# 1. INTRODUCTION

## 1.1. Hematopoiesis, general terms

Hematopoiesis is the process of producing the broad variety of blood cells which happens constantly throughout the life. It provides a mechanism of continuous damaged or aged cell replacement as well as rapid up-regulation of specific cell types in stress conditions like pathogen invasions. It is estimated that maintaining the steady state cell number requires the production of $10^{10}$ cells every hour during the life (Williams et al., 1995). This remarkable generative activity and diversity of produced cells is tightly regulated and coordinated with the current demand in the organism. Deregulations along the developmental pathway lead to various hematological diseases like anemias, immunodeficiencies or leukemias. Therefore, elucidating the mechanisms responsible for keeping this finely tuned balance is critical for understanding of both normal hematopoietic development and pathogenesis of hematopoietic diseases.

## 1.2. Hierarchy of the hematopoietic process

### 1.2.1. Hematopoietic stem cells

All blood lineages originate from one multipotent progenitor population, hematopoietic stem cells (HSC) which reside mainly in the bone marrow (Abramson et al., 1977). This population can be defined by its extensive self-renewal capacity coupled with the potential to differentiate into progenitors of all blood cell lineages. The combination of these two biological properties makes HSC population “the mother of all hematopoietic cells” and as little as one or a few of these cells are capable of repopulating the complete hematopoietic system of irradiated animals (Krause et al., 2001). Remarkably, the stem cell population which is responsible for enormous daily blood cell production is very scarce in the hematopoietic tissues (approximately 2-10 per $10^5$ marrow cells, Metcalf et al., 1999a) and moreover, mitoticaly quiescent (Ogawa et al., 1993; Morrison et al., 1994). The massive expansion of hematopoietic cells relies on the robust proliferative capacity of immediate HSC descendants.

### 1.2.2. Maturation of hematopoietic progenitor cells

The classical model of hematopoiesis delineates it as a canonical process of successive binary-choice stages which eventually lead to the development of at least eight distinct blood cell lineages (Figure 1) (Keller et al., 1985). Hematopoietic stem cells undergo series of asymmetric and symmetric divisions in order to yield committed progenitors and repopulate the stem cell compartment. Very early in the process, HSC looses the capability to self-renew but retains it multipotency. This stage is sometimes referred to as multipotent progenitor (MPP). Downstream of MPP are two developmental choices, common lymphoid progenitor (CLP), capable of giving rise to all lymphoid, but not myeloid cell types, or common myeloid progenitor (CMP), capable of giving rise to all myeloid, but not

lymphoid cell types (Kondo et al., 1997). Under normal conditions CMP and CLP do not self-renew, but proliferate to amplify the number of the specialized cell type and meet the enormous daily needs for blood cell production. With proliferation they get progressively more restricted to chosen lineages. CMP develop into bipotent granulocyte-monocyte progenitors (GMP), whose final progeny are monocytes and granulocytes, or into megakaryocyte-erythroid progenitors (MEP), which give mature thrombocytes and erythrocytes (Akashi et al., 2000). In a similar way, CLP develops to give mature B- and T-lymphocytes (Akashi et al., 1999).

The terminal cell differentiation involves the transition from bipotent progenitors into lineage-affiliated precursors, which go through several stages before finally yielding mature cells. Although most of these stages are today morphologically and phenotypicaly defined, they are for historical reasons often referred to as "colony-forming units" (CFU), based on their ability to form colonies in semisolid media (Nakahata et al., 1982a; Ogawa et al., 1982). Therefore, GMP population gives rise to granulocyte- and monocyte-colony forming units (G-CFUs and M-CFUs), but also to the mixed granulocyte/monocyte colony forming units (GM-CFUs), which produce both monocytes and granulocytes in the single colony, underlining the close developmental relatedness between these two cell types (Nakahata et al., 1982b).

The hierarchic view of the hematopoietic process, as depicted in Figure 1, risks oversimplifying of a very complex process. This linear scheme is challenged by finding of progenitors capable of producing both B-lymphoid and monocytic cells (Lacaud et al., 1998; Montecino-Rodrigues et al., 2001). Additionally, the origin of some cell types has been traced back to progenitors in both myeloid and lymphoid lineages, for example myeloid dendritic cells are produced by GMP as well as by CLP (Manz et al., 2001). There are reports of hematopoietic cell transdifferentiation into non-related lineages (Xie et al., 2004) or even non-hematopoietic cell types (Terada et al., 2002), reflecting either the non-conventional relatedness of cell types or plasticity of hematopoietic progenitor cells. However, such differentiation pathways, although possible, are extremely rare (Wagers et al., 2002). The hierarchy model of hematopoiesis presented in Figure 1 still represents the major sequence of events and therefore the useful starting point in studying hematopoiesis.

During the embryonic development, hematopoiesis first takes place in the yolk sack, then in the fetal liver and after birth, most of the mature blood cells (with the exception of late T-lymphocyte developmental stages) are produced in the bone marrow (in mice also in spleen). The progenitor cells can be isolated from the bone marrow and cultured in the presence of appropriate growth factors (reviewed in Barreda et al., 2004).

