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Ontogeny and the Development of Cells Mediating Innate and Adaptive Immunity

1. ORIGIN OF IMMUNE CELL PROGENITORS DURING EMBRYOGENESIS

All white blood cells (WBCs) are derived from a common precursor: the hematopoietic stem cell (HSC). This paradigm emerged from an elegant experiment demonstrating the repopulating of all mature WBCs in lethally irradiated adult animals subsequent to infusion of a single HSC *(135)*. This experiment demonstrated that HSCs exhibit two exquisite properties: self-renewing capacity throughout life and multipotential differentiation ability.

In all vertebrates, HSCs have two origins: extraembryonic (in the yolk sac) and intraembryonic (within the embryo itself). In lower vertebrates, the yolk sac appears to be the earliest hematopoietic organ in fish such as elasmobranches *(136)*, teleocasts such as angelfish *(137)*, and reptiles *(138)*. In rainbow trout, the HSCs produced in the yolk sac during the short larval period migrate to the intermediate cellular mass (ICM), which forms from the paraaxial mesoderm during early gastrulation. Oellacher considered that the ICM represents the site of blood cell formation in most low vertebrates *(136)*.

Studies of hematopoiesis in mouse embryos suggest that hematopoiesis begins in the mesodermal compartment of the yolk sac at d 7.5 of gestation. Extraembryonic hematopoiesis in the yolk sac is defined as primitive hematopoiesis. At d 7.5 of gestation, only primitive erythropoiesis was observed, which consisted of the generation of erythrocytes with extruded nuclei and the expression of the fetal hemoglobin gene *(139)*. Cumano et al. found only myeloid precursors within the mouse yolk sac at d 8.5 of gestation *(140)*, whereas others reported the presence of B- and T-cell precursors in the yolk sac excised on d 8.5 of gestation (before that the yolk sac and embryo became connected through circulation) *(141,142)*.

Moore and Metcalf assumed that in higher vertebrates, the blood island in the yolk sac is the first hematopoietic tissue and the early site of HSC generation *(143)*. By d 12 of gestation, the HSCs migrate to the fetal liver and later to the bone marrow and spleen. The shift of primitive hematopoiesis from the

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yolk sac to the fetal liver and then to bone marrow leads to adult-type definitive hematopoiesis.

The contribution of yolk sac hematopoiesis to definitive hematopoiesis is supported by experiments that show that the HSCs present at d 9 and 10 of gestation can repopulate hematopoiesis in conditioned newborns instead of irradiated adult mice *(144)*.

However, experiments carried out in chickens have challenged the paradigm that HSCs derive solely from the yolk sac. In these experiments, cells from quail embryos were grafted into chicken yolk sacs *in ovo* before vascularization. The hematopoiesis in this system emerged from the embryo (quail and chicken cells can be easily distinguished by size of their nucleoli) *(145)*. This *in ovo* adoptive transfer experiment showed that all cells in the thymus, spleen, and bursa of Fabricius derived from quail cells *(146)*. Histological studies showed hematopoietic foci located within the dorsal mesentery of the embryo *(147)*. In vitro experiments demonstrated that HSCs isolated from the aortic wall of the chicken embryo are able to generate erythroid and myeloid colonies *(148)*. Aortic hematopoietic clusters observed in chicken embryos were identified later in many lower and higher vertebrate species.

In lower vertebrates, HSCs derived from the embryonic mesoderm occur in distinct tissues and different locations in different species. In the Antarctic teleost pronephritic kidney, HSC progenitors were observed at 1 h posthatch, whereas in the splenic analog, they were not observed until 4 wk posthatch *(149)*. In some teleocast such as zebrafish, HSCs also were identified within the ICM *(150)*. Markers of higher vertebrate HSCs such as SCL, GATA1, c-Myb, and Flo-1 were identified in zebrafish HSCs *(151)*. In rainbow trout, the ICM progressively increases up to 12 d postfertilization and disappears by d 15 *(152)*.

In the first blood islands of early larval-stage amphibian Xenopus and Rana embryos, HSCs appear at the early neural stage in the developing dorsal aorta, postcardial veins and pronephros *(153)*, the intraembryonic mesodermic region called DLP, and VP1, which represent the sites of primitive hematopoiesis. In the late larval stage, the HSCs migrate and seed lymphoid organs during metamorphosis. At this stage, HSCs express markers characteristic of higher vertebrate HSCs—CD45, SCL, and GATA-1 *(151)*.

In the mouse embryo, hematopoiesis occurs in the aorta, gonad, and mesonephros region (AGM) and its analog, the tissues derived from the para-aortic splachnopleura (p-Sp) such as the dorsal aorta, umbilical, and omphalomesenteric arteries; the hind gut; and the septum transversum. At d 10.5 of gestation, the AGM region contains about 100 HSC precursors that exhibit long-term repopulating capacity, as assessed in transfer experiments in lethally irradiated adult mice *(154)*. Cumano et al. *(140)* showed the presence of HSCs in cultures of p-Sp harvested from mouse embryos at d 9.5 of gestation and in cultures of

AGM excised from 10.5-d-old embryos. Lymphoid precursors indicative of definitive hematopoiesis also were found in cultures of p-SP from mouse embryos *(155,156)*. By d 12, the number of precursors in this region decreased, suggesting that the AGM region is not the site of hematopoiesis throughout the entire embryonic period *(156)*. Cultures of the AGM region excised from 11.5-d-old embryos in the presence of stem cell growth factor (SGF), interleukin (IL)-3, IL-6, and erythropoietin showed an increase in the total number of colony-forming units (CFUs).The addition of oncostatin M to the culture medium increased the number of mixed colony-forming cells (granulocytes, erythrocytes, macrophages, and megakaryocytes), suggesting that oncostatin M is a key element in the generation of multipotential HSC in the AGM. These multipotential HSCs may differentiate as assessed by fluorescence-activated cell-sorter (FACS) analysis of the expression of various markers for different lineages. A major fraction of cells (50–70%) expressed Mac-1 Gr-1 Th1.2, B220, Tar119, and c-Kit. This phenotypic analysis strongly suggests the differentiation of HSCs into myeloid and lymphoid common progenitors *(156)*. It is interesting to note that oncostatin M stimulated not only the expansion of HSCs but also the formation of endothelial cell clusters *(157)*.

In humans, the HSC progenitors that produce only myeloid and natural killer (NK) cells, but not lymphoid progenitors, are detected in the yolk sac as early as d 19 of the development of the embryo, 2 d before the onset of blood circulation. The origin of these HSC progenitors has not been well-defined but could be endothelial cells *(158)*.

Th extraembryonic (yolk sac) and intraembryonic origins of HSC precursors were studied by in vitro culture of the yolk sac and p-Sp and dorsal aorta excised from embryos ranging from 19 to 48 d of age. The tissues were seeded onto the MS-5 stromal cell line, and the presence of HSC precursors was analyzed after 7 d of culture. The $CD45^+CD56^+$ $CD94^+$ NK and $CD45^+$ $CD15^+$ myeloid cell progenitors were detected in the yolk sac and p-Sp cultures from 19-d-old embryos. Although the yolk sac cultures contained no lymphoid precursors, $CD45^+$ $CD19^+$ B-cell precursors were detected in cultures of p-Sp from 24- to 25-d-old embryos, and $CD4^+$ T-cell precursors were detected in p-Sp cultures from 26- to 27-d-old embryos *(158)*.

These results clearly demonstrate that in human embryos, the precursors of NK and myeloid cells derived from the yolk sac and p-Sp, whereas the precursors of lymphoid cells were of intraembryonic origin.

Studies of the generation of HSC progenitors strongly suggest that HSCs derive from mesodermal multipotential hematoblasts immediately after gastrulation in both lower and higher vertebrate species.

More recent studies suggest that the hematoblast, which is the progenitor of hematopoietic cells, and the angioblast, which is the progenitor of endothelial

cells, are derived from a common progenitor called the hematoangioblast *(157,159,160)*.

Flk1, the fms-like receptor tyrosine kinase, which is expressed on a subset of mesodermal cells *(161)*, and CD105, which is a receptor for several members of the transforming growth factor (TGF)-β superfamily *(162)*, are markers of the bipotential hematoangioblast, the progenitor of both hematopoietic and endothelial cell lineages. Mice exhibiting targeted mutations in Flk-1 die *in utero* at day 8.5 of embryonic life with defects in blood island and vasculature formation *(161)*. In vitro cultures of Flk-1^+ cells from 8.5-d-old embryos give rise to cells expressing CD144 (cadherin), which is a marker of endothelial cells, and HSCs expressing CD45 and CD24 (heat stable antigen) *(163)*.

Angiopoietin-1 via binding to Tie-1 and Tie-2 receptors probably represents the growth factor that is required for the differentiation of the hemoangioblast into the angioblast and then into endothelial cells *(164)*. GATA-2 transcription factors are required for progression of the hemoangioblast to the hematoblast stage and then to the HSC *(165)*.

The differentiation of HSCs into mature cells that mediate innate and adaptive immunity is a multistep process with branching points for the various lineages *(166)*. This multistep process leads to the differentiation of pluripotential HSCs into lineage progenitors. Sometimes these progenitors display bipotential differentiation capacity, representing genuine branch points in differentiation processes. It is probable that different transcription factors are involved in the differentiation of cell types at branching points *(167)*. Whereas the multipotentiality and self-renewal capacity of HSCs ensure the continuous generation of white and red blood cells throughout life, the differentiation of progenitors of each lineage into mature cells leads to the death of cells. This is because of the short half-life of terminally differentiated cells. Memory lymphocytes may represent an exception.

Each step of the differentiation of a given lineage does not express a specific phenotype. However, each step requires different growth factors and cytokines, as well as activation of different transcription factors and different signaling pathways. This is probably related to the activation and inhibition of the expression of different genes in each step of the differentiation process.

2. ONTOGENY OF CELLS THAT MEDIATE INNATE IMMUNITY

Myelopoiesis is the process of the development of HSCs toward granulocyte and monocyte lineages. From the yolk sac and AGM region, the multipotential HSCs migrate to the fetal liver, which becomes the predominant hematopoietic organ during embryogenesis. Adult myelopoiesis takes place in the bone marrow *(168,169)*. In the bone marrow, the common myeloid precursor gives rise to polymorphonuclear cells. The development of mature neutro-

phils, eosinophils, and mast cells is a highly regulated process during which multipotential HSCs differentiate into different lineages. The differentiation program is guided by multiple microenvironmental factors such as stromal cells, and extracellular matrix components. Growth factors and cytokines lead to the activation of transcription factors and genes that are specifically expressed in certain steps of the differentiation process.

