of life, other DC subsets progressively appear in the spleen, as $CD4^-CD8^+$ and $CD4^+CD8^-$ DC populations. The double-negative subset that constitute the majority of neonatal DC has been shown to harbor similar properties to the $CD8^+$ DC subset, and could be considered the neonatal precursor of these cells (Dakic *et al*. 2004). Nevertheless, this lineage relationship has not been proven yet, and lineage analyses remain to be performed.

A previous study has shown that the three $CD11c^{hi}$ DC subsets have a fast turn-over of 1.5-3 days in the spleen (Kamath *et al*. 2000). These DC subsets are thus probably constantly replenished. Bone marrow precursors can differentiate into splenic DC, both *in vivo* and *in vitro* (reviewed in Shortman and Naik 2007). It has been shown that DC could differentiate from circulating blood monocytes under inflammatory conditions (Randolph *et al*. 1999; Geissmann *et al*. 2003). However, under steady state, circulating monocytes can only give rise to mucosal DC but not to splenic DC. Indeed, when transferred into MΦ- and DC-depleted recipients, monocytes replenish intestinal and lung DC but fail to do so in the spleen (Varol *et al*. 2007). It was recently shown that resident splenic DC could be repopulated from a bone marrow precursor that was isolated as $CX3CR1^+cKit^+Mac1^-$. This bipotent precursor for MΦ and DC (MDP) can reconstitute the splenic DC of irradiated recipients, under steady-state conditions (Fogg *et al*. 2006). This MDP can differentiate into splenic DC when directly injected in the spleen of DC-depleted recipients, suggesting that the MDP can progress along the DC differentiation pathway without going through a monocyte precursor (Varol *et al*. 2007), and could directly seed the spleen to achieve their differentiation.

As all lymphoid organs, the spleen consists of a non-hematopoietic cell component, also referred to as the microenvironment or stroma. In hematopoietic organs (bone marrow and thymus), the stroma provides hematopoietic progenitors with differentiation and survival signals. The spleen, although not hematopoietic during the adult life, can sustain hematopoiesis during fetal life until the two first weeks post-birth (Metcalf and Moore 1971). The splenic stroma has been shown to be involved in the differentiation and maturation of DC (Ni and O'Neill 1999). More recently, Zhang and collaborators have shown that neonatal splenic stromal cells could provide a suitable environment for the maturation of DC into regulatory DC, which can regulate the immune response in the spleen (Zhang *et al*. 2004). Recently, this group has shown that the same stromal environment could directly promote the differentiation of hematopoietic stem cells into regulatory DC, a process that involves stromal-derived IL-10 (Tang *et al*. 2006).

The lymphocyte subsets

In newborns, small follicles of B and DC cells are already formed and the T/B segregation starts to take place (Sun *et al*. 2003). These lymphoid follicles continue to grow during the first six weeks of age concomitantly to the splenic MΦ setting up. Lymphocyte populations differ between newborn and adult. These differences lead to a weak and Th2 biased immune response at the neonatal stage (reviewed in Adkins *et al*. 2004). The first thymic emigrants can be detected in the spleen after E17.5 thanks to the Thy1 antigen (Velardi and Cooper 1984). After birth, the number of splenic T cells constantly increases to reach its definitive size at 4th week post birth (Asano *et al*. 1996). The mature T populations are found in the spleen with a CD4/CD8 ratio equivalent to the adult one (Garcia *et al*. 2000). The $CD4^+$ $CD25^+CD3^+$ regulatory T cells, that negatively regulates the immune responses have been found in the spleen as soon as the third day post-birth and their percentage is stable from the 1st week of age (Asano *et al*. 1996; Dujardin *et al*. 2004). The neonatal splenic environment favors the Th2 response contrary to the LN environment (Adkins *et al*. 2000). It has been suggested that neonatal T progenitors preferentially acquire a Th2 phenotype whereas the Th1 population may be affected by selective apoptosis (Adkins *et al*. 2004; Li *et al*. 2004). B cells accumulate in the spleen until the 4th week of age (Velardi and Cooper 1984). B1 and B2 cells differ for their origins, phenotypes and functions (Montecino-Rodrique *et al*. 2005). The most frequent subset of the fetal/neonatal life consists of B1 cells whereas in the adult, most B cells belong to the B2 subset. When activated *via* TLR9, the neonate splenic B1 subset secrete IL-10 that prevent DC to trigger the Th1 response and favor the Th2 response (Sun *et al*. 2005). All B1 cells express the Mac1 marker and can be separated into the B1a ($CD5^+$) and B1b ($CD5^-$) cells. During the first two weeks of age, $CD5^+$ B1a cells represent 30 to 40% of the splenic population whereas they account for less than 5% of the adult spleen (Hayakawa *et al*. 1983; Sun *et al*. 2005). Wen and colleagues have shown that B1 cells are in contact with follicular dendritic cells and localize in the white pulp of the spleen from two week-old mice (Wen *et al*. 2005). The limited T cell dependent immune response observed during the neonatal period has been linked to the absence of germinal center until the second week of age (Pihlgren *et al*. 2003).

THE CONSTRUCTION OF THE SECONDARY IMMUNE ORGAN

The segregation of red and white pulp as well as the T/B segregation in the white pulp lies in events that could have started during the fetal and neonatal life. During fetal life, the $CD45^+CD4^+CD3^-$ cell population, named lymphoid tissue inducer cells (LTi) is required to induce the formation of lymph nodes (LN) and Peyer's patch (PP) (reviewed in Mebius 2003). The organization of secondary lymphoid organs depends on the expression of the LTα1β2 complex by LTi cells. LTi cells also express adhesion molecules such as CD44, intracellular adhesion molecule 1 (ICAM1), the α4β7 integrin and the inactive form α4β1 integrin. Thanks to α4β7 expression, LTi cells interact with blood vessels (splenic, intestinal and mesenteric) that express the mucosal addressin cell adhesion molecule 1 (MAdCAM1) and colonize the relative fetal organs (Hashi *et al*. 2001). The activation of the LTβR pathway by the LTα1β2 complex triggers the production of chemokines (CXCL12, CXCL13, CCL19, CCL21) and adhesion molecules (VCAM1 and ICAM1) by stromal cells (Dejardin *et al*. 2002) (reviewed in Chaplin and Fu 1998; Fu and Chaplin 1999; Chaplin 2002). Hence, LTi cells that express CXCR4, CXCR5 and CCR7 are attracted by the stromal compartment (reviewed in Cyster 2005). In PP anlagen, $CXCR5^+$ LTi cells are activated by CXCL13 and transactivate the inactive α4β1 complex to allow the interaction with the $VCAM1^+$ stromal cells (Finke *et al*. 2002). The LTβR signaling induces a positive feed-

back loop that enhances the expression of VCAM1 and chemokines by the stromal cells and then, recruits more LTi cells. This clustering of LTi cells with stromal organizer cells is followed by the recruitment of the first lymphocytes to finally give rise to the complex vascularized lymphoid tissues structure (reviewed in Mebius 2003).