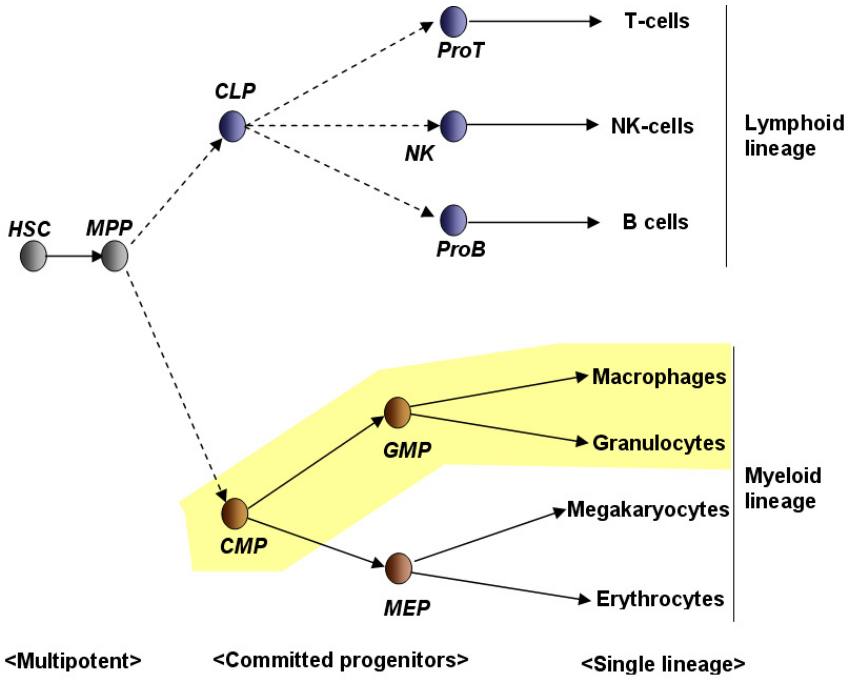


**Figure 1: Scheme of hematopoiesis** (addapted from Kondo et al, 2003)

### 1.2.3. The mature cells of the myeloid lineage

The term "myeloid cells" in its broadest sense refers to all non-lymphoid hematopoietic cells ("myeloid" means "marrow"). However, this term is often used to designate mature monocytes, granulocytes and their committed progenitors CMP and GMP (highlighted in Figure 1). In the following text, the latter definition will be used.

#### 1.2.3.1. Granulocytes

The development of mature granulocytes from bipotent GMPs comprises several stages. The earliest committed form in the granulocyte development is the myeloblast, followed by promyelocyte, myelocyte, metamyelocyte and band cell stage which finally form terminally differentiated segmented cells. A fraction of mature granulocytes is released into the blood stream, where they constitute 10-20% of all circulating leukocytes in mice. Unlike other blood cells, granulocytes form a depot of functional, mature cells in the bone marrow, which allows their rapid increase in response to infection (Nathan C, 2006).

Morphologically, granulocytes are easily recognised by their irregular, segmented nucleus (often ring-shaped in mice) and abundant cytoplasmic granules. Different staining of cytoplasmic granules enables differentiation of three morphologically and functionally distinct subtypes: neutrophils (often taken as synonymous to "granulocytes" since they are by far the most numerous type of granulocytes), eosinophils and basophils (Figure 2).

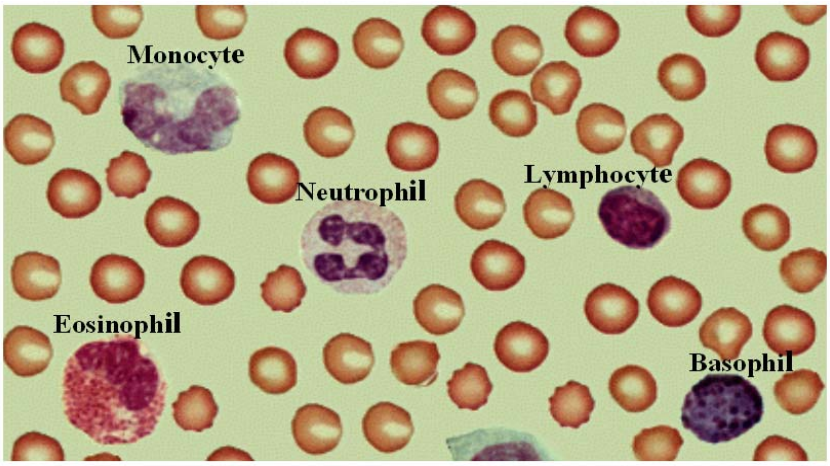


**Figure 2: Leukocytes in the peripheral blood**

Neutrophils play a central role in acute inflammatory processes. They are the first wave of immune cells invading the infection sites. Neutrophils rapidly respond to the chemotactic stimuli which lead them to the site of infection, where foreign particles, such as microbes, are recognized and phagocytozed. The process of destroying the pathogen particles is facilitated by strong bactericidal enzymes stored in the neutrophilic granules. The store of granules cannot be replenished and once it is exhausted, the cells die. Lost neutrophils are quickly replenished from the reserve population in the bone marrow. The major growth factor responsible for neutrophil development in the bone marrow is G-CSF, but additional cytokines like IL-3 and GM-CSF also contribute to expansion of the neutrophil population (reviewed in Parker et al., 2005).

Eosinophils are, like neutrophils, the first line of defense against certain types of infectious agents. The specific enzymes found in eosinophilic granules (like eosinophil peroxidase, major basic protein, arylsulphatase B, phospholipase D and histaminase) make them very efficient in defense against larger parasites. They are also abundant at the sites of immediate hypersensitivity reactions (allergy) and are considered to be involved in the pathogenesis of chronic allergic and autoimmune disorders (reviewed in Rothenberg et al., 2006). Eosinophils comprise only 1-5% of all circulating leukocytes. They are produced in the bone marrow with the help of several growth factors: IL-3, GM-CSF and IL-5. IL-5 is eosinophil specific growth factor, responsible for eosinophil proliferation and differentiation in the bone marrow, as well as their release in the circulation. (Lampinen et al., 2004).

Basophils are thought to be the circulating counterparts of the mast cells, responsible for secreting the chemical mediators of the immediate hypersensitivity reaction. Their development is governed by several growth factors, like IL-3, IL-6 and SCF (Mitre et al., 2006).