2.1. Neutrophil Development

In mice, HCSs in the fetal liver give rise to granulocyte-monocyte (GM) progenitors that are characterized by the phenotype $CD34^+$, Kit^+, Lin^-, and $IL\text{-}7R^-$. This cell population can be subdivided into Sca^+ and Sca^-. In vitro cultures of these cells in the presence of steel factor (SLF), granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin, erythropoietin, IL-3, IL-6, and IL-7 give rise to mixed CFUs. The myelomonocytic progenitors in mixed CFU cultures begin to express Fcγ R II and III. Injection of these $CD34^+$ $Fc\gamma R^+$ cells into lethally irradiated recipients gives rise to $GR\text{-}1^+$ $Mac\text{-}1^+$ myelomonocytic cells. No B- or T-cell progenitors were detected in the cultures of $CD34^+$ $Fc\gamma R^+$ fetal cells *(170)*. These observations suggest that the differentiation potential of fetal liver granulocyte-monocyte colony-forming units (GM-CFUs) are entirely restricted to the myeloid lineage.

Similarly, in human fetal livers, the HSCs give rise to GM progenitors, which are characterized by expression of CD34 and Fcγ R II and III. CD34 is a sialo-mucin associated with the membrane of hematopoietic cells that interacts with CD62 ligand, which is a C-type lectin.

The differentiation of granulocyte progenitors is regulated by various cytokines, such as IL-3, GM-CSF, and, in particular, G-CSF, which is the major growth factor required for granulocyte production. The action of G-CSF is mediated through interaction with the G-CSF receptor. The critical role of G-CSF in neutrophil generation was demonstrated by the reduction of granulocyte precursors in G-CSF knockout mice *(171)*. The direct role of G-CSF-R in granulopoiesis comes from studies of patients with severe congenital neutropenia who exhibited a truncated cytoplasmic domain of the G-CSF-R *(172,173)*. In myeloid cells, the membrane proximal domain is essential for the transduction and differentiation signals in neutrophil progenitors. Binding of G-CSF to G-CSF-R results in rapid phosphorylation of four tyrosine residues that are involved in the recruitment of STAT3 and formation of Shc/Grb2/p140 and Grb/p90 complexes. In turn, the subsequent activation of the Syk kinases and PI3K activates Akt, which is an important factor involved in cell growth *(174)*.

The migration of granulocyte progenitors to bone morrow leads to their differentiation into mature neutrophils. The differentiation process is characterized by cytological changes, activation of early myeloid genes, and some specific transcription factors.

Neutrophil maturation proceeds from differentiation of myeloid progenitors into myeloblasts and then to promyelocytes, metamyelocytes, and, finally, neutrophils. Each cytological step of differentiation is associated with the activation of specific genes.

Early myeloid gene activation is responsible for the expression of the G-CSF-R and surface markers such as CD33 (sialo-mucin) and CD13 (metalloproteinase). Other early myeloid genes activated in the process of maturation of neutrophils include mim-1, myeloperoxidase, neutrophil elastase, myeloblastin, and lysozyme. The myelocyte stage is characterized by the expression of genes that encode for proteins contained in secondary granules such as lactoferrin and gelatinase *(174,175)*. Activation of these genes and the progression of different precursors toward neutrophil maturation are regulated by different transcription factors.

Several transcription factors are expressed and activated in HSCs:

a. c-Myb is a transcription factor that recognizes a sequence known as the Myb response element. c-Myb contains an N-terminal DNA-binding domain, a central transactivating domain, and a C-terminal region that contains an evolutionary ESVES-conserved negative regulatory domain. Its role in hematopoiesis is clearly demonstrated in studies carried out using knockout mice that lack myeloid, lymphoid, and erythroid precursors *(176)*.
b. Core binding factor (CBF) is a heterodimeric protein that contains three CBFα subunits that are bound to a CBFβ subunit. CBF is expressed in multipotential bone marrow HSCs. Animals bearing targeted mutated genes that encode both subunits lack all lineages of definitive hematopoiesis *(177)*.
c. SCL is a helix–loop–helix transcription factor that is considered a key element of the regulation of hematopoiesis required for generation of multipotent HSCs *(178)*.
d. GATA-2 is a transcription factor found only in hematopoietic cells. It mediates the differentiation of HSCs toward granulocyte differentiation by regulating C/EBPα levels *(179)*.

   Different transcription factors are required for the differentiation of granulocyte progenitors and activation of various genes during the process of maturation. Thus, c-Myb is involved not only in the growth of HSCs but also in the activation of the CD13 promoter and the activation of the myeloperoxidase gene in response to G-CSF in early myeloid cells *(180)*.
e. Homeobox proteins possess a specific helix–turn–helix DNA-binding domain and comprise a multiple-member family. HoxB4, which is one member of the Homeobox family, is expressed in the earliest HSCs. Hox A9 and Hox A10 are highly expressed in human $CD34^+$ cells; however, their expression is downregulated during hematopoiesis *(181)*.

Other transcription factors specifically regulate the expression of various genes in earlier phases of myelogenesis:

a. C/EBP is a member of a large leucine zipper 4 family. C/EBP is composed of six factors that bind to a similar consensus DNA sequence. Among them, C/EBPα is

detected in human $CD33^+$ $CD34^+$ myeloid progenitors, and its expression increases during the development of myeloid progenitors and their maturation into neutrophils *(181)*. Mice with a disrupted *C/EBPαα* gene show an early block in granulocyte differentiation. Cell lines from these mice express c-Kit but do not express Gr-1, CD34, or G-CSF-R markers. Transfection of fetal liver C/EBPα-/- cells with G-CSF or IL-6 restores the ability of these cells to generate neutrophils *(182)*.
C/EBPα plays a role in the activation of early myeloid genes. This was effectively demonstrated using the human U937 monocytoid line, which, after transfection with *C/EBPα* gene, expressed transcripts of *G-CSF-R, lactoferrin*, and *neutrophil collagenase* genes *(182)*. The expression of mim-1 also is regulated by this transcription factor *(174)*.

b. PU.1 is a member of the ETS transcription factor family that contains a conserved DNA-binding domain located in the C-terminal. PU.1 is phosphorylated on multiple serine residues by ERK1 and JNK1 kinases. PU.1 is expressed at high levels in murine myeloid progenitors and increases during granulocyte differentiation of $CD34^+$ cells in bone marrow *(181)*.
The role of this transcription factor in the maturation of neutrophils is supported by results derived from knockout mice that showed that fetal liver cells exhibit a complete defect in generating G-CFU and lack the expression of CD11b, CD18, and Gr-1 markers. PU.1 regulates the expression of *myeloperoxidase, elastase, lysozyme*, and *c-fes* genes during early phases of neutrophil development.
c. Myeloid zinc finger 1 is preferentially expressed in myeloid progenitors, and reduction of its expression prevents the formation of G-CFU *(183)*. This factor activates the CD34 promoter *(184)*.
d. Notch signaling via RBP-J Jak 3 promotes myeloid differentiation characterized by an increased number of neutrophils that express Fcγ R II and III and Gr-1 marker *(185)*.

In summary, these findings show that the differentiation of myelocyte progenitors into neutrophils is a multistep process in which different genes that encode receptors and specific markers are expressed subsequent to the activation of specific transcription factors.

2.2. Eosinophil Development

HSCs in the fetal liver and later in the bone marrow differentiate into common myeloblast progenitors (CMPs). In bone marrow, CMPs that express GM-CSF-R and G-CSF-R differentiate into monocyte and neutrophil progenitors as well as into $CD34^+$ eosinophil and/or eosinophil-basophil progenitors. In humans, eosinophil progenitors also are found in the cord blood. The events associated with the branching of CMP to the eosinophil lineage are not completely understood because, to date, no eosinophil growth factor or corresponding receptor has been discovered. Therefore, it is thought that some cytokines and transcription factors play a role in the maturation of eosinophil progenitors. Eosinophils that express Fcε R1 can differentiate in vitro from cord blood $CD45^+$ CMP in cultures supplemented with IL-3, GM-CSF, and IL-5 *(186)*.

These cells express proteins contained in eosinophilic granules such as Charcot–Lyeden crystal (16–18%), eosinophil peroxidase (7–8%), and eosinophil-derived neurotoxin (2–4%) *(187)*. Lundahl et al. *(188)* showed that the differentiation of $CD45^+$ CMP into eosinophils is associated with the upregulation of β*7-integrin* and *complement receptor* genes. The expression of EOS47, which is an early specific marker of eosinophil differentiation in the chicken hematopoietic system, is stimulated by GATA-1, c-Myb, Ets-1, and C/EBPα transcription factors. In transfection experiments, Nagny et al. *(189)* demonstrated that Ets-1 and C/EBPα proteins are physically associated with the EOS47 promoter. Overexpression of GATA-1 and GATA-2 in CMP promotes the differentiation and complete maturation of eosinophils, suggesting that these transcription factors play a crucial role in the maturation process of eosinophils *(190)*.

It is worth noting that an increased number of eosinophils were observed by addition of cysteinyl leukotrienes to cultures of bone marrow cells in the presence of GM-CSF, IL-3, and IL-4 *(191)*. However, the addition of IL-12 inhibited the differentiation of bone marrow cells cultured under the same conditions *(192)*.

Taken together, these findings suggest that some cytokines and the activation of some transcription factors direct the differentiation of eosinophil progenitors into mature eosinophils.

2.3. Development of Basophils and Mast Cells

Basophils and mast cells are derived from $CD34^+$ common myeloid progenitors. The differentiation of basophils from progenitors is independent of a defined lineage-specific growth factor. Therefore, one may argue that the differentiation of basophils and mast cells from progenitors results from a "default" pathway resulting from lack of a growth factor. This implies that other environmental factors, particularly cytokines, play an important role in the development of basophils and mast cells.

In vitro studies suggest that the basophils and mast cells represent different sublineages. Whereas basophils are derived from bone marrow $CD34^+$ HSC, the mast cells are derived from a progenitor that expresses CD34, c-Kit, and CD13 *(193)*. However, a recently prepared monoclonal antibody that is specific for CD203 binds to the progenitor of both basophilic and mast cells *(194)*. This information challenges the currently held concept that the basophil and mast cell are derived from different progenitors.

The basophils identified by a specific marker, namely, histamine decarboxylase, have been cultured in vitro subsequent to incubation of mixed, bipotential CFU progenitors (CFU eosinophil-basophil and CFU megakaryocyte-basophil) or unipotential precursors with different cytokines or growth factors *(193)*.

Several known cytokines allow the differentiation of bone marrow and human cord blood $CD45^+$ myeloid precursors into basophils:

a. IL-3 alone or together with stem cell factor or TGF-β induces the differentiation of basophils from CMP *(195)*. The IL-3 receptor CD123 is strongly expressed on basophils, and the SCF receptor CD117 is expressed on mast cells *(196)*. IL-3 functions as a basophilopoietin without evident effect on the maturation of mast cells.
b. IL-5 and eotaxin are involved in the differentiation of eosinophils and basophils from mixed CFU eosinophil-basophil progenitors *(193,197)*.
c. GM-CSF stimulates the production of histamine in monkey bone marrow cell cultures, suggesting that it may play a role in the differentiation of basophils *(193)*.