LTi cells can be found in the anlagen of all future secondary organs. They have been detected in cervical lymph nodes as soon as E12.5, in E13.5 fetal spleen, in the blood at E14.5 and in E16.5 mesenteric LN and PP. In contrast, they are absent from the fetal thymus and liver (Mebius *et al*. 1997; Yoshida *et al*. 1999; Eberl *et al*. 2004). LTβR$^+$ stromal cells also express MadCAM1, VCAM1 and ICAM1 in E16.5 mesenteric LN and E17.5 intestine (Honda *et al*. 2001; Cupedo *et al*. 2004).

The LTi cells originate from lymphoid-restricted fetal liver progenitors (IL7Rα$^+$α4β7$^+$) that migrate *via* the mesenteric vessels between E11.5 and E15.5 to colonize the gut and LN (Yoshida *et al*. 1999; Mebius *et al*. 2001). Id2 and a specific isoform of the retinoic acid orphan-related receptor γ, called RORγt, are the two transcription factors that are known to be strictly required for LTi generation (Yokota *et al*. 1999; Eberl *et al*. 2004). Indeed, LTi cells are absent from Id2$^{-/-}$ or RORγ$^{-/-}$ mice (Yokota *et al*. 1999; Sun *et al*. 2000; Eberl *et al*. 2004). This failure in LTi generation causes the absence of PP, mesenteric and inguinal LN indicating their absolute requirement to generate these secondary lymphoid tissues. As far as spleen architecture is concerned, the white pulp is not affected by the lack of LTi since the adult spleen of Id2$^{-/-}$ mice present normal follicular B/T segregation, MZ MΦ and GC repartition (Yokota *et al*. 1999). The importance of LTi cells in the splenic organization is not yet known and we discuss herein some recent data on their hypothetical functions in this developmental process.

Is the early step of the fetal spleen structure LTi-dependent?

Firstly colonized by myeloid cells, the 13.5 dpc fetal spleen hosts several hematopoietic progenitors and CD4$^+$IL7Rα$^+$CD3$^-$ LTi cells (Desanti, unpublished observations). These progenitors are circulating hematopoietic cells, that probably originates from the fetal liver and reach the spleen *via* Flk-1$^+$MadCAM1$^+$ endothelial cells (Mebius *et al*. 1997; Hashi *et al*. 2001; Eberl *et al*. 2004). The presence of LTi cells in early spleen is probably due to an *in situ* differentiation rather than colonization from circulating LTi cells. However, these two possibilities are not mutually exclusive. LTi precursors have been isolated from fetal spleen, as Lin$^-$CD4^{int}RAG2$^{-/lo}$ cells and fetal spleen organ cultures (FSOC) have proved their capacity to sustain the differentiation and proliferation of Lti cells *in situ* (Desanti, unpublished observations).

Histological studies have demonstrated that the early organization of spleen mainly consists of numerous F4/80$^+$ MΦ, erythrocytes and few isolated lymphoid progenitors scattered throughout the whole organ (Morris *et al*. 1991; Bertrand *et al*. 2006). The first sign of any organization occur through the accumulation of LTi cells around the central arterioles (Eberl *et al*. 2004). This may reflect the first step of splenocyte segregation. In mesenteric LN and PP, LTi cells are attracted by CXCL13 expressing stromal cells to establish the positive feedback loop that result in the secondary lymphoid organ formation (reviewed in Mebius 2003; Cyster 2005). Since E13.5 fetal spleen contains LTi cells and lymphoid progenitors, we propose that LTi cell constitute the first pool of cells that interact with the stroma to activate this positive feedback loop (Mebius *et al*. 1997; Godin *et al*. 1999; Bertrand *et al*. 2006). Indeed, LTβR, CXCL13 and VCAM1 transcripts are expressed by E14.5-E15.5 fetal spleen stroma (Desanti, unpublished observations). However, these first structural steps may be triggered by an LTβR-independent pathway. Lane and collaborators have recently shown that E15.5 splenic LTi cells are highly potent to trigger the expression of CCL21 and VCAM1 on adult splenic lymphoid stromal cells from LTα$^{-/-}$ mice (Kim *et al*. 2007). At E16.5, LTi cell foci enlarged around the arteriolar cavities and the first splenic B cells are detected (Spear *et al*. 1973; Velardi and Cooper 1984; Eberl *et al*. 2004). Despite the normal spleen architecture in mice devoid of LTi cells, we suggest that LTi cells may prime the stroma of the future lymphoid area. It implies a redundancy in the cells that are able to activate the TNF family pathways. Besides, B progenitors that develop in the fetal spleen could upregulate the LTα1β2 complex and participate to the feedback loop necessary for the white pulp construction (Ngo *et al*. 2001). As hematopoietic cells are colonizing the spleen, they may be retained to increase the size of the lymphoid foci that surrounds the growing arteriole. Then, the first splenic structural steps are the growing lymphoid area from the arteriole lumen that pushes back the myeloid area that represents the future red pulp. We postulate for an action of LTi cells during these early steps of organogenesis.

Structuration of the spleen as a secondary immune organ

The follicular T/B segregation starts between 3 to 7 days after birth and is maintained throughout life by permanent molecular cross-talk between splenic B, T and stromal cells. The molecules involved in the maintenance of the B/T segregation are identical to those implied in the white pulp generation (LTα1β2, LTβR, TNFR, TNFα, CXCL13, CCL19, CCL21, CCR5, CCR7) (reviewed in Tumanov *et al*. 2003; McCarthy *et al*. 2006). Two weeks after birth, the MZ appears around the B and T follicles and delineate the white from the red pulp (Takeya and Takahashi 1992). This structure is maintained throughout life according to similar LTβR mechanisms probably activated by B cells expressing LTα1β2 (Yu *et al*. 2002). This induction of chemokine production is thought to occur when B cells home to the MZ or migrate through it to terminate in the white pulp (reviewed in Mebius and Kraal 2005). The upregulation of CCL19 and CCL21 are essential for the localization of the MZ MΦ next to the endothelial marginal sinus (Ato *et al*. 2004).