#### 1.2.3.2. Monocytes/macrophages

The development of monocytes, like that of granulocytes, starts with the common granulocyte-macrophage progenitor (GMP). The earliest morphologically recognizable cell committed to the macrophage lineage is the monoblast, which develops further into promonocyte and monocyte.

Monocytes are not completely differentiated cells, but they already posses migratory, chemotactic and phagocytic activity. They are released in the peripheral circulation where they can easily be recognized as large, round cells with abundant cytoplasm and bean-shaped nucleus (Figure 2). After engrafting in the tissues, monocytes finalize their maturation into macrophages. Major cytokine regulating development and activation of macrophages is M-CSF, but GM-CSF has largely overlaping effects with it. Activated macrophages are extremely efficient phagocytic and antigen presenting cells, central to the innate immunity and important in the antigen presentation and effector phase of the acquired immunity. Absence of the normal macrophage function leads to immunodeficiency (reviewed in Takahashi et al., 1996)

## 1.3. Regulation of hematopoiesis

### 1.3.1. Molecular basis of hematopoietic lineage commitment

The exact molecular nature of "lineage commitment" is not completely understood. Today, it is generally excepted that each distinct cell type expresses a distinct set of genes which determines its unique developmental, morphological and functional features (Cross et al., 1994). The classical model of hematopoiesis presented above suggests that these cell type specific molecular signatures are reached by orderly changes in gene expression during the development: HSC express the genes that encode its characteristic properties, like self-renewal and multipotency and as they differentiate, stem cell genes are gradually silenced and replaced with the genes encoding the properties of the selected lineage (Rosmarin et al., 2005). Nevertheless, numerous studies show that changes in gene expression patterns in hematopoiesis are not linear and that HSC and their progeny express rather unexpected combinations of lineage restricted genes (Akashi et al., 2003). This "promiscuous" gene expression does not seem to be completely random, since the genes of unrelated developmental programs (i.e. myogenic profiles) are not expressed (Hu et al., 1997). Hence, there are mechanisms that distinguish hematopoietic and non-hematopoietic directions. Rosmarin et al. describe this multilineage gene activation in immature cells as "hematopoietic identity crisis, rather than a state of global confusion" (Rosmarin et al, 2005). But if lineage restricted genes, including many crucial regulatory factors, are already activated in the stem cell, how do we define the lineage commitment on the molecular level and identify the factors that initiate this process?

Intensive debates over these questions shaped several general theories dealing with the initiation of cell commitment. On one side, there are models proposing that hematopoietic lineage determination is driven externally, through interactions with growth factors (Till et al., 1980; Metcalf et al., 1993; Skalnik et al., 2002), bone marrow stroma or other environmental influences which instruct the cell toward a particular lineage (Roberts et al., 1988; Watt et al., 2000). These instructive theories are opposed by stochastic models, which suggest that commitment is a random event decided by the accumulation of critical intrinsic factors (Nakahata et al., 1982; Ogawa et al., 1993). Recently, new models are emerging which incorporate the elements of both classical approaches and suggest a

more dynamic system where progenitor cells do not follow linear developmental pathways hierarchically organized in a hematopoietic scheme, but instead retain certain plasticity and trans-differentiation potential which change with the chromatin remodeling during each cell cycle (Colvin et al., 2004; Quesenberry et al., 2005).

Up to date, a consensus model of hematopoietic development was not reached. However, it remains clear that two types of regulatory molecules play crucial role in the regulation of this process: cytokines and transcription factors.

### 1.3.2. The role of cytokines in controlling hematopoiesis

Recombinant cytokines are today routinely used in experimental laboratories as well as in clinical praxis to stimulate the growth and maturation of hematopoietic cells or modulate their function (Table 1, additional reviews in Metcalf D., 1999b; Thomas et al., 2004).

| Cytokine | Primary effects | Therapeutic use |
|---|---|---|
| Interleukin-1 | mediates acute phase inflammatory response; knock-out mice show impaired acute phase response and resistance to fever development (Zheng et al., 1995; Alheim et al., 1998) | recombinant nonglycosilated form of IL-1Ra used in clinical trials for rheumatoid arthritis therapy (Zwerina et al., 2005) |
| Interleukin-2 | major autocrine T-cell growth factor; stimulates NK cells and B-lymphocytes; knock-out mice show polyclonal expansion of B- and T-cells and fail to eliminate autoreactive T-lymphocytes (Schörle et al., 1991) | anti-IL-2R antibodies used for organ-allograft rejection treatment; clinical trials in leukemia treatments (Waldmann TA., 2006) |
| Interleukin-3 | promotes the expansion of early progenitors of all lineages; in later stages stimulates more specifically development of eosinophils, mast cells and basophils; knock-out mice have no gross hematological abnormalities (Nishinakamura et al., 1996; Barreda et al., 2004) | clinical trials showed limited efficacy in treatment of the bone marrow failure (Kurzrock et al., 2005) |
| Interleukin-4 | proliferation and stimulation factor of Th2 lymphocytes; blocks the Th1 responses; required for IgE isotype switching; stimulates expression of adhesion molecules on vascular endothelial cells; deletion of IL-4 blocks the production of other Th2 cytokines (Kopf et al., 1993) | implications for tuberculosis vaccine design (Rook et al., 2005) |
| Interleukin-5 | major proliferation and differentiation factor for eosinophils; knock-out mice show normal basal eosinophil levels, but cannot elicit eosinophilia (Kopf et al., 1997); mice transgenic for IL-5 show constitutively high eosinophil counts (Dent et al., 1990) | anti-IL-5 antibody in clinical trials for treatment of hypereosinophilic syndromes (Sutton et al., 2005) |
| Interleukin-6 | mediates acute-phase inflammatory response; stimulates late B-cell differentiation; costimulator with other cytokines for growth of early hematopoietic precursors; knock-out mice develop normally, but show defective acute-phase response to infections (Kopf et al., 1994) | anti-IL-6 and anti IL-6R antibodies used in clinical trials for rheumatoid arthritis and Castelman's disease therapy (Zwerina et al., 2005) |
| Interleukin-7 | stimulates proliferation and differentiation of progenitors committed to B- or T-lymphocyte lineage; knock-out mice show impairment of B-lymphopoiesis and block in δγ T-cell development (von Freeden Jeffry et al.,1995) | suggested clinical applications in lymphopenia treatment and stem cell transplantation (Krawczenko et al., 2005) |