It is noteworthy that the presence of histamine decarboxylase, the synthesis of histamine, and the expression of Igε R are the most faithful markers used to identify the differentiation and the maturation of basophils and mast cells.

The subtle events involved in the differentiation of CMP into basophils and mast cells are not well understood. However, apparently the cytokine milieu, rather than specific growth factors, regulates the differentiation of common myeloid precursors into basophils and mast cells.

2.4. Development of the Monocyte-Macrophage System

The mononuclear phagocyte system, a major arm of innate immunity involved in the defense reaction against microbes and clearing of apoptotic cells, is evolutionarily conserved.

In both invertebrate and vertebrate species, the monocyte-macrophage lineage derives from the mesoderm. In insects, macrophages represent a subpopulation of blood cells called hemocytes. Like vertebrate macrophages, the hemocytes are derived from the mesoderm of the head or that associated with the aorta and heart *(198)*. In Drosophila, the hemocytes express CD36 and scavenger receptors, suggesting that they are the ancestors of vertebrate macrophages *(199)*.

In nematodes such as *Caenorhabitidis elegans*, which do not have blood cells, macrophage-like cells exhibit similar properties to fetal vertebrate macrophages and play a role in morphogenesis. Phagocytosis of apoptotic cells requires the expression of cc-7, which is homologous to murine ABC1 expressed in fetal macrophages *(200)*.

In lower vertebrates like Zebra fish, macrophages are derived from the yolk sac and, as in vertebrates, they are involved in the defense reactions against bacteria *(201)*.

In Xenopus larvae, macrophages are not derived from blood islands but from the mesoderm associated with the embryonic head located anterior to the heart *(153)*.

Study of the development of the monocyte system in mice suggests that, whereas fetal macrophages derive from the yolk sac *(202)*, the monocyte-macrophage lineage derives from the embryonic mesoderm.

Using colony-forming assays, Moore and Metcalf demonstrated that in mice, the precursors of macrophages are located in the yolk sac, which is the site of primitive hematopoiesis, on day 9 of gestation *(143)*. By d 10 of embryonic life, these precursors mature into macrophages, which leave the blood islands, enter the mesenchyme, and then migrate to various tissues via circulation. Fetal macrophages of yolk sac origin express F4/80 antigen *(203)* but are devoid of peroxidase activity, which is characteristic of adult macrophages *(204)*.

The monocyte-macrophage lineage derives from the embryonic hematoangioblast expressing Flk-1 and CD105 markers. The hemocytoblast differentiates into HSCs that migrate to the liver and give rise to common myeloblast progenitors (CMPs). Commitment of CMPs to monocyte progenitors coincides with granulocyte commitment because the bipotent GM-CFU that expresses the GM-CSF receptor can give rise to G-CFU as well as to M-CFU. In the fetal liver or later in the bone marrow, the M-CFU progenitor stimulated by G-CSF and other cytokines differentiates into monoblasts. In the bone marrow, the monoblast that expresses the M-CSF receptor (a product of c-fens protooncogene), lysozyme, and FcRγ II and III differentiate into promonocytes, which, in the blood stream, give rise to monocytes.

Mature blood monocytes express additional markers such as macrosialin, CD14, CD11b, and CD18 and possess strong phagocytic properties *(175)*.

Terminal differentiation of CMPs into monocytes is associated with the activation of Fes protein. This was demonstrated by studying the gain-of-function markers in the bipotential human U937 monocytoid line transfected with the C-fps/fes protooncogene. Transfected cells acquired macrophage cytological features and CD11b, CD11c, CD18, CD14, and M-CSFR marker expression characteristics for monocytes *(205)*. Maturation of monocytes also is associated with the ERK-MAP kinase pathway, as shown by increased phosphorylation of MEK1/2 and ERK1/2 in HL-60 cells treated with PMA *(206)*. Transcription factors regulate the expression of genes in various stages of the maturation of monocytes. Whereas some transcription factors are quite specific for the monocyte lineage, others activate genes in granulocyte progenitors. Egr-1, a member of the zinc-finger transcription family, is activated in various bipotential cell lines or bone marrow cells, which differentiate into macrophages after exposure to phorbol ester or cytokines *(181)*. Ectopic expression of Egr-1 in myeloid progenitor lines increases the expression of the M-CFU receptor *(207)*. The binding of C/EBP, PU.1, and c-Jun transcription factors to their corresponding DNA-binding motifs activates the promoter of the *GM-CSF receptor* gene and of the *lysozyme* gene that is expressed in monoblasts *(208,209)*.

It is noteworthy to point out that there are a few transcription factors that are specific for a given maturation step of the monocytic or granulocytic lineages. The majority of transcription factors are activated in CMP and in later phases

Table 13
Target Genes of Transcription Factors During Various Stages of the Development of Cells From Common Myeloid Precursors

Myeloid precursors	
Transcription factors	Target gene
C/EBP-α, PU.1	GM-CSF R
C/EBP-α, PU.1	G-CSF R
C/EBP-α, PU.1, c-Myb	M-CSF R
C/EBP-α, PU.1 c-MybL	Lysozyme
C/EBP-α, c-Myb	Min-1
C/EBP-α, PU.1, c-Myb, MZF-1	Myeloperoxidase
C/EBP-α, PU.1 CBF, c-Myb, SP-1	Neutrophil elastase
PU.1, SP-1	c-fes
C/EBP-α, PU. 1, c-Myb	Myeloblastin
C/EBP-α, PU.1, EPS	CD13
HoxA10	p21
C/EBP-α	p27
MZF-1	CD34
Mature cells	
Neutrophils	
C/EBP-α, CDP, Sp-1, MZF-1	Lactoferrin
Eosinophils	
GATA-1	Unknown
Ets-1, C/EBP-α	EOSA7 (birds)
Monocytes	
PU.1, c-Jun	Macrosialin
Egr-1	Unknown
C/ EBP-α, Sp-1	CD14
PU.1, SP-1, GABP	CD18
PU.1, Sp-1	CD11b
ICSBP	IL-12
Dendritic cells	
PU.1	CD11c

Adapted from refs. *162, 174, 175, 179, 181.*

of maturation. They can bind to the promoters of different genes. In addition, there is an extensive redundant effect of transcription factors on the activation of a single gene because DNA-binding motifs of different transcription factors can be present on the promoter of a single gene. Conversely, a single transcription factor might bind to the promoter of different genes. An example of redundancy of transcription factors is illustrated in Table 13.

Blood monocytes migrate to various tissues and mature into macrophages. In spite of the fact that tissue macrophages may display various cytological and distinct features, they express some common markers such as CD11b, IL-1β, FcγR, scavenger receptors, and CD11b, CD14, and CD18 markers.

An elegant demonstration of the capacity of mouse bone marrow monoblasts to differentiate into distinct types of macrophages was provided by Servet-Delpart et al. *(210)*. They showed that bone marrow immature monocytes expressing Flt3 ligand could differentiate in vitro into osteoclasts, microglia, or dendritic cells depending on culture conditions. For instance, bone marrow $Flt3^+$ progenitors expressing CD11b after 6 d of culture in the presence of M-CSF and RANKL or tumor necrosis factor (TNF) give rise to osteoclasts. Similarly, the progenitors that express CD11b and F4/80 antigen cultured for 11 d in the presence of M-CSF and glial-conditioned medium differentiate into microglial macrophages.

There is recent evidence that blood monocytes in humans can differentiate into dendritic cells. In initial studies, it was shown that human blood monocytes cultured in the presence of GM-CSF and IL-4 differentiated into immature dendritic cells characterized by downregulation of the expression of CD14 and acquisition of CD1a antigen *(79)*. Upon in vitro stimulation of these cells with LPS, TNF-α, IL-1, and CD40L, they exhibit the phenotype of mature dendritic cells. Randolph et al. *(211)* showed that the differentiation of blood monocytes into dendritic cells can be achieved in the absence of cytokines subsequent to culturing the monocytes on an endothelial cell layer grown on a collagen matrix. Only the monocytes transmigrating across the endothelial layer into the collagen matrix acquire dendritic cell markers such as CD83, CD86, and dendritic cell-LAMP and lose CD14 and CD64 monocyte differentiation antigen.

The growth factors, cytokines, and cellular requirements of the differentiation of monocytes in various types of tissue macrophages are not completely elucidated and are the subject of intensive research. Recently, it was shown that the activation of caspases-3 and -9, which play a crucial role in apoptosis, are activated in blood monocytes during differentiation into macrophages, a process not associated with apoptosis *(212)*.

2.5. Development of Dendritic Cells

Dendritic cells were identified in the blood and in tissues such as peripheral lymphoid organs and thymus, skin and epithelia (Langerhans cells [LC]), and other tissues (e.g., interstitial dendritic cells).

It is difficult to identify lineage ontogeny of dendritic cells in the fetus because (a) there is not a specific dendritic cell-growth factor, and, consequently, there is not a receptor for a dendritic cell-specific growth factor that can be used as a cell marker; (b) the differentiation of common monocyte pre-

cursors, from which it is supposed that dendritic cells emanate, give rise to monocyte intermediates that can not be distinguished from dendritic cells; and (c) newly discovered markers of mature dendritic cells such as DEC10 in mice or dendritic cell-LAMP in humans are not expressed on the most primitive dendritic cell progenitor, which differentiates from CMP or CLP.

Major insights into dendritic cells have come from in vitro studies because new methods aimed at characterizing the progenitor of dendritic cells have been perfected. Senju et al. developed a new method to generate dendritic cells from 10-d-old embryo murine HSCs by culturing the HSCs on feeder cell layers of OP9 cells in the presence of GM-CSF *(213)*. Seven days after culture, the occurrence of irregularly shaped floating cells possessing strong phagocytic properties and expressing class II, CD11c, CD80, and CD86 antigen has been observed. Jackson et al. studied ex vivo differentiation of murine $Scal^{+}$ Lin^{-} HPCs into dendritic cells grown in the presence of GM-CSF alone or in various combinations with IL-4 and TNF-α *(214)*. Scal+ HPC cultured for 9 d in the presence of a low dose of GM-CSF alone or in combination with IL-4 and/or TNF-α induced the differentiation of precursors into immature dendritic cells *(214)*. The cells generated in cultures exhibited the immature dendritic cell phenotype: $CD11b^{bright}$, $CD11c^{mod}$, $CD86^{low}$, class II^{low}, $DEC\ 250^{low}$, and $CD4^{-}$. The stimulation of these immature dendritic cells with LPS or CD40L resulted in dendritic cells exhibiting the mature phenotype $CD40^{high}$, secretion of IL-β and IL-12, and the ability to be strong stimulators in a mixed lymphocyte culture.