Several knock-out mice have uncovered the disparities in the formation of secondary lymphoid tissues (**Table 4**). These differences highlight that diverse mechanisms are activated to generate the lymphoid tissues. For instance, the mesenteric and peripheral LN organogenesis are both dependent on LTβR although their stromal phenotypic differences have been related to supplemental individual regulation (Cupedo *et al*. 2004). Despite the presence of LTi cells in the white pulp and NALT of normal embryos, these two lymphoid tissues could be formed independently of LTi cells (Yokota *et al*. 1999; Harmsen *et al*. 2002). NALT formation is even more confusing since NALT is present in RORγ$^{-/-}$ but absent from Id2$^{-/-}$ mice (Fukuyama *et al*. 2002). The example of NALT organogenesis highlights the complex interaction of the different pathways involved in lymphoid tissue formation. In conclusion, the regulation of the lymphoid tissue is complex and new pathways should be discovered to explain peculiarities of each lymphoid tissue.

The splenic white pulp is normally segregated into B and T areas in Id2$^{-/-}$ mice (Yokota *et al*. 1999). The hematopoietic capacities of the fetal spleen may partially explain that LTi cells are not required to white pulp but LN and PP generation. Hence, neonatal spleen already possesses several LTα1β2-expressing cell types when the architecture into white pulp and B/T segregation is initiated. Then, the LTi deficiency is probably complemented by other hematopoietic cells. It was shown that B and T lymphocytes express the LTα1β2 complex and are actively participating to the maintenance of the white pulp structure (Tumanov *et al*. 2002). This probable supply is reinforced by a recent study showing the capacity of neonatal LTi cells to restore the B/T segregation in LTα$^{-/-}$ mice (Kim *et al*. 2007).

Table 4 Knock-out mice presenting defects in secondary lymphoid tissues.

Genes deleted	Cells affected	PLN[1)]	MLN	PP	NALT[2)]	LTi	Reference
Id2	hema	-	-	-	-	-	Yokota *et al.* 1999
Rorγ / Rorγt	hema	-	-	-	↓	-	Sun *et al.* 2000; Kurebayashi *et al.* 2000; Eberl *et al.* 2004
Ltα	hema	-	- / ↓	-	↓	+	Banks *et al.* 1995; Koni *et al.* 1997; De Togni *et al.* 1994; Eberl *et al.* 2004
Ltβ	hema	- / ↓ C	↓	- / +	↓	N.D	Alimzhanov *et al.*1997; Koni *et al.* 1997; Kuprash *et al.* 1999
Tnf	hema	+	+	↓	↓	N.D	Körner *et al.* 1997; Pasparakis *et al.* 1997
Light	hema	+	+	+	N.D	N.D	Scheu *et al.* 2002
Ltβ/Tnf	hema	- / ↓	↓	-	N.D	N.D	Kuprash *et al.* 1999
Ltα+/- / Ltβ+/-	hema	+	+	-	N.D	N.D	Koni *et al.* 1998
Tnf/Ltα	hema	-	-	-	↓	N.D	Körner *et al.* 1997
Light / Ltβ	hema	-	<*Ltβ*$^{-/-}$	-	N.D	N.D	Scheu *et al.* 2002
TRANCE-R	hema	-	-	↓	↓	N.D	Dougall *et al.* 1999
Traf6	hema	-	-	+	N.D	↓	Naito *et al.* 1999; Yoshida *et al.* 2002
Il7rα	hema	- / ↓ B	↓	-	↓	↓	Adachi *et al.* 1998; Luther *et al.* 2003
γc	hema	↓ B, F	↓	-	N.D	N.D	Cao *et al.* 1995; Luther *et al.* 2003
Rnf110 / Phc2	hema	N.D	N.D	↓	N.D	↓	Sato *et al.* 2006
Cxcr5	hema	- B, C, F	+	↓	N.D	+	Ansel *et al.* 2000; Ohl *et al.* 2003
Ccr7	hema	+	+	+	N.D	+	Ohl *et al.* 2003
Cxcr5 / Ccr7	hema	-	+	>*Cxcr5*$^{-/-}$	N.D	N.D	
Ltβ / Tnfr1	hema/stroma	-	-	-	N.D	N.D	Koni *et al.* 1998
Trance	hema/stroma	-	-	↓	↑	↓	Kong *et al.* 1999; Kim *et al.* 2000
Il7rα / Cxcl13	hema/stroma	-	-	-	N.D	↓	Luther *et al.* 2003
Tnfr1-/-	stroma	+	+	↓	↓	+	Neumann *et al.* 1996; Pasparakis *et al.* 1997; Alcamo *et al.* 2001
Tnfr1 / Rela	stroma	-	-	-	N.D	+	Alcamo *et al.* 2001
Aly/Aly	stroma	-	-	-	↓	+	Miyawaki *et al.* 1994
Ltβr	stroma	-	-	-	N.D	N.D	Fütterer *et al.* 1998
Nik	stroma	-	-	-	N.D	N.D	Yin *et al.* 2001
Nfkb1	stroma	↓ A, B, C, F, PA	+	+	+	N.D	Weih *et al.* 2003; Lo *et al.* 2006
Nfkb2	stroma	↓ A, B, C, F, PA	+	-	↓	N.D	Weih *et al.* 2003; Lo *et al.* 2006
Nfkb1 / Nfkb2	stroma	-	-	-	N.D	N.D	Lo *et al.* 2006
Traf2 / Traf5	stroma	N.D	+	↓	N.D	N.D	Piao *et al.* 2007
Plt/Plt	stroma	+	+	+	+	+	Luther *et al.* 2003; Fukuyama *et al.* 2006
Cxcl13	stroma	↓ C, F	+	↓	+	+	
Cxcl13 / Plt/Plt	stroma	↓	+	-	+	+	

[1)] Each kind of peripheral lymph nodes (PLN) cited are listed by their first letter as follow: A: axillary; B: brachial; C: cervical; F: facial; I: Iguinal; P: popliteal; PA: periaortic; R: renal; S: sacral. The PLN that are not affected by the knock-out are indicated by their corresponding letters.
[2)] NALT organogenesis has been mainly observed in Harmsen *et al.* 2002; Fukuyama, Kiyono *et al.* 2002.
+: similar to wild-type; -: absent; ↓: smaller in size and/or number; ↑: larger in size; <*Ltβ*$^{-/-}$: less than in *Ltβ*$^{-/-}$; > *Cxcr5*$^{-/-}$: more than in *Cxcr5*$^{-/-}$; N.D: not determined; hema: hematopoietic; PLN: peripheral lymph nodes, MLN: mesenteric lymph nodes.

Are accessory and LTi cells related?