| | | |
|---|---|---|
| Interleukin-9 | Th2 type cytokine, T-lymphocytes and mast cells growth factor; promotes IgE production by B-cells; transgenic mice develop thymic lymphomas Renauld et al., 1994; Demoulin et al., 1998) | suggested clinical applications in therapy of Hodgkin and T-cell lymphoma (Knoops et al., 2004) and asthma (Zhou et al., 2001) |
| Interleukin-10 | inhibits Th1-cell and macrophage produced cytokines; knock-out mice develop chronic bowel inflammation due to uncontrolled immune responses (Kuhn et al., 1993; Moore et al., 2001) | IL-10 upregulating agents are in clinical trial for allograft rejectio and autoimmune diseases therapy (Zhou et al., 2005) |
| Interleukin-11 | stimulates megakaryopoiesis and synergizes with other cytokines in promoting early progenitors of various lineages (Du et al., 1997) | low-dose IL-11 in clinical trials in cases of bone marrow failure (Kurzrock et al., 2005) |
| Interleukin-12 | strong activator of NK cells; stimulates production of IFNγ; activates CD8+ lymphocytes to differentiate into CTLs; knock-out mice show defective IFNγ production and Th1 type responses (Magram et al., 1996) | anti-IL-12R (CD122) antibodies in clinical trial for treatment of inflammatory bowel disease (Korzenik et al., 2006) |
| Interleukin-13 | promotes Th2 cytokine production; knock-out mice fail to develop Th2-type responses and hyperreactivity reactions (McKenzie et al., 1998) | IL-13 inhibiting agents are suggested in asthma therapy (Blease et al., 2003) |
| Interleukin 15 | proliferation and differentiation of NK-, B- and T-cells; maintenance of memory T-cells; knock-out mice show marked reduction of NK and memory T-cells (Ohteki et al., 2002) | anti-IL-15 antibodies in clinical trials for treatment of autoimmune diseases (Waldmann et al., 2006) |
| Interleukin-18 | cooperates with IL-12 in inducing Th1 mediated inflammatory response; knock-out mice show reduced IFNγ production, NK- and Th1-cell activity (Takeda et al., 1998) | preclinical studies of anti-IL-18 antibodies and small inhibiting molecules in rheumatoid arthritis therapy (McInnes et al., 2005) |
| Interferon α/β | inhibits viral replication; increases lytic potential of NK-cells; increases MHC I, and reduces MHC II molecule expression (Katze et al., 2002) | recombinant IFNα/β routinely used in treatments of viral infections, malignant and autoimmune diseases |
| Interferon γ | strong activator of mononuclear phagocytes; increases MHC I and MHC II expression; promotes Th1 subset and suppresses Th2 cell subset; activates neutrophils and NK-cells (Farrar et al., 1993) | IFNγ blocking agents used in therapy of Th1-mediated autoimmune diseases (Skurkovich et al., 2005) |
| TNFα | strong activation of Th1 and macrophage cytokine production; endogenous pyrogen; stimulates the production of acute-phase proteins in liver; activates the coagulation system; inhibits bone marrow progenitor proliferation; reduces tissue perfusion (Watts T., 2005) | TNFα blocking agents used in therapy of Th1-mediated autoimmune diseases (Skurkovich et al., 2005; Feldmann et al., 2001) |
| TGFβ | inhibits T-cell proliferation and CTL maturation; causes synthesis of extracellular matrix molecules; knock-out mice develop uncontrolled inflammatory reactions (Li et al., 2006) | inhibiting agents used locally or systemically in clinical trials for treatment of various malignancies (Bierie et al., 2006) |
| Stem cell factor (c-kit ligand) | increases stem cells responsiveness to other growth factors, but does not promote growth by itself; strong synergy with G-CSF in expanding HSC and primitive progenitors; promotes mast cell development; deletion of SCF or its receptor c-kit causes perinatal lethality (Chabot et al., 1998 ; Smith et al., 2001) | used for *ex-vivo* expansion of stem cells for transplantation; clinical trials in enhancing the progenitor cell mobilisation after transplantation, along with G-CSF (Duarte et al., 2001) |