Studies of the differentiation of dendritic cells from bone marrow have advanced the hypothesis of a dual origin of dendritic cells from common myeloid and lymphoid progenitors. Several lines of experimental evidence support this concept. Inaba et al. showed that murine dendritic cells could be generated along with monocytes from cells within a single CFU after in vitro culture with GM-CSF but not with G-CSF or M-CSF *(215)*. dendritic cells generated in an ex vivo system expressed MHC class II molecules and were able to prime naïve T cells. In adoptive transfer experiments, it was shown that these dendritic cells homed to the T zone of lymphoid organs. Reid et al. demonstrated ex vivo differentiation of dendritic cells from human bone marrow bipotential $CD34^{+}$ cells and mixed CFU cultured with GM-CSF and TNF-α *(216)*. The dendritic cells recovered from the colony did not express CD14 but exhibited strong staining with anti-MHC class II, CD80, and CD86 monoclonal antibodies. Caux et al. showed that $CD34^{+}$ HSCs present in human cord blood could also differentiate into dendritic cells *(217)*.

The transcription factors NF-κB/Rel and PU.1 play an important role in the differentiation of dendritic cells of myeloid origin. Data indicating a reduced number of $CD8\alpha^{-}$ dendritic cells in mice with disrupted Rel or PU.1 genes strongly support this concept (reviewed in ref. *218*).

LCs are derived from blood dendritic cells. Strunk et al. demonstrated that $CD34^+$ progenitors expressing a skin homing receptor cultured in the presence of GM-CSF, IL-4, and TGF-β can differentiate into LCs expressing CD1a, langerin, E-cadherin, and Bierbeck granules *(219)*.

In human blood, Ito et al. identified another subset expressing CD1a and CD11c that can differentiate into LCs after culturing with GM-CSF, IL-4, and TGF-β *(86)*. The dendritic cells emanated from cultures also expressed markers characteristic of LCs such as langerin, E-cadherin, and Bierbeck granules. It is interesting to note that the development of dendritic cells from CMPs requires the activation of the Rel transcription factor. However, the LCs were present in Rel knockout mice. This observation raises interest regarding the myeloid origin of LCs *(220)*.

The concept of the lymphoid origin of a subset of dendritic cells arises from the observation that thymic, and some splenic, dendritic cells express markers of lymphoid cells such as CD8α, CD2, CD4, CD25, B220, and BP1 *(220–222)*. The concept of lymphoid origin is supported by adoptive transfer experiments of $CD4^{low}$ lymphoid precursors, which, after injection in lethally irradiate mice, gave rise to $CD8\alpha^+$ cells exhibiting a plasmoid morphology *(223)*. In mice, the precursors of plasmacytoid $CD8\alpha^+$ dendritic cells also are found in the bone marrow. These precursors can differentiate in ex vivo cultures supplemented with Flc-3 ligand as the sole growth factor *(224)*. Hochrein et al. showed that in vitro differentiated $CD8\alpha^+$ dendritic cells have the capacity to produce type I IFN *(225)*. In humans, the equivalent of murine $CD8\alpha^+$ plasmacytoid dendritic cells is characterized by the expression of IL-3R and CD68 (macrosialin); lack of expression of CD11b, CD11c, CD14, CD13, and CD33; and a high capacity to produce INF but not IL-12 *(226)*.

Human plasmacytoid dendritic cells can be generated in vitro from $CD34^+$ progenitors from fetal liver, cord blood, and bone marrow cultured with Flt-3 ligand alone or from $CD34^+$ $CD38^-$ fetal liver progenitors cultured for 7 d with murine stromal cells *(227)*. Liu et al. demonstrated that the thymus also contains plasmacytoid dendritic cells that develop from $CD34^+$ precursors *(228)*. Unlike interstitial dendritic cells of myeloid origin, the plasmacytoid dendritic cells do not require GM-CSF for their development.

In humans, $CD34^+$ HSCs that are able to generate dendritic cells are detected in the fetal liver until approx 20 wk after gestation after which they are found mainly in the bone marrow. After birth, 1–3% of cord blood cells express CD34 and, therefore, can be considered as multipotential HSCs. During the differentiation of $CD34^+$ $CD45RA^-$ progenitors into pre-dendritic cells, they gradually lose CD34 antigen and express CD4, CD45RA, IL-3R, and major histocompatibility complex (MHC) class II antigens *(228)*.

Immature dendritic cells differentiate into mature dendritic cells after stimulation by various agents. Thus, CpG and CD40 ligands induce the maturation of preplasmacytoid dendritic cells, which in turn are able to induce the differentiation of Th1 cells producing INF-γ. Stimulation with viruses induces the maturation of plasmacytoid dendritic cells that are able to activate regulatory T cells producing IFN-γ and IL-10. Stimulation with IL-3 induces the maturation of plasmacytoid dendritic cells that are able to induce the differentiation of Th2 cells producing IL-4, IL-5, and Il-10 *(228)*.

The prompt synthesis of IFNs by mature plasmacytoid dendritic cells after recognition of pathogens may represent a master function of these cells in innate immunity during development and throughout entire life. First, IFNs can activate other cells involved in the innate immune defense reactions, such as macrophages and NK cells. Second, IFNs may favor the initiation of the adaptive immune response by virtue of their pleiotropic effects on T and B cells, thereby triggering a whole spectrum of specific immune responses *(229)*.

More recently, the concept of different origins of the two subsets of dendritic cells was challenged by a report suggesting the presence in the bone marrow of a dendritic cell-progenitor that can differentiate into either $CD8\alpha^-$ or $CD8\alpha^+$ mature dendritic cells. These dendritic cell-progenitors are devoid of myeloid or lymphoid differentiation potential inducible by various growth factors *(230)*.

2.6. Development of NK Cells

NK cells are generated during fetal life from lymphoid common precursors that arise from the differentiation of HSCs. Mature NK cells originate from NK unipotential progenitors found in the fetal liver or from bipotential progenitors (NK and T cells) found in the fetal thymus. During adult life, NK progenitors are present within bone marrow.

Apparently, IL-15 secreted by stromal cells is the major growth factor directing the differentiation of NK precursors into mature NK cells. In the murine system, NK progenitors were identified in 14-d-old fetal livers. They express NK1.1 antigen, CD94 protein, and later Ly49E receptors that recognize $Qa1^b$ nonclassical MHC antigen. A significant proportion of fetal NK cells binds the Qa1 tetramer *(231)*. The expression of Ly49E receptors significantly decreases after birth. Other members of the Ly49 receptor family such as Ly49A, C/1 D, G2, and H are almost indetectable during fetal life and in the first week after birth *(232)*.

Leclercq et al. demonstrated that in fetal thymus the NK cells arose from bipotential T/NK progenitors *(233)*. These bipotential progenitors differentiate into mature lytic NK cells upon in vitro culture with IL-15. A detailed kinetic

and phenotypic analysis of clones derived from a 14-d-old fetal thymus showed that CD94 and NKG2 were expressed earlier than LY49E, which was acquired in a progressive and stochastic manner independent of the expression of CD94/NKG2 *(234)*.

In murine bone marrow, NK cells are derived from two different subsets of multipotential HSCs that express different phenotypes. The first subset, with both lymphoid and myeloid differentiating ability, expresses the phenotype Scal1$^+$, c-Kit$^+$, CD43^{high}, Ftl-3^{high}. In adoptive transfer experiments, it was shown that this cell subset gave rise to NK cells *(235)*. The second subset, which represents 1% of total bone marrow cells, displays a different phenotype: Scal2$^+$, c-Kit$^+$, CD44^{high}, HSA^{int}. This subset does not express Ly49, CD2, B220, Gr1, CD11b, NK1.1, CD4, CD8, and CD3 markers. These cells, when cultured for 5–6 d in medium containing IL-6, IL-7, SCF, and Ftl-3L and then for additional 4–5 d with IL-15, gave rise to NK1.1$^+$ cells displaying lytic activity *(235)*. These results suggest that the Scal 2$^+$ progenitors cultured in medium with a mixture of cytokines acquired the IL-15R and that IL-15 caused the terminal differentiation in mature NK cells that express the NK1.1 marker and later the Ly49 receptors. This observation suggests that the occurrence of lytic activity and the expression NK1.1 antigen precede the expression of the Ly49 receptor. In bone marrow culture, stromal cells are required for the induction of Ly49 *(236)*. In the absence of stromal cells, the progenitors dedifferentiate into NK1.1$^+$ cells that display cytotoxic abilities but lack Ly49 expression *(237)*.

In contrast to the expression of Ly49 during NK development, little is known about the expression of CD94/NKG2 during differentiation of NK from embryonic HSCs. NKG2 represents a family of genes. Whereas NKG2A associated with CD94 represents an inhibitor receptor complex, CD94/NKG2C and NKG2E are thought to be activating receptor complexes *(238)*. Lian et al. showed that there is a programmed order of the acquisition of CD94/NKG2 receptors during in vitro differentiation of embryonic HSCs into NK cells *(239)*. First, the CD94 transcript is detected in HSCs at the beginning of culture. A few days later, NKG2D transcript is detected, and at approx 8 d of culture, the transcripts of NKG2A and NKG2E are expressed. The NKG2C transcript was detected by RT-PCR at 10 d after culture. Thus, it appears that during the differentiation of NK cells, the inhibitory receptors that prevent autoagression and favor self-tolerance are expressed first, and the activating receptors are expressed later *(240)*.

In humans, CD56$^+$ NK cells develop from fetal livers, cord blood, and adult bone marrow CD45$^+$ HSCs cultured in vitro in the presence of IL-15. The differentiation of CD34$^+$, Ftl3$^+$, c-Kit$^+$ NK progenitors into CD34^{bright} IL-2R$^+$ IL-15 R$^+$ requires Ftl-3 and KL ligands that induce the expression of the IL-2 and

IL-15 receptors. These precursors in the presence of the stromal bone marrow environment or subsequent to stimulation by IL-2 and IL-15 give rise to mature NK cells characterized by the $CD56^+$, $IL\text{-}2R^+$, $IL\text{-}15R^+$ NKR^+ phenotype *(106)*. It is still not clear which factors are required for the expression of KIR receptor family.

Studies carried out both in mouse and human systems clearly demonstrated that the NK progenitors are derived from HSCs that differentiate into NK progenitors. Further, the maturation of NK precursors is dependent on cytokines, particularly IL-15 secreted by stromal cells.

Figure 26 illustrates the pathways of the differentiation of WBCs of myeloid origin.

3. ONTOGENY OF LYMPHOCYTES

Mature lymphocytes are derived from common lymphoid progenitors that arise from pluripotent HSCs, which differentiate from hematongioblasts of mesodermal origin. The commitment of HSCs to the lymphoid lineage is a stepwise process leading to irreversible differentiation into T and B lymphocytes. The commitment of HSCs to a given lineage follows a genetic program. However, the mechanisms underlying the accomplishment of the genetic program are unclear because they depend on a multitude of factors. Among these factors, one may cite (a) the cellular microenvironment, in which the expression of adhesion molecules and chemokines receptors might play a role; (b) the growth factors, which are required for the self-renewal of progenitors and their differentiation; and (c) cytokines, which may provide the activation signal or suppress the expression of genes at certain stages of the stepwise differentiation process. Because lymphopoiesis is a stepwise differentiation process, certain genes expressed in early phases can be silenced in more advanced stages of differentiation. Similarly, other genes can be activated in certain stages of irreversible commitment to a lineage. This may be related to activation and/or upregulation of certain transcription factors. The lineage commitment requires the stabilization of expression of transcription factors during the progressive differentiation genetic program of a lineage.