Recently, it has been described that splenic LTi cells could evolve into a new $CD4^+CD3^-$ cell compartment called "accessory cells". These cells are found in one week-old newborns and express OX40L and CD30L co-stimulatory molecules (Ki *et al*. 2005). The *in vitro* acquisition of CD30L depends on the IL-7/IL-7R signaling while OX40L acquisition depends on TL1A (Kim *et al*. 2005, 2006). Accessory cells seem to retain a tissue inducer activity since their injection into LTα$^{-/-}$ mice restore the splenic T/B segregation (Kim *et al*. 2007). Accessory cells express the genes of the TNF family and it was shown that RORγt, LTβ and LIGHT mRNA levels are lower than those of their fetal counterpart (cultured for 5 days with IL7) (Kim *et al*. 2006). It was proposed that accessory cells derive from fetal LTi cells since TL1A was shown to induce the down-regulation of RORγt, LTβ and LIGHT from splenic neonate $CD4^+CD3^-$ *in vitro* (Kim *et al*. 2006). However, a mice model that allows to track the fetal LTi hematopoietic progeny has suggested that no progeny could be generated from LTi cells (Eberl *et al*. 2004). The accessory cells play a role in immune response through expression of OX40 and CD30L. Located in B cell follicles and GC, they are supposed to sustain the survival of T-helper 2 cells that are expressing OX40 and CD30 (Kim *et al*. 2003).

CONCLUSION

The spleen is found in all vertebrates despite variations in its architecture. In mammals, only slight differences could be observed like the absence of the marginal sinus in human (Steiniger *et al*. 2006). On the contrary, the architecture could be highly different depending on species, such as the complete loss of red and white pulp segregation observed in Urodela amphibians (Tooze and Davies 1967). The red pulp area is the only structure to be maintained in all species. As the main filter of blood, the spleen contains all myeloid subtypes commonly found in circulation. It is a reservoir of erythrocytes where MΦ participates to the iron recycling via erythrophagocytosis. The cleaning capacity of the spleen is maintained through evolution. The MZ is a specific acquisition of mammals with a unique organization and composition that is essential in cases of infection to encapsulated bacteria (Kraal 1992). As a secondary lymphoid organ, the spleen also possesses a dynamic architecture into primary and secondary follicles. Hence, the spleen is competent to answer to both the innate and adaptative immune responses. White pulp, germinal centers and adaptive immune functions are not conserved in evolution whereas

hematopoietic processes are sustained. In mice, hematopoiesis is observed from the early fetal stages until the firsts weeks after birth. In urodela and anouran amphibians, the Ikaros, Rag and TdT genes are expressed in tadpole and adult spleen (reviewed in Hansen and Zapata 1998). Similarly, Rag transcripts were detected in adult spleen of sharks exposing the hematopoietic capacities of this organ as a quasi-universal characteristic (Bernstein *et al*. 1994). We speculate that this organ was gained in the common vertebrate ancestor in parallel to the acquisition of the Rag genes and the events that generate the Ig repertoire diversity. Furthermore, in the abnormal situation of metaplasia, the mammal adult spleen upregulates its hematopoietic capacities suggesting the maintenance of this function by the stromal compartment.

REFERENCES

Adachi S, Yoshida H, Honda K, Maki K, Saijo K, Ikuta K, Saito T, Nishikawa SI (1998) Essential role of IL-7 receptor α in the formation of Peyer's patch anlage. *International Immunology* **10**, 1-6

Adkins B, Bu Y, Cepero E, Perez R (2000) Exclusive Th2 primary effector function in spleens but mixed Th1/Th2 function in lymph nodes of murine neonates. *Journal of Immunology* **164**, 2347-2353

Adkins B, Leclerc C, Marshall-Clarke S (2004) Neonatal adaptive immunity comes of age. *Nature Review Immunoly* **4**, 553-564

Akazawa H, Komuro I, Sugitani Y, Yazaki Y, Nagai R, Noda T (2000) Targeted disruption of the homeobox transcription factor *Bapx1* results in lethal skeletal dysplasia with asplenia and gastroduodenal malformation. *Genes and Cells* **5**, 499-513

Alcamo E, Hacohen N, Schulte LC, Rennert PD, Hynes RO, Baltimore D (2002) Requirement for the NF- κB family member RelA in the development of secondary lymphoid organs. *Journal of Experimental Medicine* **195**, 233-244

Angelin-Duclos C, Domenget C, Kolbus A, Beug H, Jurdic P, Samarut J (2005) Thyroid hormone T3 acting through the thyroid hormone α receptor is necessary for implementation of erythropoiesis in the neonatal spleen environment in the mouse. *Development* **132**, 925-934

Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, Browning JL, Lipp M, Cyster JG (2000) A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* **406**, 309-314

Asano M, Toda M, Sakaguchi N, Sakaguchi S (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *Journal of Experimental Medicine* **184**, 387-396

Asayesh A, Sharpe J, Watson RP, Hecksher-Sorensen J, Hastie ND, Hill RE, Ahlgren, U (2006) Spleen versus pancreas: strict control of organ interrelationship revealed by analyses of *Bapx1*$^{-/-}$ mice. *Genes and Development* **20**, 2208-2213

Ato M, Nakano H, Kakiuchi T, Kaye PM (2004) Localization of marginal zone macrophages is regulated by C-C chemokine ligands 21/19. *Journal of Immunology* **173**, 4815-4820

Banks TA, Rouse BT, Kerley MK, Blair PJ, Godfrey VL, Kuklin NA, Bouley DM, Thomas J, Kanangat S, Mucenski ML (1995) Lymphotoxin-α-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *Journal of Immunology* **155**, 1685-1693

Bartram U, Wirbelauer J, Speer CP (2005) Heterotaxy syndrome – asplenia and polysplenia as indicators of visceral malposition and complex congenital heart disease. *Biology of the Neonate* **88**, 278-290

Becker-Herman S, Lantner F, Shachar I (2002) Id2 negatively regulates B cell differentiation in the spleen. *Journal of Immunology* **168**, 5507-5513

Bernstein RM, Schluter SF, Lake DF, Marchalonis JJ (1994) Evolutionary conservation and molecular cloning of the recombinase activating gene 1. *Biochemical and Biophysical Research Communications* **205**, 687-692

Bertrand JY, Desanti GE, Lo-Man R, Leclerc C, Cumano A, Golub R (2006) Fetal spleen stroma drives macrophage commitment. *Development* **133**, 3619-3628

Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A, Godin I (2005) Three pathways to mature macrophages in the early mouse yolk sac. *Blood* **106**, 3004-3011

Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, Bellusci S, Scharfmann R (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* **128**, 5109-5117

Brendolan A, Ferretti E, Salsi V, Moses K, Quaggin S, Blasi F, Cleary ML, Selleri L (2005) A *Pbx1*-dependent genetic and transcriptional network regulates spleen ontogeny. *Development* **132**, 3113-3126

Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN (2007) Development and function of the mammalian spleen. *Bioessays* **29**, 166-177

Cao X, Shores,EW, Hu-Li J, Anver MR, Kelsall BL, Russell SM, Drago J, Noguchi M, Grinberg A, Bloom ET, Paul WE, Katz SI, Love PE, Leonard WJ (1995) Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity* **2**, 223-238