| | | |
|---|---|---|
| Flt3-ligand | like SCF, stimulates primitive progenitors in combination with other growht factors; stimulates dendritic cells; flt3 or flt3-ligand knock-out mice show deficiencies in primitive progenitor function (Mackarehtschian et al., 1995; McKenna et al., 1996) | used for *ex-vivo* stem cell exspansion; clinical trials in recovery after stem cell transplantation and in cancer immunotherapy (Wodnar-Filipowicz et al., 2003) |
| Erythropoietin (Epo) | promotes the survival, proliferation and differentiation of erythroid progenitor cells; deletion of erythropoietin or its receptor results in severe anaemia and death (1991; Wu et al., 1995) | recombinant protein or its derivates are routinely used for treatment of anaemias (Kaushansky et al., 2006) |
| Thrombopoietin (TPO) | supports the survival and proliferation of megakaryocyte progenitors; important cofactor in survival and expansion of HSC; elimination of TPO gene or its receptor strongly reduces the number of platelets and HSC (Ramsfjell et al., 1996; Sitnicka et al., 1996; de Sauvage et al., 1996) | small molecule mimetics are used in clinical trials for mild thrombocytopenia treatment (Kaushansky et al., 2006) |
| GM-CSF | promotes the proliferation and differentiation of neutrophils, macrophages and dendritic cells (in higher concentrations also eosinophils) from myeloid progenitor cells; knock-out mice of GM-CSF or its receptor have normal steady-state hematopoiesis, but reduced response in infections (Stanley et al., 1994; Zhan et al., 1998) | recombinant GM-CSF in trials for use after bone marrow transplantations, in treatment of various tumors and inflam. bowel disease (Lofts et al., 1998; Korzenik et al., 2006) |
| G-CSF | stimulates the survival and differentiation of neutrophils; mobilizes hematopoietic stem cells from bone marrow into peripheral blood; knock-out mice develop chronic neutropenia and show reduced number of GMP (Lieschke et al., 1994; Barreda et al., 2004) | routinely used to accelerate recovery after bone marrow transplantation and in treating different neutropenic conditions (Kaushansky et al., 2006; Duarte et al., 2002) |
| M-CSF | promotes the maturation of monocytes and macrophages from their committed precursors; strong activation factor of macrophages; absence of M-CSF causes severe deficiency of macrophages and osteoclasts, resulting in abnormal bone formation (Barreda et al., 2004) | recombinant M-CSF is used as consolidation therapy in AML, ovarian cancer and in treating invasive fungal infections (Motoyoshi et al., 1998; Mizutani et al., 2003; Nemunaitis J., 1998) |

**Table 1: Hematopoietic growth factors**

Decades of experimental work indisputably prove that their activity strongly affects the hematopoietic cell survival, proliferation and activation (Table 1). Cytokines are indispensable mediators coordinating the production of cells with the current demand in the organism and deregulation of their function leads to inappropriate output of corresponding cell types (Barreda et al., 2004). However, the role of cytokines in determining the lineage choice remains disputable.

Cytokines have dramatic effect on the differentiation outcome of immature, uncommitted progenitors. However, this does not regard the question whether cytokines actively switch the fate of uncommitted cells or, alternatively, just provide the survival signals for particular cell types in the randomly generated lineage progenitors, “rescuing” them from alternative lineage fates (Fairbairn et al., 1993). The experiments with ectopic cytokine receptor expression in unrelated hematopoietic cells were meant to reconcile these two possibilites. However, they led to ambiguous conclusions. On one side, M-CSF receptor expression in B-cell lineages was able to switch their development into macrophages (Borzillo et al., 1990) and GM-CSF receptor expression in committed lymphoid

precursor (CLP) was able to drive the cells toward myeloid cell fate (Iwasaki-Arai et al., 2003; Kondo et al., 2000). Still, given the possibility of close relatedness of myeloid and B-lymphoid cells (discussed in Section 1.1.1), these experiments may not reflect the true alteration of the cell fate. In accordance with this, similar experiments with more distantly related lineages failed to induce the switching. For example, erythropoetin receptor expression in macrophage precursors stimulated colony formation in response to erythropoietin, but the formed colonies consisted of macrophages, not erythroid cells (McArthur et al., Blood, 1995). In reverted experiment M-CSF stimulated erythroid colony formation (McArthur et al., 1994), indicating that external stimuli do not effect the cell fate choice.

It has been shown that cytokines and their receptors do not regulate wide enough spectrum of biological events necessary to ensure the cell development and functionality. The work of Anderson et al. (Anderson et al., 1998a; Anderson et al., 1998b) demonstrated that myeloid cell line deficient for M-CSF receptor, G-CSF receptor and transcription factor Pu.1 showed limited survival, growth and differentiation properties when only the cytokine receptors were restored. In contrast, restoration of the Pu.1 transcription factor resulted in both expression of functional cytokine receptors as well as in development of terminally differentiated and functional cells of either lineage. Therefore, Pu.1 fulfills additional requirements of hematopoietic development to those mediated by the cytokine receptors.

Another example shaking the view of cytokines as critical regulators of cell commitment is the work from Wu et al. and Lin et al. who showed the presence of early erythroid progenitors in the mice with targeted deletion of erythropoietin, i.e. erythropoietin receptor, demonstrating that neither the growth factor nor its receptor were required for the commitment to erythroid lineage (Wu et al., 1995; Lin et al., 1996). Many other knock-out mouse models of various cytokines or their receptors show deregulations in the production of corresponding cell types, but not their complete absence, indicating that cytokines rather modulate the production of cells than initiate their lineage commitment.

### 1.3.3. Transcriptional control of hematopoiesis

Regardless of what triggers the initial commitment event in the progenitor cell (external influences or intrinsic factors), it is has became evident over the years that the progenitor cells combine multiple processes like cell-cell or cell-matrix contacts, signals from growth factors, activation molecules or intrinsic modulators in order to assure the appropriate biological response (Morrison et al., 1997). The nodal point where all these signals integrate are transcription factors. They invoke the expression of maturation specific genes, growth factor receptors and often they control their own expression through positive regulatory loops, which reinforces the differentiation decision (Shivdasani et al., 1996; Barreda et al., 2001). Therefore, understanding of transcription factor function is essential to the study of differentiation.