3.1. Development of B Cells

In the mouse, B cells are derived from HSCs of the yolk sac and the body of embryo. The HSCs migrate at d 12 to the fetal liver, which contains 1600–2000 antibody-forming precursors. These precursors migrate to bone marrow at d 15, which then becomes the major central organ in which the precursors mature. Such B cells exhibit cytodifferentiation antigen, including surface IgM and IgD, and the rearrangement of V genes that encode the specificity of the B-cell receptor (BCR). Early studies of bone marrow precursors of B cells showed

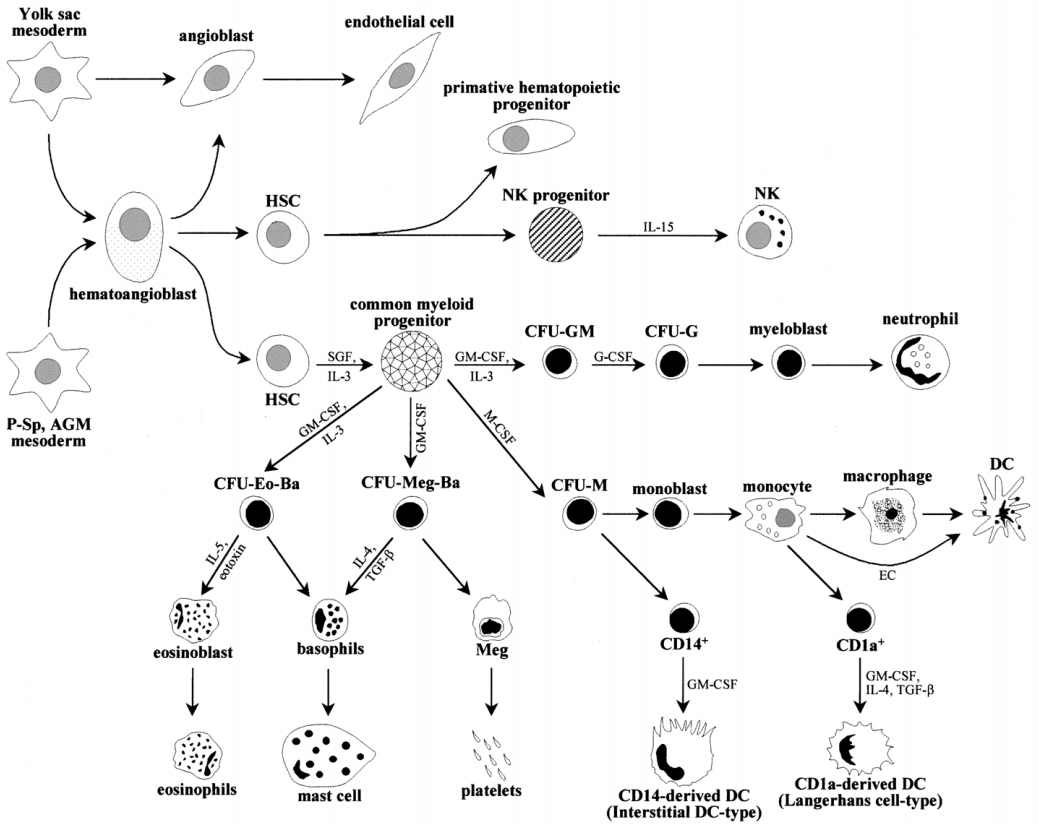

Fig. 26. Development of hematopoietic lineage during ontogeny, leading to generation of cells involved in innate immunity.

that some of them are devoid of the surface Ig receptor and express the μ heavy chain in the cytoplasm and that the V genes are not rearranged. This type of cell, called a pre-B cell, is considered to represent one element of the stepwise maturation process of B-cell progenitor development into mature B cells *(241)*.

Extensive studies led to the proposal of a unified model of B lymphopoiesis in which each step of differentiation process is characterized by the expression of various cell markers, Ig genes, and some specific transcription factors *(242)*.

The B-lymphocyte development model consists of several stages: pro-B cells derived from a stem cell lymphoid progenitor, pre B-1 cells represented by large cells that exhibit dividing capacity, leading to pre B-2 small cells, which differentiate into immature B cells and finally into mature B cells.

This model of B lymphopoiesis originates mainly from studies carried out in mice and humans and is consistent with the comparative phylogenetic studies of B-cell development in vertebrate species with species-specific differences related to the duration of pregnancy and the appearance of lymphoid organs during fetal life.

In lower vertebrate species, the tissues harboring early B-cell progenitors are still not well defined.

From a phylogenetic point of view, the elasmobranchs, which are cartilaginous fish such as sharks and skates, are the earliest species having a lymphoid system as defined by the presence of V genes that encode the BCR. During embryonic life, the kidney could be the initial site for the development of B-cell progenitors *(243)*. Later, when the embryo grows (5–10 cm long), renal lymphopoietic capacity declines and B-cell development switches to other tissues that may be considered to be an equivalent of bone marrow, i.e., Leyding organ, intestinal spinal valve, and spleen.

In the Aleutian skate (*Bathyreja aleutica*), cells expressing Ig were found in the embryonic spleen. Their number increases during embryonic development *(244)*. In another cartilaginous fish species, *Raja eglanteria*, the transcripts for IgM and IgX were found abundantly in 8-wk-old embryos in the spleen, Leyding organ, liver, gonad, and thymus *(245)*.

It is interesting to note that in these species, *in situ* chromosomal hybridization using Ig probes identified multiple IgX and IgM loci, suggesting that the *V* genes encoding Ig might be located on various chromosomes.

In teleosts, the kidney pronephros and mesonephros are the major sources of B cells. The presence of pre-B cells in the kidney is supported by the identification of non-rearranged multiple IgL transcripts in the pronephros of Atlantic cod and rainbow trout *(246)* and the high expression of Ikaros and RAG genes involved in the development and rearrangement of V genes in B cells *(247, 248)*. The differentiation of B-cell progenitors in the pronephros occurs later in ontogeny *(249,250)*. The origin of B-cell progenitors in the pronephros is

unknown. Thompson et al. hypothesized that in zebrafish, the c-Myb$^+$ progenitors of the dorsal aorta move to the kidney *(251)*.

In Atlantic cod, the head of the kidney and the spleen appear as the first lymphoid organs at the time of hatching *(252)*. In catfish, the first B lymphocytes are observed in renal hematopoietic tissue and later in the spleen between d 7 and 14 after hatching *(253)*.

From these observations, several conclusions might be drawn: (a) In fish, pre-B cells and IgM- and IgX-bearing B cells are present during embryonic life; (b) the kidney appears to be the source of hematopoiesis and of B-cell progenitors; and (c) during embryonic development, the Leyding organ, liver, and gonad may be the site of B lymphopoiesis, which eventually may be considered the equivalent of bone marrow in higher vertebrates.

In amphibia, the larvae hatch 2 d after fertilization. The stem cells, lymphopoietic progenitors migrating from the lateral plate of the mesoderm, are detected in the liver and thymus during the first week of development. Larval B-cell development can be divided into two stages. The first stage starts at d 4 when RAG transcripts and B-cell precursors are detected, whereas by d 8, most IgM$^+$ B cells are pre-B cells lacking a rearranged light chain *(254)*.

The second phase extends from d 12 to 50, corresponding to the end of metamorphosis. Near the end of metamorphosis, the number of pre-B cells in the liver and spleen decline, and B lymphopoiesis switches to the bone marrow *(255)*.

Studies of the ontogeny of B cells in reptiles (e.g., Chalcides ocellatus lizard) indicate that the embryonic liver is the site for the differentiation of B cells *(256)* and that by d 40–41, 40–50% of spleen cells express cytoplasmic and surface Ig *(257)*.

In contrast to cold, lower vertebrate species, the avian species have a lymphoid organ called the bursa of Fabricius, which is the equivalent of bone marrow in mammalian species. The bursa of Fabricius has two major functions: (a) expansion of B-cell progenitors, and (b) the diversification of the antibody repertoire. The role of the bursa was elegantly demonstrated by Cooper et al. *(258)*, who showed that a bursectomy carried out on a 17-d-old embryo results in an agammaglobulinemic chicken. The bursa develops from the hindgut at day 4 of embryonic life from the endodermal bud surrounded by the mesoderm *(259)*. Using two monoclonal antibodies specific for stromal cells, Olah et al. showed that mesenchymal stromal cells migrate to the surface of the bursal epithelium around d 12 of embryonic life, preceding follicular formation *(260)*. Some of the stromal cells remain at the luminal surface and others migrate to the medulla. The stromal cells play an important role in the maturation of B-cell progenitors after interaction with HSCs.

The ontogeny of avian B cells can be divided into three phases. In the extrabursal stage, B-cell progenitors are derived from the intraembryonic mes-

enchyme *(261)*. This stage exists only during embryonic life and ceases after hatching. The most primitive B-cell progenitors are detectable in the yolk sac by d 5–6 and express only a D-J rearrangement characteristic for pre-B cells *(262)*. Complete VDJ rearrangement in the extrabursal progenitors is virtually complete by 15 d after incubation *(263)*. In birds, B-cell progenitors expressing Bu-1 antigen seem to be irreversibly committed to the B-lymphoid lineage prior to migration to the bursa *(264)*. The bursal stage is characterized by migration to and seeding of B-cell progenitors in the bursa, where the maturation of the precursors takes place after interaction with bursal stromal cells *(266)*. The postbursal stage begins during the late phase of embryonic life and continues at hatching when mature B cells (in the case of ducklings) migrate to the liver, bone marrow, and then lymph nodes and Harderian glands *(265)*. The colonization of peripheral organs takes place 10–17 d after hatching *(266)*.

Thus, the major differences between the ontogeny of avian B cells and those of the lower vertebrates and mammals consists of the generation of B cells from a single set of precursors during embryonic life and the maturation of B-cell progenitors in the bursa from which they colonize the peripheral lymphoid organs.

Murine mature B cells are divided into two subsets: (a) B-1 expressing CD5, which produces mainly IgM-polyreactive antibodies; and (b) the B-2 $CD5^-$ subset, which produces antibodies specific for foreign antigen and pathogenic autoantibodies *(107,109)*. Both subsets are derived from common lymphoid HSCs.

In mice, pluripotential HSCs move from splanchnopleura to the fetal liver at d 12 of gestation. The fetal liver and the spleen represent the major sites of B lymphopoiesis during embryonic life, which continue until the first week after birth. The B cells arise from a common lymphoid precursor population that expresses $Sca1^{low}$ $c\text{-}Kit^{low}$ and IL-7Rα^+ and is identified at day14 in the fetal liver. These precursors are able to reconstitute all lymphoid lineages when injected into sublethally irradiated newborn mice. After culture on a stromal cell layer, in a medium supplemented with IL-7, they differentiate into immature $CD19^+$ IgM^+ B cells *(267)*.