Carlyle JR, Zuniga-Pflucker JC (1998) Requirement for the thymus in αβ T lymphocyte lineage commitment. *Immunity* **9**, 187-197

Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H, Chisholm O, Hofstetter W, Pollard JW, Stanley ER (1994) Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* **120**, 1357-1372

Chaplin DD (2002) Regulation of spleen white pulp structure and function by lymphotoxin. *Advances in Experimental Medicine and Biology* **512**, 49-56

Chaplin DD, Fu Y (1998) Cytokine regulation of secondary lymphoid organ development. *Current Opinion of Immunology* **10**, 289-297

Christensen JL, Wright DE, Wagers AJ, Weissman IL (2004) Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biology* **2**, E75

Cupedo T, Vondenhoff MF, Heeregrave EJ, De Weerd AE, Jansen W, Jackson DG, Kraal G, Mebius RE (2004) Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *Journal of Immunology* **173**, 2968-2975

Cyster JG (2005) Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annual Review of Immunology* **23**, 127-159

Dakic A, Shao QX, D'Amico A, O'Keeffe M, Chen WF, Shortman K, Wu L (2004) Development of the dendritic cell system during mouse ontogeny. *Journal of Immunology* **172**, 1018-1027

De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, Smith SC, Carlson R, Shornick LP, Strauss-Schoenberger J (1994) Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* **264**, 703-707

Dear TN, Colledge WH, Carlton MB, Lavenir I, Larson T, Smith AJ, Warren AJ, Evans MJ, Sofroniew MV, Rabbitts TH (1995) The *Hox11* gene is essential for cell survival during spleen development. *Development* **121**, 2909-2915

Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li ZW, Karin M, Ware CF, Green DR (2002) The lymphotoxin β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity* **17**, 525-535

DiMartino JF, Selleri L, Traver D, Firpo MT, Rhee J, Warnke R, O'Gorman S, Weissman IL, Cleary ML (2001) The Hox cofactor and proto-oncogene *Pbx1* is required for maintenance of definitive hematopoiesis in the fetal liver. *Blood* **98**, 618-626

Djaldetti M, Bessler H, Rifkind RA (1972) Hematopoiesis in the embryonic mouse spleen: an electron microscopic study. *Blood* **39**, 826-841

Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon, Schuh J (1999) RANK is essential for osteoclast and lymph node development. *Genes and Development* **13**, 2412-2424

Dujardin HC, Burlen-Defranoux O, Boucontet L, Vieira P, Cumano A, Bandeira A (2004) Regulatory potential and control of *Foxp3* expression in newborn CD4$^+$ T cells. *Proceedings of the National Academy of Sciences USA* **101**, 14473-14478

Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR (2004) An essential function for the nuclear receptor RORγt in the generation of fetal lymphoid tissue inducer cells. *Nature Immunology* **5**, 64-73

Edlund H (2002) Pancreatic organogenesis – developmental mechanisms and implications for therapy. *Nature Reviews Genetics* **3**, 524-532

Eloranta ML, Alm GV (1999) Splenic marginal metallophilic macrophages and marginal zone macrophages are the major interferon-α/β producers in mice upon intravenous challenge with herpes simplex virus. *Scandinavian Journal of Immunology* **49**, 391-394

Ferlicot S, Emile JF, Le Bris JL, Cheron G, Brousse N (1997) Congenital asplenia. A childhood immune deficit often detected too late. *Annals of Pathology* **17**, 44-46

Finke D, Acha-Orbea H, Mattis A, Lipp M, Kraehenbuhl J (2002) CD4$^+$CD3$^-$ cells induce Peyer's patch development: role of α4β1 integrin activation by CXCR5. *Immunity* **17**, 363-373

Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F (2006) A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* **311**, 83-87

Fu YX, Chaplin DD (1999) Development and maturation of secondary lymphoid tissues. *Annual Review of Immunology* **17**, 399-433

Fukuyama S, Hiroi T, Yokota Y, Rennert PD, Yanagita M, Kinoshita N, Terawaki S, Shikina T, Yamamoto M, Kurono Y, Kiyono Y (2002) Initiation of NALT organogenesis is independent of the IL-7R, LTβR, and NIK signaling pathways but requires the *Id2* gene and CD3$^-$CD4$^+$CD45$^+$ cells. *Immunity* **17**, 31-40

Fukuyama S, Nagatake T, Kim DY, Takamura K, Park EJ, Kaisho T, Tanaka N, Kurono Y, Kiyono H (2006) Cutting edge: Uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. *Journal of Immunology* **177**, 4276-4280

Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K (1998) The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* **9**, 59-70

Garcia AM, Fadel SA, Cao S, Sarzotti M (2000) T cell immunity in neonates.

Immunological Research **22**, 177-190

Geissmann F, Jung S, Littman DR (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71-82

Germing U, Perings C, Steiner S, Peters AJ, Heintzen MP, Aul C (1999) Congenital asplenia detected in a 60 year old patient with septicemia. *European Journal of Medical Research* **4**, 283-285

Gilbert B, Menetrey C, Belin V, Brosset P, de Lumley L, Fisher A (2002) Fa-milial isolated congenital asplenia: a rare, frequently hereditary dominant condition, often detected too late as a cause of overwhelming pneumococcal sepsis. Report of a new case and review of 31 others. *European Journal of Pediatry* **161**, 368-372

Godin I, Cumano A (2002) The hare and the tortoise: an embryonic haematopoietic race. *Nature Review of Immunology* **2**, 593-604

Godin I, Garcia-Porrero JA, Dieterlen-Lievre F, Cumano A (1999) Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *Journal of Experimental Medicine* **190**, 43-52

Green MC (1967) A defect of the splanchnic mesoderm caused by the mutant gene dominant hemimelia in the mouse. *Developmental Biology* **15**, 62-89

Halbertsma FJ, Neeleman C, Weemaes CM, van Deuren M (2005) The absent and vanishing spleen: congenital asplenia and hyposplenism – two case reports. *Acta Paediatrica* **94**, 369-371

Hansen JD, Zapata AG (1998) Lymphocyte development in fish and amphibians. *Immunological Review* **166**, 199-220

Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, Zou YR, Littman DR, Cyster JG (2001) A coordinated change in chemokine responsiveness guides plasma cell movements. *Journal of Experimental Medicine* **194**, 45-56

Harmsen A, Kusser K, Hartson L, Tighe M, Sunshine MJ, Sedgwick JD, Choi Y, Littman DR, Randall TD (2002) Cutting edge: organogenesis of nasal-associated lymphoid tissue (NALT) occurs independently of lymphotoxin-α (LTα) and retinoic acid receptor-related orphan receptor-γ, but the organization of NALT is LTα dependent. *Journal of Immunology* **168**, 986-990