Numerous experiments show that manipulation of transcription factor expression can direct the developmental choice of cells. For example, the expression of the erythroid transcription factor

Gata1 in hematopoietic stem cells induces the differentiation along the megakaryocyte-erythroid lineage (Aurigemma et al., 1992). Moreover, Gata1 suppresses myeloid promoting factor Pu.1 and reprograms the progenitors already committed to lymphocyte or myelocyte lineage to develop into erythrocytes (Kulessa et al., 1995; Heyworth et al., 2002). Conversely, induction of Pu.1 in multipotent progenitor cells suppresses erythroid promoting factor Gata1, suppresses erythroid lineage choice and causes irreversible myeloid differentiation (Nerlov et al., 1998; Zhang et al., 2000). Apart from supporting the idea of deterministic role of transcription factors in hematopoietic fate choice, these experiments show that the lineage commitment, on a molecular basis, depends on the repression of alternative developmental choices as much as it depends on positive action toward a particular fate. One convenient way to coordinate the synchronous up-regulation of one set of genes and down-regulation of the other is the employment of one or few pivotal transcription factors which activate genes of the chosen lineage, while repressing the factors of alternative fate choices. These pivotal factors are sometimes regarded as “master regulators”, which may not be fully appropriate. The definition of a “master regulator” implies that the transcription factor is restricted to a specific lineage and that its expression alone is sufficient to direct differentiation and repress alternative choices (Rosmarin et al., 2005). This is not the case with either Pu.1, Gata1 or any other candidate hematopoietic factor. Namely, apart from erythroid lineage, Gata1 expression is important for the development of certain myeloid cells (eosinophils and mast cells) and Pu.1 is necessary for the development of B-lymphoid cells. Additionally, the antagonism of these two factors, which is thought to impart the erythroid versus myeloid cell fate (Rekhtman et al., 1999) does not seem to be the general rule. Eosinophils simulataneously express both Gata1 and Pu.1 and certain eosinophil-specific genes are synergistically driven by these two factors (Du et al., 2002). Therefore, the lineage specific genetic programs are more likely initiated and maintained by specific combinations of transcription factors, than by a single, master regulator (Skalnik D, 2002). Indeed, the interactions with other transcription factors and differential use of co-activator proteins seems to be decisive point enabling one transcription factor to activate different genetic programs (reviewed in Cantor et al., 2001). For example, interaction of Gata1 with C/ebpβ induces the expression of the eosinophil major basic protein (Mbp) (Yamaguchi et al., 1999). On the other side, Gata1 interaction with Fog (Friend-of-Gata) is necessary for the activation of erythroid restricted genes, but inhibits Mbp expression (Yamaguchi et al., 1999) and suppresses eosinophil differentiation (Querfurth et al., 2000).

Several other mechanisms involved in the regulation of lineage specific gene expression during hematopoiesis were suggested (reviewed in Schivdasani et al., 1996; Barreda et al., 2001). For one, the accessibility of DNA sequences defines the set of available target regions and thereby directs the activation of a specific genetic program. For example, there are at least three DNAseI hypersensitive regions (HS) detected in the proximal (hematopoietic specific) promoter of Gata1 and each one of them functions as an enhancer element in different cell types. Targeted disruption of the HSI region demonstrated its requirement for the Gata1 expression in megakaryocytes (Shivdasani et

al., 1997), mast cells (Migliaccio et al., 2003) and erythroid cells (Vyas et al., 1999; Onodera et al., 1997). The deletion of the HSII, on the other side, showed that it is dispensable for erythroid or megakarycyte expression, but absolutely required for the eosinophil Gata1 expression (Yu et al., 2002). The chromatin accessibility represents the basis of the "lineage priming" theory of hematopoietic cell commitment, excepted by many authors today (suggested by Weintraub H., 1985; reviews Enver et al., 1998; Orkin S., 2003).

Another example showing the importance of *cis*-elements in the transcriptional regulation of hematopoiesis is the redirection of a B-cell line to develop into macrophages in the presence of the DNA-demethylating agent azacytidine (Boyd et al., 1982).

The complexity of transcription factor combinations which determin the cell identity is further increased by the fact that the their expression does not function like "all-or-nothing" switch. On the contrary, finely graded concentration of transcription factors seems to be crucial in the developmental fate choice. For example, the *EOS47* promoter, which induces the expression of an early eosinophil differentiation marker in the chicken hematopoiesis is activated by low, but repressed by high Gata1 concentration (McNagny et al., 1998). Similarly, multipotent hematopoietic progenitors subjected to low concentrations of Pu.1 developed into B-lymphocytes, while high Pu.1 concentrations promoted macrophage differentiation (DeKoter et al., 2000).

From the examples mentioned above, it is evident that the lineage specific gene expression and subsequent differentiation of cells are based on a complex network of coordinated regulatory processes. In order to properly exhibit their function, transcription factors require a defined set of interaction partners, "primed" critical regions of chromatin structure, finely tuned expression levels and adequate posttranscriptional and postranslational processing. The term "cellular context" is often used to describe the plethora of different influences in the intracellular environment which affect the final outcome of the developmental process.

### 1.4. The study of transcription factors in the myeloid development

The manipulation of the transcription factor expression in hematopoietic cells represents an irreplaceable laboratory tool for studying their biological function. Numerous studies exploiting over-expression or targeted deletion aproaches have been described and in most cases they showed gross defects in the cell development, like inhibition of proliferation, blocked differentiation or altered lineage commitment (some examples are given in Table 2).

| Transcription factor | Role in hematopoiesis | References |
|---|---|---|
| Pu.1 | essential for the development of both myeloid and lymphoid lineages; in contrast, down-regulation of Pu.1 is necessary for erythroid differentiation | McKercher et al., 1996; Scott et al., 1997; Scott et al., 1994; DeKoter et al., 1998 |
| C/ebpα | critical regulator of granulocyte development; knock-out mice show impaired granulocyte and eosinophil development, but other lineages are not affected | Radomska et al., 1998; Zhang et al., 1997 |