The differentiation of B lymphocytes from multipotential progenitors is a stepwise process characterized by the expression of certain antigen and rearrangement of *V* genes. Pro-B cells that are characterized by the new phenotype $B220^+$ $c\text{-}Kit^+$, $CD43^-$ TdT^- and the V pre-B receptor (encoded by the genes *VpreB1*, *VpreB2*, and λ5) arise from the progenitors committed to differentiation into the B lineage. The ligand for this receptor, as well as its function, is poorly defined. However, it plays an important role in furthering the differentiation process because *VpreB1*, *VpreB2*, and λ5 triple-deficient mice show impaired B-cell development *(268)*. Pro-B cells differentiate into pre-B-1

exhibiting the phenotype CD43$^+$, B220$^+$, c-Kit$^+$ VpreB$^+$ and the rearrangement of D and J segments of V genes. Both RAG1 and RAG2, which mediate the recombination of V germline gene segments, are expressed in pro-B cells. In contrast to fetal liver pre-B1 cells, the pre-B1 cells in bone marrow do not express TdT, which may explain why the fetal liver B cells lack N-addition in *V* genes *(269)*. The pre-B1 cells differentiate further into pre-B2, which are large dividing cells that exhibit complete VDJ gene rearrangement and express cytoplasmic μ-heavy chain. The RAG activity strongly decreases in pre-B1 cells in the next step, and pre-B2 small cells begin to rearrange the genes that encode k and λ-light chains, leading to the expression of surface IgM. In this stage, the RAG activity is again increased and the expression of CD43 antigen is lost. These cells differentiate into immature B cells, which are characterized by a B200$^+$, sIgM$^+$ phenotype. *(269)*.

Little is known about the fetal differentiation pathway of CD5$^+$ B1 cells. Clarke et al. proposed that the B1 phenotype results from the upregulation of CD5 on mature B2 cells during self-antigen-driven expansion *(270)*. The process of development of B2 cells is viewed as a transition from CD23$^+$ B200^{high} IgM^{low} IgD^{high} CD5$^-$ to CD5$^+$ B220^{low} IgM$^+$. Hayakawa and Hardy consider that signaling via the B-cell receptor may play a role in the differentiation of the B1 subset *(271)*.

In rabbits, B-cell lymphopoiesis begins in the fetal liver and switches to the bone marrow late in fetal life and continues in the bone marrow throughout life. Pre-B cells are detected in the bone marrow beginning at d 14 of gestation in the fetal liver, reaching a peak at d 17–19, and disappearing at day 10 after birth. In fetal bone marrow, pre-B cells are detected at d 25. Pre-B2 small cells are detected in the fetal spleen at d 29 of gestation (data reviewed in ref. *272*). Previously, we showed that an extract from *Nocardia opaca* (NWSM) is a polyclonal stimulator of rabbit B cells just as LPS is a polyclonal stimulator of murine B cells *(273)*.

We found that B cells from fetal liver harvested at d 17–29 of gestation proliferate upon stimulation with NWSM and, at d 22, proliferate subsequent to stimulation with antiallotype antibodies. At birth (1-d-old pups), although the NWSM and antiallotype proliferative responses disappeared in fetal liver cells, a significant increase in NWSM and antiallotype responses was observed in spleen cells. Whereas B cells from the fetal liver harvested at d 17 of gestation lack the ability to synthesize Ig, Ig synthesis was observed at d 22 and gradually increased until d 29. In 1-d-old pups, whereas the liver cells were no longer able to synthesize Ig, the spleen cells from newborn rabbits exhibited the ability to spontaneously synthesize Ig, a process that was considerably enhanced after stimulation with NWSM *(274)*. Pre-B cells also were identified in rabbit fetal and postnatal omentum *(275)*. Apparently, at birth, the B cells are

able to function in an immune response. This concept is supported by an experiment where the transfer of newborn lymphocytes into an adult rabbit was able to respond to a challenge with Shigella antigen *(276)*. In rabbits, the first peripheral organs colonized with B cells after birth are gut-associated lymphoid organs, such as the appendix, Peyer's patches, and sacculus rotundus, which is an organ found at ileal-cecal junction *(272)*.

In pigs, the first pre-B cells exhibiting VDJ rearrangement appear at d 29 of gestation in the yolk sac and at d 30 in the fetal liver, which, thereafter, is the major site of B-cell lymphopoiesis *(277)*.

B-cell development in the lamb is particularly interesting because the immune system matures rapidly during gestation, and the lamb can actually develop an antibody response after fetal immunization. Hematopoiesis occurs in the yolk sac at d 19–27 of the 145-d gestation period. The first B1 and B2 cells were detected in the spleen at approx d 81 and 48 of gestation, respectively *(278,279)*. The maximum proliferation of IgM^+ cells in the spleen was prominent between d 60 and 70. Little information exists concerning the origin of pre-B cells during lamb gestation. If pre-B cells exist, they likely have a very short life because of the rapid differentiation into IgM^+ B cells. Similar to pigs, the gut-associated lymphoid system is the first organ colonized with B cells after birth *(279)*.

There is little information on nonhuman primate B-cell development. A recent study carried out on Rhesus monkey fetuses demonstrated that the first IgM^+ B cells were identified at d 65 of gestation (the term of gestation in Rhesus monkey is 165 d). The $CD20^+$ $CD5^+$ B cells expressing class II molecules appear in large numbers in the spleen, mesenteric lymph nodes, and small intestine at d 65 of gestation, and their number increases until birth. The most likely origin of $CD5^+$ B cells in monkeys is the omentum and fetal liver. By d 145, $CD20^+$ $CD5^-$ B2 cells become predominant in the B-cell follicles in various organs. They express not only surface IgM but also IgG and IgA *(280)*. These observations indicate that immune competence in monkeys may be achieved in the last trimester of fetal life, permitting the occurrence of an immune response soon after birth.

The human immune system, including B cells, is fully developed at birth. Throughout gestation, more than 90% of B cells in the fetal liver and spleen are $CD5^+$. The origin of the precursors of fetal $CD5^+$ B cells is not well defined, but it is believed to be the yolk sac, omentum, and fetal liver *(281)*.

HSCs, from which B cells arise, are present in the fetal liver, cord blood, and later in bone marrow. Apparently, the $CD45^+$ that express B-cell progenitors are retained in the fetal liver and bone marrow until differentiation into IgM^+/CD79 cells. Data showing that $CD34^+$ B-cell precursors are undetectable in cord blood support this concept *(282)*. However, another study that found B

cells that express CD34 and CD10 or CD19 in the blood challenged this concept *(283)*. The differentiation of B cells from $CD34^+$ B-cell progenitors was studied in vivo in severe combined immunodeficiency (SCID) mice infused with human fetal cells and in vitro in stromal-dependent culture systems detailing development of HSC from cord blood or bone marrow.

In these systems, it was shown that in humans, similarly to mice, B-cell development is a genetically programmed stepwise process characterized by (a) the expression of regulatory genes; (b) occurrence and disappearance of certain membrane antigen; (c) somatic rearrangements of germline genes containing the information for the specificity of Ig receptor; and (d) signaling molecules associated with the BCR, such as Ig β-B29, Ig α-mb-1, and B-lymphoid tyrosine kinase (Blk). The prevailing concept of human B-cell differentiation consists of progressive changes to the genetic program during the differentiation of $CD35^+$ multipotent HSCs that represent the most primitive HSCs from which $CD34^+$, $CD38^+$ B-cell progenitors are derived *(284)*. From these progenitors arise pre-pro-B cells expressing CD10 and CD19. Pro-B cells express a surrogate light chain receptor encoded by two very homologous *V-pre-B* genes and the λ*5* gene. Pro-B cells do not exhibit any rearrangement of V_H and V_L genes but have the enzymes that mediate the recombination of V-gene segments such as RAG1 and RAG2. Progression to the pre-B1 stage is associated first with the somatic rearrangement of D-J gene segments of the V heavy chain gene and with the expression of CD29 and mb-1 required for the transduction signals. In the next stage of the development, the large dividing pre-B1 cells bear an in-frame productive VDJ μ chain and maintain the expression of pseudo λ, CD10, and CD19. However, the pre-B1 cells downregulate the expression of CD34, RAG, and TdT enzymes *(284–286)*. In the pre-B2 small cells, the CD34 gene is completely silenced and the rearrangement of light chain genes is completed, allowing for the expression of IgM-CD79 complexes on the surface of immature B cells *(287)*.

As we have seen, in both lower and higher vertebrate species, B-cell development is a stepwise process composed of various stages, which are irreversible after the beginning of the differentiation process. This process is characterized by the activation of certain genes expressed in all the stages of maturation, the activation of new genes or silencing of other genes in certain stages, and the activation in early stages of enzymes that mediate the rearrangement of V genes and a large number of proteins composing pre-B- and B-cell receptors (IgL pre-B λ5, mb1, B29, Blk). All of these processes follow a genetic program controlled by some genes, which are specifically required for B-cell development, and by transcription factors involved in transcriptional control of the promoters of various genes expressed or silenced during B-cell development.

The expression of some genes such as *Ikaros*, and some transcription factors such as E47, E12, EBF, BSAP, PAX-5, and PU.1 are required for the survival of HSC-common lymphoid precursors and are critical for B-cell differentiation.

The critical role of *Ikaros* gene expression in early B-cell development in mice was demonstrated by two distinct Ikaros-targeted mutations. These mice lack not only B cells but also T cells, NKs, and dendritic cells *(288,289)*. The *Ikaros* gene encodes a zinc-finger transcription factor that acts as a master regulator of the development of the B lineage *(288)*. A mutation that deletes the N-terminal zinc-finger DNA-binding domain blocks B-cell development in early stages *(288)*. The Ikaros protein has two C-terminal zinc-finger domains that interact with four N-terminal zinc-finger domains. The homo- or heterodimerization of Ikaros proteins with DNA-binding activity requires the interaction of at least three zinc-finger domains *(290)*. A second strain of mice with Ikaros-targeted mutations was obtained by making different deletions of the C-terminal domain that prevent dimerization. These mice lack both fetal B1 and adult B2 cells. The differentiation of B cells in these mice is blocked at the level of fetal and postnatal HSCs *(289)*. A third strain was obtained via homologous recombination by introducing an in-frame β galactosidase sequence into exon 2 of the *Ikaros* gene. These mice lack B cells in the fetal liver and display a marked reduction of pre- and pro-B cells but also have B1 cells ($B220^+$, $CD5^+$ IgM^+, and Mac-1^+) in the peritoneal cavity *(291)*. B cells from these mice exhibit a defect in IL-7-dependent proliferation and the progression of IgM^- to IgM^+ B cells. Taken together, the results strongly suggest that the *Ikaros* gene is required for optimum proliferation and differentiation of B cells.