Hashi H, Yoshida H, Honda K, Fraser S, Kubo H, Awane M, Takabayashi A, Nakano H, Yamaoka Y, Nishikawa S (2001) Compartmentalization of Peyer's patch anlagen before lymphocyte entry. *Journal of Immunology* **166**, 3702-3709

Hayakawa K, Hardy RR, Parks DR, Herzenberg LA (1983) The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *Journal of Experimental Medicine* **157**, 202-218

Hecksher-Sorensen J, Watson RP, Lettice LA, Serup P, Eley L, de Angelis C, Ahlgren U, Hill RE (2004) The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by *Bapx1*/*Nkx3.2*. *Development* **131**, 4665-4675

Herzer U, Crocoll A, Barton D, Howells N, Englert C (1999) The Wilms tumor suppressor gene *wt1* is required for development of the spleen. *Current Biology* **9**, 837-840

Honda K, Nakano H, Yoshida H, Nishikawa S, Rennert P, Ikuta K, Tamechika M, Yamaguchi K, Fukumoto T, Chiba T, Nishikawa SI (2001) Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *Journal of Experimental Medicine* **193**, 621-630

Hummler HD, Pohlandt F, Essig A (2005) Fulminant pneumococcal sepsis in a 13-months old child with congenital asplenia. *Clinical Pediatrics* **217**, 274-275

Ivemark BI (1955) Implications of agenesis of the spleen on the pathogenesis of conotruncus anomalies in childhood; an analysis of the heart malformations in the splenic agenesis syndrome, with fourteen new cases. *Acta Paediatrics* **44**, 7-110

Kamath AT, Pooley J, O'Keeffe MA, Vremec D, Zhan Y, Lew AM, D'Amico A, Wu L, Tough DF, Shortman K (2000) The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *Journal of Immunology* **165**, 6762-6770

Kanzler B, Dear TN (2001) *Hox11* acts cell autonomously in spleen development and its absence results in altered cell fate of mesenchymal spleen precursors. *Developmental Biology* **234**, 231-243

Kiel MJ, Iwashita T, Yilmaz OH, Morrison SJ (2005) Spatial differences in hematopoiesis but not in stem cells indicate a lack of regional patterning in definitive hematopoietic stem cells. *Developmental Biology* **283**, 29-39

Kim D, Mebius RE, MacMicking JD, Jung S, Cupedo T, Castellanos Y, Rho J, Wong BR, Josien R, Kim N, Rennert PD, Choi Y (2000) Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *Journal of Experimental Medicine* **192**, 1467-1478

Kim MY, Anderson G, White A, Jenkinson E, Arlt W, Martensson IL, Erlandsson L, Lane PJ (2005) OX40 ligand and CD30 ligand are expressed on adult but not neonatal $CD4^+CD3^-$ inducer cells: evidence that IL-7 signals regulate CD30 ligand but not OX40 ligand expression. *Journal of Immunology* **174**, 6686-6691

Kim MY, Gaspal FM, Wiggett HE, McConnell FM, Gulbranson-Judge A, Raykundalia C, Walker LS, Goodall MD, Lane PJ (2003) $CD4^+CD3^-$ accessory cells co-stimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. *Immunity* **18**, 643-654

Kim MY, McConnell FM, Gaspal FM, White A, Glanville SH, Bekiaris V, Walker LS, Caamano J, Jenkinson E, Anderson G, Lane PJ (2007) Function of $CD4^+CD3^-$ cells in relation to B- and T-zone stroma in spleen. *Blood* **109**, 1602-1610

Kim MY, Toellner, KM, White A, McConnell FM, Gaspal FM, Parnell SM, Jenkinson E, Anderson G, Lane PJ (2006) Neonatal and adult $CD4^+CD3^-$ cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A. *Journal of Immunology* **177**, 3074-3081

Kim SK, Selleri L, Lee JS, Zhang AY, Gu X, Jacobs Y, Cleary ML (2002) Pbx1 inactivation disrupts pancreas development and in *Ipf1*-deficient mice promotes diabetes mellitus. *Nature Genetics* **30**, 430-435

Koehler K, Franz T, Dear TN (2000) *Hox11* is required to maintain normal *Wt1* mRNA levels in the developing spleen. *Developmental Dynamics* **218**, 201-206

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, KhooW, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**, 315-323

Koni PA, Flavell RA (1998) A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin β. *Journal of Experimental Medicine* **187**, 1977-1983

Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA (1997) Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity* **6**, 491-500

Koo GC, Peppard JR, Hatzfeld A (1982) Ontogeny of $Nk\text{-}1^+$ natural killer cells. I. Promotion of $Nk\text{-}1^+$ cells in fetal, baby, and old mice. *Journal of Immunology* **129**, 867-871

Kraal G. (1992) Cells in the marginal zone of the spleen. *International Review of Cytology* **132**, 31-74

Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK (2001) Identification of the haemoglobin scavenger receptor. *Nature* **409**, 198-201

Kuprash DV, Alimzhanov MB, Tumanov AV, Anderson AO, Pfeffer K, Nedospasov SA (1999) TNF and lymphotoxin beta cooperate in the maintenance of secondary lymphoid tissue microarchitecture but not in the development of lymph nodes. *Journal of Immunology* **163**, 6575-6580

Kuprash DV, Alimzhanov MB, Tumanov AV, Grivennikov SI, Shakhov AN, Drutskaya LN, Marino MW, Turetskaya RL, Anderson AO, Rajewsky K, Pfeffer K, Nedospasov SA (2002) Redundancy in tumor necrosis factor (TNF) and lymphotoxin (LT) signaling *in vivo*: mice with inactivation of the entire TNF/LT locus versus single-knockout mice. *Molecular and Cellular Biology* **22**, 8626-8634

Kurebayashi S, Ueda E, Sakaue M, Patel DD, Medvedev A, Zhang F, Jetten AM (2000) Retinoid-related orphan receptor gamma (RORγ) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proceedings of the National Academy of Sciences USA* **97**, 10132-10137

Kuroda K, Han H, Tani S, Tanigaki K, Tun T, Furukawa T, Taniguchi Y, Kurooka H, Hamada Y, Toyokuni S, Honjo T (2003) Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity* **18**, 301-312

Lettice LA, Purdie LA, Carlson GJ, Kilanowski F, Dorin J, Hill RE (1999) The mouse bagpipe gene controls development of axial skeleton, skull, and spleen. *Proceedings of the National Academy of Sciences USA* **96**, 9695-9700

Li L, Lee HH, Bell JJ, Gregg RK, Ellis JS, Gessner A, Zaghouani H (2004) IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. *Immunity* **20**, 429-440