| | | |
|---|---|---|
| C/ebpβ | important in macrophage development and activation; increased during neutrophil differenationation; plays a role in B-lymphocyte development | Yamanaka et al., 1998; Hu et al., 1998; Querfurth et al., 2000 |
| C/ebpδ | up-regulated during myelopoiesis; in macrophages it regulates the expression of inflammatory cytokines | Zafarana et al., 2000 |
| C/ebpε | regulates eosinophil, neutrophil and macrophage terminal differentiation; knock-out mice show defects in eosinophil and neutrophil development | Angerer et al., 1999; Yamanaka et al., 1997 |
| Mzf-1 | induction of granulopoiesis, possibly expansion of myeloid precursors prior to terminal differentiation | Bavisotto et al., 1991; |
| Wt-1 | highly expressed in HSC, down-regulation is necessary for myeloid development | Svedberg et al., 1998 |
| Fog-1 | along with Gata1, fundamental role in the development of erythroid and megacariocytic lineage | Holmes et al., 1999; Querfurth et al., 2000 |
| HoxA5 | positively regulates myeloid differentiation, while it needs to be down-regulated to allow erythropoiesis | Fuller et al., 1999 |
| HoxA9 | important role in the development of myeloid, erythroid and B-cell progenitors | Lawrence et al., 1997 |
| HoxA10 | amplification of early hematopoietic precursors; down-regulation necessary for myeloid and B-cell differentiation | Thorsteinsdottir, 1997 |
| HoxB3 | down-regulation important for normal B- and T- lymphoid differentiation; over-expression promotes myeloid differentiation | Sauvageau et al., 1997 |
| HoxB8 | positive regulation of macrophage development and negative regulation of granulocyte development | Krishnaraju et al., 1997 |
| Egr-1 | up-regulation is associated with differentiation along macrophage lineage; negatively regulates granulocyte development; knock-out mice show unimpared hematopoiesis | Nguyen et al., 1993 Lee et al., 1996 |
| Stat1 | central role in interferon signaling; promotes macrophage differentiation | Coccia et al., 1999 |
| Icsbp | promotes differentiation of bipotent myeloid progenitors to macrophages on the expense of granulocytes | Holtscke et al., 1996; Tamura et al., 2000 |
| MafB | induction of macrophage differentiation program; repression of erythroid specific genes in myeloid cells | Sieweke et al., 1996 |
| Gata1 | key role in the development of erythrocytes and megakaryocytes; deletion leads to the block in the primitive erythropoiesis; negative regulation of myeloid lineage, but in the presence of C/ebpβ induces eosinophil development | Pevny et al., 1991 Fujiwara et al., 1996 Yamaguchi et al., 1998 |
| Gata2 | early stages of hematopoiesis; cooperates with Gata1 in stimulating erythroid and eosinophil development | Tsai et al., 1994 Weiss et al., 1995 |
| p53 | in myelopoiesis: ectopic expression induces monocytic differentiation in bipotent cell lines: knock-out mice develop normaly | Soddu et al., 1996; Lowe et al., 1993 |

**Table 2: The transcription factors involved in the control of myeloid development**
(adapted from Barreda et al., 2001)

Although providing us with strong evidence of the transcription factor function in vivo, knock-out or transgenic models are not without limitations. First, many biological systems ensure the survival of the organism by supporting the critically important processes by alternative mechanisms. Therefore, crucial parts of the system, like transcription factors or growth factors and their receptors often show functional redundancy. For example, up-regulation of the zinc-finger transcription factor Egr-1 was shown to be necessary for the terminal differentiation of macrophages (Nguyen et al., 1993). However, macrophage development in Egr-1 knock-out mice was fully uncompromised (Lee et al., 1996), suggesting the compensation by alternative pathways. Another example are the C/ebp transcription factors. Many genes harboring the CCAAT sequence in their promoter can be regulated by several proteins of this family (Hu et al., 1998), therefore, knock-out model of a single factor does not reveal all of its targets.

The additional limitation of knock-out models is that they show only the earliest block in the pathway, so the roles in later developmental stages of the cell or in cell types which exert their function later in the development might not be noted. For example, mice deficient for Gata1 die due to the block in primitive erythropoiesis (Pevny et al., 1991). The indispensable role of Gata1 in eosinophil development was first noted years later (Kulessa et al., 1995) and confirmed *in vivo* after more then a decade (Yu et al., 2002; Hirasawa et al., 2002).

Important experimental breakthrough in resolving such problems and opening new perspectives for studying hematopoietic processes was identification, isolation and characterization of hematopoietic precursors (Weissman et al., 2001). The comparative analysis of gene expression patterns in purified progenitor populations gives us the possibility to follow the developmental process step by step and analyze the hierarchy among regulatory factors (Terskikh et al., 2003).

Finally, a lot of information that we have today about the role of certain transcription factors comes from observations made in leukemia patients. Remarkably, the same factors that regulate normal hematopoietic development are found to participate in the malignant transformation of cells. One of the numerous examples is Pu.1 (Moreaugachelin et al., 1988; Rosenbauer et al., 2004). These findings once again underline the importance of regulatory processes that govern hematopoiesis.

Taken together, the regulation of hematopoiesis relies on a combinatorial effect of multiple regulatory factors. The identification of these regulatory factors and elucidating the molecular events that underlie commitment and maturation of hematopoietic cells are crucial for understanding of both normal hematopoiesis and diseases of the hematopoietic system.

### 1.4.1. Icsbp

In past years the work in our laboratory was concentrated on studying the development of myeloid cells. Identifying factors that play a role in this process and analyzing the mechanism of their action is crucial for our understanding of normal myelopoiesis. One of the factors identified by our

group as important in determining the fate of myeloid cells is Interferon Consesus Sequence Binding Protein (Icsbp, Irf-8).

Icsbp is a transcription factor belonging to the Interferon Regulatory Factor (Irf) family (Nelson et al., 1993). This family is involved in controlling the signaling of interferons, potent immune system modulators, affecting thus multiple biological processes of the immune system such as pathogen response, cytokine signaling, cell growth regulation and hematopoietic development (Taniguchi et al., 2001).