High expression of *Ikaros* was observed in the pronephros and mesonephros of Atlantic cod and rainbow trout *(292)*. In these species, the kidney is the major source of B cells. The expression of Helios, another member of the Ikaros family, was detected during the neural stage in 15-d-old embryos of Mexican axolotl, and both Helios and Ikaros transcripts were detected in the ventral area containing HSCs at d 38. This observation indicates that during lymphoid development of Mexican axolotl, the expression of Helios precedes the expression of Ikaros *(293)*.

Ikaros genes exert a positive and negative regulatory effect on various genes expressed during multiple stages of the differentiation of B cells. Thus, it was shown that Ikaros binds to regulatory elements in the promoters of TdT and λ5. *Ikaros* also binds to the 5'-upstream regulatory region of the pre-*VB1* and pre-VB2 genes *(294)* and to the TATA-less promoter of B28 *(295)*.

This information strongly suggests that the Ikaros gene family plays a critical role in the development of lymphoid precursors and of pro- and pre-B cells in lower and higher vertebrate species.

Other transcription factors, including E2A and EBF, also play an important role in the early stages of B-cell development. The *E2A* gene encodes two factors, E12 and E47, which arise through alternative splicing. *E2A* belongs to the basic helix–loop–helix family of transcription factors, which is not B-cell specific *(296)*. However, E2A knockout mice exhibit a complete block at the pro-B-cell stage because of a lack of expression of RAG1 and RAG2 and the structural components of the pre-B receptor *(297)*. Binding sites for these factors were identified on the promoter of various genes—on the111bp promoter of the *RAG1* gene, which can bind to E47 and E12, and on the λ5 TATA-less promoter/enhancer, which may bind to E47 *(297)*.

Early differentiation B factor (EBF) is another transcription factor that exhibits a broad regulatory effect on the expression of various genes during B-cell development. Mice bearing EBF-targeted mutations exhibit a blockage at the pro-B-cell stage *(298)*. EBF is a B-cell restricted factor that binds as a homodimer via a large binding domain motif containing a zinc-coordination element *(299)*. EBF binding motifs were identified in the upstream segment of the λ5 promoter, 5'-upstream region of V-pre-B1 and V-pre-B2 promoters, and the promoter of B29 and mb1 *(300)*. Recently, the promoter of human V pre-B was cloned and sequenced. It shows 56% homology with its mouse counterpart and has three binding sites for EBF. The ectopic expression of the human V-pre-B promoter in HeLa cells induces the activity of a reporter gene under the control of V-pre-B promoter. This effect is enhanced by E47, suggesting a synergistic effect between EBF and E47 on V-pre-B-promoter activity *(300)*. In human B cells, E47 also controls the expression of the *mb-1* and *B29* genes that encode proteins required for the signaling pathways induced by BCE *(300)*.

B-cell-specific activator protein (BSAP) transcription factor plays a role in the regulation of antigen expressed on the membrane of B cells in various stages of differentiation. Thus, BSAP may be involved in the expression of CD19 because a promoter fragment of 280bp of *CD19* gene contains a binding site for BSPA and Ets *(301)*. Similarly, the promoter of the *CD72* gene contains a segment located between –162 to –196, which has putative BSPA and PU.1 binding sites *(302)*. In contrast, the expression of CD20, which is a pre-B-cell specific factor, may be regulated by PU.1 because the 280bp promoter of this gene contains binding sites for the PU.1 and Oct transcription factors *(303,304)*. Finally, the 5'-upstream region of the promoter of CD22 that is associated with the BCR contains binding sites for BSAP and NF-κB *(305)*.

PAX5 is another essential transcription factor for B-lineage commitment. The absence of PAX5 arrests B-cell development at the pre-B and immature B-cell stages *(306)*. In these mice, pro-B cells express CD43, c-Kit, HSA, and IL-7R but fail to express CD25 and BP-1 required for the expression of a surrogate pre-B receptor. In addition, they do not exhibit V (D) J rearrangements. Stud-

ies in PAX-5-deficient embryos showed that the fetal liver B-cell development is blocked at the pro-B-cell stage. It noteworthy that HSCs from PAX 5-deficient mice retained a broad lymphomyeloid differentiation capacity *(307,308)*. During the late phase of the maturation of B cells, PAX-5 is involved in the expression of CD19 and the switching of IgM-producing cells to IgE-producing cells. DNA-binding motifs for PAX-5 were identified in the promoter of CD19 *(308)* and the promoter of IgE *(309)*.

PAX-5 and EBF are expressed in prebursal committed B-cell progenitors prior to colonization of the embryonic bursa of Fabricius *(310)*. These observations demonstrate that PAX-5 and EBF play an important role in the differentiation process of the B-cell lineage in various vertebrate species.

Table 14 summarizes the effect of various transcription factors on genes during B-cell development.

B-cell development is a stepwise differentiation process in which some genes are activated whereas others are silenced in various stages. Both the activation and silencing of genes is genetically regulated by genes that encode transcription factors specific for B lineage and others that exhibit pleiomorphic effects. A certain degree of redundancy among the transcription factors exists because the DNA-binding motifs for a given transcription factor might be shared by various genes expressed at multiple stages of the differentiation of B cells. Table 14 illustrates the role of various transcription factors in the expression of various genes during B-cell development.

3.2. Thymus Organogenesis and Embryonic Development of α/β T Cells

In all vertebrate species, T cells arise from the common lymphoid progenitor (CLP), which can be located in different tissues in different species. The Ikaros gene family plays a crucial role in the differentiation of CLPs. This concept is supported by a seminal observation showing that Ikaros knockout mice lack T cells as well as other cells derived from CLPs such as B cells, NKs, and dendritic cells. The CLPs migrate to the thymus, where T lymphocytes mature and differentiate. The generation of T cells is under the strict control of nonlymphocytic thymic cells comprised mainly of epithelial and mesenchymal cells. The thymus gland is derived from endoderm and associated mesodermal tissue of the pharyngeal pouches and the ectoderm of the brancheal clefts. The ontogeny of the thymus gland is best studied in higher vertebrate species. Thymic organogenesis can be divided into five sequential stages: (a) formation of the thymic primordium; (b) colonization by CLPs, which migrate from various tissues in different species; (c) proliferation of thymocytes; (d) location of thymocytes in different zones of the thymus; and (e) apoptosis of thymocytes as the result of negative selection and migration of mature T cells to peripheral lymphoid organs.

Table 14
Transcription Factors Involved in the Development of B-Cell Lineage

A. Transcription factors required for transition in various stages of differentiation

Transition	Transcription factor
HSCs->common lymphoid precursor	Ikaros, PU.1, AML-1, NFkb-RelA, Myb, GATA-2, E2A SCl-Tal
Common lymphocyte progenitor-> pro-B	Ikaros, E2a,EBF
Pro-B-> pre-B	Ikaros, EBF
Pre-B-> immature B cells	BSAP, Sox-4

B. DNA binding motif for transcription factor identified on the promoter of various genes present in B cells in various stages of development

Promoter of gene	Transcription factors
IgH	Oct-2, OCA-B, NFIL-6
Igκ	EBF, OCA-B
λ5	Ikaros, BSAP, EBF, E47, PU.1, elf-1, ets-1
Vpre-B	Ikaros, E47, BSAP, EBF
mb1	PAX-5, EBF, BSAP, elf-1, ets-1
B29	Ikaros, EBF, Oct, elf-1, ets-1
RAG	Ikaros, PU.1, NF-Y/CBf, E47, E12, elf-
TdT	Ikaros, E47, E12, elf-1
BLK	BSAP, NFκB
CD19	PAX-4, BSAP, E47
CD20	PU.1, Oct
CD22	BSAP, NFkB
CD72	BSAP, PU.1

In mice, the epithelial cells in the thymus primordium are formed at d 10.5 of gestation from the pharyngeal endoderm of the third pharyngeal pouch and neural crest mesoderm *(311–313)*.

Early studies suggested that thymic epithelial cells of the cortex are derived from ectodermal stem cells, whereas those from the medulla are formed from the endoderm of third pharyngeal pouch *(313)*. Gill et al. provided direct evidence for thymic progenitor cells, giving rise to both medullary and cortical epithelial cells *(314)*. These authors generated the monoclonal antibody MTS24, which detects a mucin-like glycoprotein on the surface of epithelial cell progenitors. These cells are characterized by their capacity for self-renewal and differentiation potential into other lineages. The $MTS24^+$ cells from thymi of 15.5-d-old embryos coexpress cytokeratins-5 and -8, which are markers of adult cortical and medullary thymic epithelial cells. Ectopic grafts of purified

MTS24$^+$ epithelial cells can differentiate into cortical and medullary epithelial cells. The epithelial compartment in the thymus is unique, because it cannot be classified as stratified layers as can the skin. Based on the expression of keratin genes, a genetic marker of epithelial cell progenitors was divided into two subsets. The subset K8$^+$K18$^+$ K5$^-$ is located in the cortex; and the subset K5$^+$, K14$^+$ is restricted to the medulla *(315)*. The epithelial cell progenitors were competent and sufficient to support the development and maturation of T cells within the thymus.

In the thymus, the T cells exhibit a major expansion phase from days 11 to 15 of embryonic life. At this stage, the undifferentiated T cells (pro-T cells) localized in the cortex do not express CD3, CD4, or CD8 (triple-negative). Later, they express Pgp-1, Thy1, CD5, and CD25 (IL-2R). At d 13 of ontogeny, CD4$^-$CD8$^-$ cells (double-negative) begin to rearrange the genes that encode the TCR, a process preceded by the activation of *RAG* genes. In mouse thymocytes, T cell receptor (*TCR*) genes are in the germline configuration until d 14, when the first rearrangements of V γ and δ are detectable and the *CD3* gene is transcribed. The TCR heterodimer, which consists of V γ/δ and CD3, can be detected on the surface of cortical thymocytes at days 14–15. The Vβ and Vα gene transcripts are detectable at ds 16 and 17 *(316,317)* and of fetal life, respectively. TCR$^-$ or TCR$^-$ low double-negative thymocytes move from the cortex to the medulla at ds 17 and 18 and express both CD4 and CD8 (double-positive). Between d 18 and birth (d 21), they differentiate into two major mature subsets: TCR^{high} CD4$^+$ CD8$^-$ and TCR^{high} CD4$^-$ CD8$^+$ T cells, which emigrate from the thymus and colonize peripheral lymphoid organs.

In fish, the thymus is the first organ to become a lymphoid, followed by the kidney and the spleen. However, there are distinct organogenesis patterns of thymus development between marine and river fish species.

In trout embryo, a thymic primordium is evident between d 5 and 8 prehatching *(318,319)*. It appears as a thickened area of pharyngeal epithelial cells containing few T cells *(314)*. At hatching, the thymus consists of a few layers of epithelial cells, and at d 1 prehatching, the lymphocytes expressing Ikaros and RAG proteins become predominant *(320)*. Complete rearrangement of the *TCR-V2* gene and the presence of TdT are detected at d 15 postfertilization, a time that coincides with the occurrence of a thymic analog *(320)*. In trout embryos, the T cell is probably derived from the yolk sac and ventral aorta.