Liepinsh DJ, Grivennikov SI, Klarmann KD, Lagarkova MA, Drutskaya MS, Lockett SJ, Tessarollo L, McAuliffe M, Keller JR, Kuprash DV, Nedospasov SA (2006) Novel lymphotoxin alpha (LTα) knockout mice with unperturbed tumor necrosis factor expression: reassessing LTα biological functions. *Molecular and Cellular Biology* **26**, 4214-4225

Lioubinski O, Muller M, Wegner M, Sander M (2003) Expression of *Sox* transcription factors in the developing mouse pancreas. *Developmental Dynamics* **227**, 402-408

Lo JC, Basak S, James ES, Quiambo RS, Kinsella MC, Alegre ML, Weih F, Franzoso G, Hoffmann A, Fu YX (2006) Coordination between NF-κB family members p50 and p52 is essential for mediating LTβR signals in the development and organization of secondary lymphoid tissues. *Blood* **107**, 1048-1055

Lu J, Chang P, Richardson JA, Gan L, Weiler H, Olson EN (2000) The basic helix-loop-helix transcription factor capsulin controls spleen organogenesis. *Proceedings of the National Academy of Sciences USA* **97**, 9525-9530

Luther SA, Ansel KM, Cyster JG (2003) Overlapping roles of CXCL13, interleukin 7 receptor α, and CCR7 ligands in lymph node development. *Journal of Experimental Medicine* **197**, 1191-1198

Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene *Nkx2-5*. *Genes and Development* **9**, 1654-1666

McCarthy DD, Summers-Deluca L, Vu F, Chiu S, Gao Y, Gommerman JL (2006) The lymphotoxin pathway: beyond lymph node development. *Immunological Research* **35**, 41-54

McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ (1996) Targeted disrup-

tion of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO Journal* **15**, 5647-5658

Mebius RE (2003) Organogenesis of lymphoid tissues. *Nature Review Immunology* **3**, 292-303

Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature Review Immunology* **5**, 606-616

Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, Akashi K (2001) The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, $CD45^+CD4^+CD3^-$ cells, as well as macrophages. *Journal of Immunology* **166**, 6593-6601

Mebius RE, Rennert P, Weissman IL (1997) Developing lymph nodes collect $CD4^+CD3^-$ $LT\beta^+$ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* **7**, 493-50

Metcalf D, Moore MAS (1971) Haematopoietic cells. In: Neuberger A, Tatum EL (Eds) *Frontiers in Biology*, Amsterdam, North Holland Publish Co., **24**, pp 172-271

Migliaccio AR, Adamson JW, Stevens CE, Dobrila NL, Carrier CM, Rubinstein P (2000) Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. *Blood* **96**, 2717-2722

Miyawaki S, Nakamura Y, Suzuka H, Koba M, Yasumizu R, Ikehara S, Shibata Y (1994) A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *European Journal of Immunology* **24**, 429-434

Montecino-Rodriquez E, Min H, Dorshkind K (2005) Reevaluating current models of thymic involution. *Seminars in Immunology* **17**, 356-361

Morris L, Graham CF, Gordon S (1991) Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development* **112**, 517-526

Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue JI (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes to Cells* **4**, 353-362

Nakano H, Mori S, Yonekawa H, Nariuchi H, Matsuzawa A, Kakiuchi T (1998) A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. *Blood* **91**, 2886-2895

Neumann B, Luz A, Pfeffer K, Holzmann B (1996) Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *Journal of Experimental Medicine* **184**, 259-264

Ngo VN, Cornall RJ, Cyster JG (2001) Splenic T zone development is B cell dependent. *Journal of Experimental Medicine* **194**, 1649-1660

Ni K, O'Neill H (1999) Spleen stromal cells support haemopoiesis and *in vitro* growth of dendritic cells from bone marrow. *British Journal of Haematology* **105**, 58-67

Ohl L, Henning G, Krautwald S, Lipp M, Hardtke S, Bernhardt G, Pabst O, Forster R (2003) Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *Journal of Experimental Medicine* **197**, 1199-1204

Pabst O, Forster R, Lipp M, Engel H, Arnold HH (2000) NKX2.3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen and mucosa-associated lymphoid tissue. *The EMBO Journal* **19**, 2015-2023

Pabst O, Zweigerdt R, Arnold HH (1999) Targeted disruption of the homeobox transcription factor *Nkx2-3* in mice results in postnatal lethality and abnormal development of small intestine and spleen. *Development* **126**, 2215-2225

Patterson KD, Drysdale TA, Krieg PA (2000) Embryonic origins of spleen asymmetry. *Development* **127**, 167-175

Piao JH, Yoshida H, Yeh WC, Doi T, Xue X, Yagita H, Okumura K, Nakano H (2007) TNF Receptor-Associated Factor 2-Dependent canonical pathway is crucial for the development of Peyer's Patches. *Journal of Immunology* **178**, 2272-2277

Pihlgren M, Tougne C, Bozzotti P, Fulurija A, Duchosal MA, Lambert PH, Siegrist CA (2003) Unresponsiveness to lymphoid-mediated signals at the neonatal follicular dendritic cell precursor level contributes to delayed germinal center induction and limitations of neonatal antibody responses to T-dependent antigens. *Journal of Immunology* **170**, 2824-2832

Pillai S, Cariappa A, Moran ST (2005) Marginal zone B cells. *Annual Review of Immunology* **23**, 161-196

Quong MW, Martensson A, Langerak AW, Rivera RR, Nemazee D, Murre C (2004) Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. *Journal of Experimental Medicine* **199**, 1101-1112

Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells *in vivo*. *Immunity* **11**, 753-761

Sadahira Y, Mori M (1999) Role of the macrophage in erythropoiesis. *Pathology International* **49**, 841-848

Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, Yamaguchi T, Yamamoto G, Seo S, Kumano K, Nakagami-Yamaguchi E, Hamada Y, Aizawa S, Hirai H (2003) Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* **18**, 675-685

Sanchez-Sanchez N, Riol-Blanco L, Rodriguez-Fernandez JL (2006) The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *Journal of Immunology* **176**, 5153-5159

Sato T, Endoh M, Yoshida H, Yasuo S, Katsuno T, Saito Y, Isono K, Koseki H (2006) Mammalian Polycomb complexes are required for Peyer's patch development by regulating lymphoid cell proliferation. *Gene* **379**, 166-174

Scheu S, Alferink J, Potzel T, Barchet W, Kalinke U, Pfeffer K (2002) Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin β in mesenteric lymph node genesis. *Journal of Experimental Medicine* **195**, 1613-1624

Shortman K, Liu YJ (2002) Mouse and human dendritic cell subtypes. *Nature Review Immunology* **2**, 151-161

Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. *Nature Review Immunology* **7**, 19-30

Sock E, Rettig SD, Enderich J, Bosl MR, Tamm ER, Wegner M (2004) Gene targeting reveals a widespread role for the high-mobility-group transcription factor *Sox11* in tissue remodeling. *Molecular and Cellular Biology* **24**, 6635-6644

Spear PG, Wang AL, Rutishauser U, Edelman GM (1973) Characterization of splenic lymphoid cells in fetal and newborn mice. *Journal of Experimental Medicine* **138**, 557-573

Steiniger B, Timphus EM, Barth PJ (2006) The splenic marginal zone in humans and rodents: an enigmatic compartment and its inhabitants. *Histochemistry and Cellular Biology* **126**, 641-648

Sun CM, Deriaud E, Leclerc C, Lo-Man R (2005) Upon TLR9 signaling, $CD5^+$ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* **22**, 467-477

Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R (2003) Ontogeny and innate properties of neonatal dendritic cells. *Blood* **102**, 585-591

Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, Mebius RE, Littman DR (2000) Requirement for RORγ in thymocyte survival and lymphoid organ development. *Science* **288**, 2369-2373

Suzuki N, Suwabe N, Ohneda O, Obara N, Imagawa S, Pan X, Motohashi H, Yamamoto M (2003) Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. *Blood* **102**, 3575-3583

Takeda K, Cretney E, Hayakawa Y, Ota, T, Akiba H, Ogasawara K, Yagita H, Kinoshita K, Okumura K, Smyth MJ (2005) TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* **105**, 2082-2089

Takeya M, Takahashi K (1992) Ontogenic development of macrophage subpopulations and Ia-positive dendritic cells in fetal and neonatal rat spleen. *Journal of Leukocyte Biology* **52**, 516-523

Tang H, Guo Z, Zhang M, Wang J, Chen G, Cao X (2006) Endothelial stroma programs hematopoietic stem cells to differentiate into regulatory dendritic cells through IL-10. *Blood* **108**, 1189-1197

Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, Suzuki A, Nakano T, Honjo T (2002) Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nature Immunology* **3**, 443-450

Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S (2005) Macrophage receptors and immune recognition. *Annual Review of Immunology* **23**, 901-944

Thiel GA, Downey H (1921) The development of the mammalian spleen with special reference to its hematopoietic activity. *American Journal of Anatomy* **28**, 279-333

Tooze J, Davies HG (1967) Light- and electron- microscope studies on the spleen of the newt *Triturus cristatus*: the fine structure of erythropoietic cells. *Journal of Cell Science* **2**, 617-640

Tribioli C, Lufkin T (1999) The murine *Bapx1* homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development* **126**, 5699-5711

Tumanov A, Kuprash D, Lagarkova M, Grivennikov S, Abe K, Shakhov A, Drutskaya L, Stewart C, Chervonsky A, Nedospasov S (2002) Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues. *Immunity* **17**, 239-250

Tumanov AV, Grivennikov SI, Shakhov AN, Rybtsov SA, Koroleva EP, Takeda J, Nedospasov SA, Kuprash DV (2003) Dissecting the role of lymphotoxin in lymphoid organs by conditional targeting. *Immunological Review* **195**, 106-116

van Rooijen N (1990) Antigen processing and presentation *in vivo*: the microenvironment as a crucial factor. *Immunology Today* **11**, 436-439

van Rooijen N, Kors N, Kraal G (1989) Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. *Journal of Leukocyte Biology* **45**, 97-104

Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S (2007) Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *Journal of Experimental Medicine* **204**, 171-180

Velardi A, Cooper MD (1984) An immunofluorescence analysis of the ontogeny of myeloid, T, and B lineage cells in mouse hemopoietic tissues. *Journal of Immunology* **133**, 672-677

Weih F, Caamano J (2003) Regulation of secondary lymphoid organ development by the nuclear factor-κB signal transduction pathway. *Immunological Review* **195**, 91-105

Wen L, Shinton SA, Hardy RR, Hayakawa K (2005) Association of B-1 B cells with follicular dendritic cells in spleen. *Journal of Immunology* **174**, 6918-692

Willimann K, Legler DF, Loetscher M, Roos RS, Delgado MB, Clark-Lewis I, Baggiolini M, Moser B (1998) The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7. *European Journal of Immunology* **28**, 2025-2034

Witmer-Pack MD, Hughes DA, Schuler G, Lawson L, McWilliam A, Inaba K, Steinman RM, Gordon S (1993) Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *Journal of Cell Science* **104**, 1021-1029

Wolber F, Leonard E, Michael S, Orschell-Traycoff C, Yoder M, Srour E (2002) Roles of spleen and liver in development of the murine hematopoietic system. *Experimental Hematology* **30**, 1010

Yanai N, Matsuya Y, Obinata M (1989) Spleen stromal cell lines selectively support erythroid colony formation. *Blood* **74**, 2391-2397

Yilmaz ZB, Weih DS, Sivakumar V, Weih F (2003) RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF. *The EMBO Journal* **22**, 121-130

Yin L, Wu L, Wesche H, Arthur CD, White JM, Goeddel DV, Schreiber RD (2001) Defective lymphotoxin-β receptor-induced NF-κB transcriptional activity in NIK-deficient mice. *Science* **291**, 2162-2165

Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P (1999) Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor *Id2*. *Nature* **397**, 702-706

Yokoyama T, Copeland NG, Jenkins NA, Montgomery CA, Elder FF, Overbeek PA (1993) Reversal of left-right asymmetry: a *situs inversus* mutation. *Science* **260**, 679-682

Yoshida H, Honda K, Shinkura R, Adachi S, Nishikawa S, Maki K, Ikuta K, Nishikawa SI (1999) IL-7Rα^+ CD3$^-$ cells in the embryonic intestine induces the organizing center of Peyer's patches. *International Immunology* **11**, 643-655

Yoshida H, Naito A, Inoue J, Satoh M, Santee-Cooper SM, Ware CF, Togawa A, Nishikawa S (2002) Different cytokines induce surface lymphotoxin-αβ on IL-7Rα cells that differentially engender lymph nodes and Peyer's patches. *Immunity* **17**, 823-833

Yu P, Wang Y, Chin RK, Martinez-Pomares L, Gordon S, Kosco-Vibois MH, Cyster J, Fu YX (2002) B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *Journal of Immunology* **168**, 5117-5123

Zhang M, Tang H, Guo Z, An H, Zhu X, Song W, Guo J, Huang X, Chen T, Wang J, Cao X (2004) Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. *Nature Immunology* **5**, 1124-1133

Zhang N, Guo J, He YW (2003) Lymphocyte accumulation in the spleen of retinoic acid receptor-related orphan receptor γ-deficient mice. *Journal of Immunology* **171**, 1667-1675