In zebrafish, a thymic primordium lacking lymphocytes and consisting of two layers of epithelial cells is observed as early as 60 h postfertilization. The first lymphoid cells in the thymic analog were observed at 65 h postfertilization, and at d 4 postfertilization, the number of lymphocytes increased considerably *(321)*. It noteworthy that in zebrafish the thymus becomes colonized with lym-

phocytes before the pronephros becomes a hematopoietic organ. It is likely that the T lymphocytes in zebrafish derived from HSCs located in the ICM and dorsal aorta *(320)* that express the *Ikaros* and *RAG* transcripts *(331)*.

In *Xenopus*, the thymus primordium is evident at d 3 postfertilization, and 1 d later, it is seeded with lymphoid cells. At this embryonic stage, the lymphocytes express RAG, and 1 d later, *TCR* genes are rearranged. Sometime during d 4–12 postfertilization, a second seeding takes place, which extends into the juvenile and adult periods *(322)*. The T-cell progenitors derive from fetal liver colonized by HSCs from VBI and DLP mesodermal tissues proper to embryo *(323)*. After the first wave of colonization of the thymus with lymphoid progenitors, 20% of thymocytes express XTLA-1 T-cell-specific antigen, and at 12 d postfertilization, 65% express CD8 and the vast majority expresses CD5 antigen *(324)*.

There is little information of thymus organogenesis in reptiles. El Deeb and Zada, using an anti-T-cell antibody, reported the presence of T cells in 40- to 41-d-old embryos *(258)*.

Similar to amphibians, the thymus primordium in birds is colonized by several waves during embryonic life. T cells derive from HSCs beginning on d 4 of embryogenesis in the para-aortic mesenchymal tissue *(325)*. The first and second waves of colonization of the thymus with HSCs occurs at d 6 and 7 of embryonic life, respectively *(326,327)*. In birds, the rearrangement of V genes that encode the TCR occurs exclusively in the thymus. Whereas *Vb1* gene rearrangements were observed at d 12 in thymocytes, the Vb2 rearrangements were observed at d 14 of embryonic life *(328)*.

In pigs, as in birds, the thymus primordium, which appears at the end of the first trimester of intrauterine life (d 38 of gestation), is sequentially colonized by T-cell progenitors that express CD45 antigen. The T-cell progenitors do not express CD3, CD4, or CD8 (triple-negative). The differentiation of thymocytes expressing the α/β TCR occurs progressively from $CD3^-$, $CD4^+$, $CD8^+$ during the first 10 d of embryonic life. At d 55 of embryonic life, the T cells are fully mature and express CD25 *(329)*.

In humans, the thymus develops from the third brachial pouch at about 6 wk of gestation *(330)*. The pathway of the differentiation T-cell progenitors in the human thymus was studied by various methods that consisted of phenotypic analysis of the expression of various membrane markers and transcription of genes that encoded the TCR in thymus samples harvested from fetuses, the infusion of fetal liver, thymus, cord blood cells in NOD-SCID mice, and the growth and differentiation of cells in fetal thymus organ culture on thymic stromal cells. The most primitive T-cell progenitor expresses high levels of CD34 and CD45RA and lacks expression of CD38, CD2, and CD5 *(331,332)*. Important phenotypic changes occurring during the early stages of $CD34^+$

$CD38^-$ fetal liver cells in fetal organ cultures showed that they differentiate into two populations: $CD4^+$ $CD3^-$ $CD7^-$ and $CD4^-$ $CD7^-$ $CD3^-$. Although the $CD4^+$ subset differentiates into dendritic cells, the other subset differentiates into $CD4^+$ $CD7^+$ $CD3^+$ cells. After 11 d of culture, they are $CD2^+$ and the expression of CD1a is upregulated. At this stage, the rearrangement of TCR genes is initiated *(332)*. In the thymus, $CD34^+$ $CD1a^+$ T cells become double positive, expressing CD4 followed by CD8α before the acquisition of CD8β. This double-positive subset is the subject of positive selection, and the cells that do not die express activation markers (CD69 followed by CD27) *(333)*. Productive rearrangements of the TCR *V*β gene were detected in $CD34^+$ $CD1a^-$ cells, whereas pre Tα and CD3 leading a functional TCR were present in double-positive T cells *(334–336)*. In vitro studies of the differentiation of $CD34^+$ cells isolated from human and Rhesus monkey cord blood cultured on thymic stromal cells showed that the differentiation of HSCs into mature T cells recapitulates T-cell in vivo ontogeny *(337)*.

3.3. Fetal Development of γ/δ T Cells

A minority of T cells in the lymphoid organs expresses an alternative TCR that is encoded by Vγ and Vδ genes. These T cells have been found in many species. The γ/δ T cells display various functions, such as cooperation with α/β T cells in the immediate hypersensitivity reaction, secretion of cytokines, Ig subclass switching, non-MHC-restricted cytotoxicity, and antimicrobial defense reactions. They accumulate at the site of infections by bacteria, viruses, and parasites and in granulomas *(338)*.

In birds, the γ/δ T cells are the first T cells generated in the thymus during ontogeny, and, in adult life, they represent about 50% of blood T lymphocytes *(339)*.

In mice, cells that express γ/δ TCR transcripts are detectable in the fetal thymus at d 14 of gestation *(340)*. The analysis of the expression of Vγ genes in the murine fetal thymus suggests the ordered and selective occurrence of distinct γ/δ T-cell subsets *(341)*. In mice, γ/δ T cells predominate in the skin, gut, lungs, and female reproductive organs.

A discrete subset expresses a TCR encoded by Vγ3 and Vδ4. In fetal life, mature $V\gamma3^{high}$ HSA^{low} T cells mature from immature thymic $V\gamma3^{low}$ HSA^{high} *(342)*. Interestingly, these cells, found in fetal thymus and in the skin of adult mice, express the Ly49E and CD94/NKG2 inhibitory receptors of NK cells *(344)*. The expression of NK inhibitory receptors correlates with the expression of CD44, 2B4, and IL-2R and the absence of CD25 *(343)*. This observation clearly shows that γ/δ T cells do not derive from a common precursor of T cells because no α/β T cells expressing inhibitory receptors of NK cells were identified in α/β T cells. Thus, the concept is strengthened by other observa-

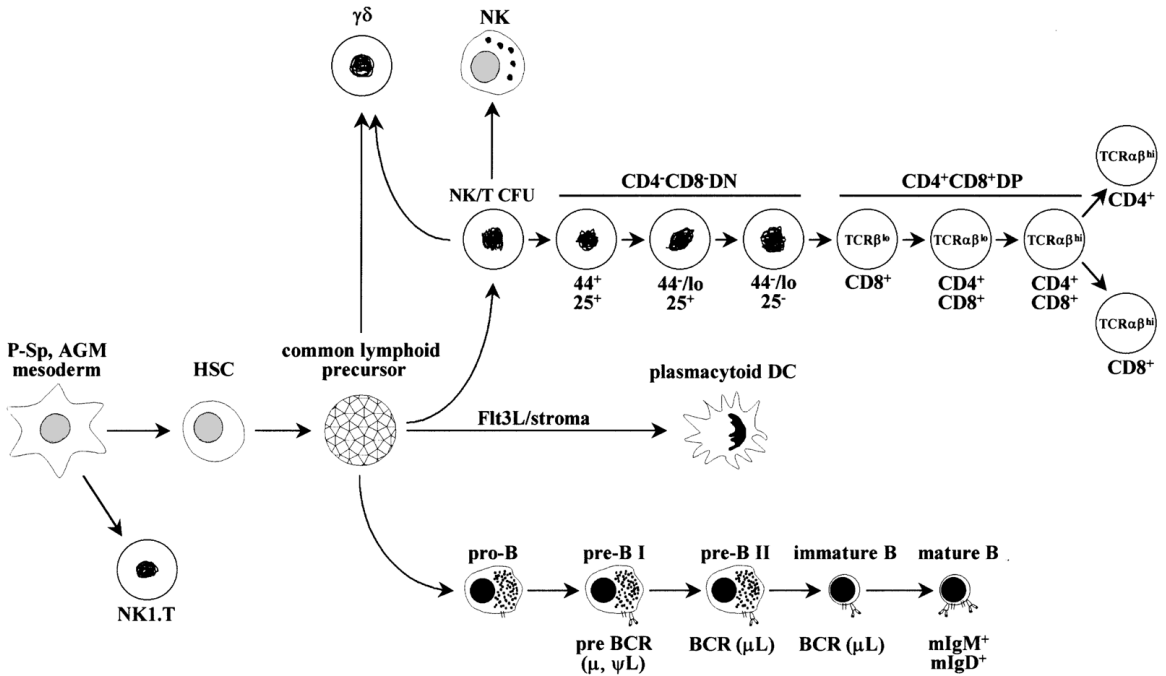

Fig. 27. Development of lymphoid lineage during ontogeny, leading to the generation of B and T cells and plasmatoid dendritic cells.

tions that demonstrate that γ/δ T cells develop normally in α/β TCR and β2-microglobulin knockout mice *(345)*. The γ/δ T cells might develop extrathymically because, in nude mice, they constitute the majority of gut intraepithelial lymphocytes *(346)*.

Human cells expressing γ/δ TCR and CD3 transcripts were identified in fetal liver at 6–8 wk of gestation, before the occurrence of the thymus primordium *(348)*. McVay et al. found transcripts of V genes in primitive gut between 6 and 9 wk of gestation, whereas they were detected in the fetal thymus at a later stage of gestation *(347)*. This raises the hypothesis of extrathymic origin of some TCR-γ/δ TCR T cells.

Taken together, these studies provide evidence that in various vertebrate species, different fetal tissues support the development of γ/δ T cells.

3.4. Ontogenetic Development of NK T Cells

In mice, a discrete subset of T cells called NK1 T cells express α/β TCR and the NK1.1 antigen characteristic for NK cells. The TCR of NK T cells is encoded by an invariant *V*α gene, resulting from the recombination of Vα14 and Jα28 segments. The invariant *V*α gene can pair with *V*β8, *V*β7, or *V*β2 genes. These cells recognize peptide in association with CD1 molecules *(110)*. Whereas the vast majority of T cells expressing α/β TCR develop around day 15 of gestation in the thymus, NK T cells preferentially develop extrathymically. Makino et al. have found transcripts of invariant Vα14 in embryos at d 9.5 of gestation and in the yolk sac and fetal liver at d 11.5–13.5 of gestation, respectively *(349)*. Fluorescent phenotypic analysis of these cells showed that they express a TCR composed of Vα14 and Vβ8 chains and CD3. It is believed that NK T cells are derived from the AGM at d 8.5–9 of embryogenesis and migrate later to fetal liver and then to thymus around day 15 of gestation.

Figure 27 illustrates the differentiation of B, T, and plasmacytoid dendritic cells from common lymphoid progenitor.

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