Icsbp is expressed mainly in the cells of hematopoietic system. In mature hematopoietic cells, it is found in monocytes, B lymphocytes, and activated T lymphocytes, while it is not detectable in granulocytes and resting T lymphocytes (Driggers et al., 1990; Nelson et al., 1996). Icsbp is also shown to be expressed in early multipotent progenitor cells (in lineage marker negative population: Tsujimura et al., 2002; in CD34+ population: Qian et al., 2002) and in progenitors of the myeloid lineage (Terszowski et al., 2005). At the final branching towards monocytes or granulocytes at the GMP stage, the expression of Icsbp remains high in the monocytes lineage, but commitment into granulocyte lineage is connected with Icsbp silencing (Figure 3).

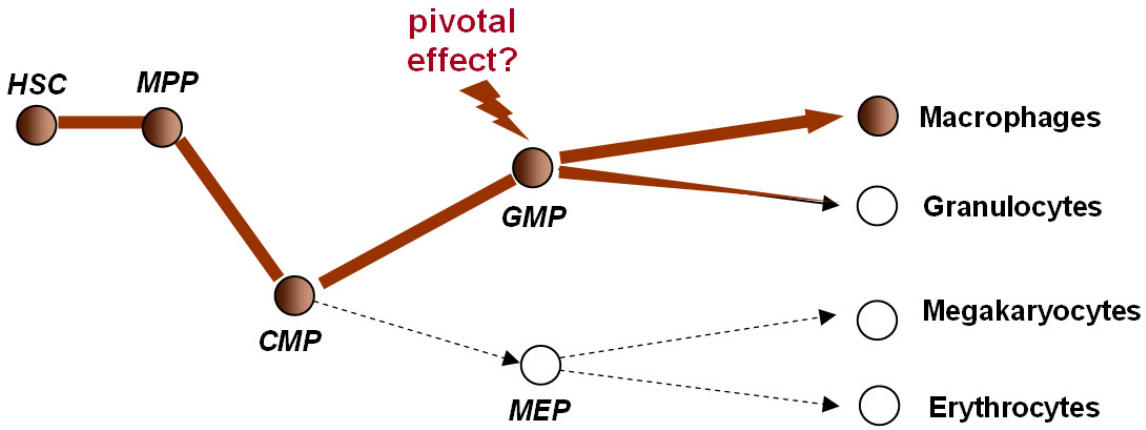


**Figure 3: Expression pattern of Icsbp in mature myeloid cells and their precursors**

#### 1.4.1.1 Mice with a targeted deletion of Icsbp

Like other members of Irf family, Icsbp is involved in the regulation of genes induced with both interferon α/β and interferon γ. Mice with a null mutation for Icsbp fail to develop a Th1-driven immune response and interferon γ-dependent host defense, which results in increased susceptibility to various pathogens, including viruses, parasites, intra- and extracellular bacteria (Holtschke et al., 1996; Fehr et al., 1997; Scharton-Kersten et al., 1997; Hein et al., 2000; Ko et al., 2002)

In addition to immunodeficiency, targeted deletion of Icsbp revealed a previously unsuspected role of this transcription factor in the development of myeloid cells. Icsbp$^{-/-}$ mice show a myelopoiesis misbalance reflected in striking increase of granulocytes, while macrophages and monocytes are decreased and functionally impaired (Holtschke et al., 1996). Those abnormalities have been traced to myeloid progenitor cells (Scheller et al., 1999) where Icsbp plays a pivotal role in deciding the fate of

progenitors, directing them towards monocytic lineage on the expense of the granulocytic lineage (Tamura et al., 2000).

Due to the enhanced production of granulocytes and their accumulation in all hematopoietic tissues, Icsbp$^{-/-}$ mice develop a syndrome similar to chronic myelogenous leukemia (CML) with 100% penetrance (Holtschke et al., 1996). This is another example showing that aberrant expression of a transcription factor involved in normal hematopoiesis leads to the hematological disease. Connection between ICSBP deregulation and human CML has also been described (Schmidt et al., 1998; Qian et al., 2002; Larson et al., 2005).

#### 1.4.1.2. Pursuing the mechanism of Icsbp action in myeloid development – global gene expression analysis

In spite of the dramatic consequences of *Icsbp* gene disruption observed in mice, the mechanism of Icsbp function in determining the fate of myeloid cells and in preventing leukemogenesis remains largely unknown. The Icsbp expression pattern shown in Figure 3 suggests that the most probable stage where Icsbp exerts its pivotal effect in myelopoiesis is the bipotent granulocyte-monocyte progenitor (GMP). In order to analyze the molecular networks that Icsbp is involved with and identify the downstream targets that mediate the effects of Icsbp, we isolated GMP cells from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice and performed comparative analysis of gene expression by Affymetrix technology. More than 1600 genes were found by this method to be differentially expressed in Icsbp$^{-/-}$ cells, among them other transcription factors like *HoxA9, Meis1a, Gata2* or *Klf4*, all of them (except *Klf4*) previously shown to play important role in hematopoiesis (Table 2). This finding, together with general principals of hematopoietic processes discussed before, suggested that the hematopoietic defects seen in Icsbp$^{-/-}$ mice are more likely caused by deregulation of multiple transcription factors, than by absence of Icsbp alone. In order to follow this hypothesis further, we decided to analyze the contribution of other transcription factor deregulations to the phenotype of Icsbp$^{-/-}$ mice.

### 1.5. Klf4

The global gene profiling of purified hematopoietic precursors performed in our laboratory identified Klf4 (Gklf, Gut-enriched Krüppel-like Factor) as strongly expressed gene in CMP and GMP population (unpublished data). Additionally, experiment performed in cooperation with G.Terszowski (Departemnt for Immunology, University of Ulm, Germany) outlined Klf4 as one of most highly differentially expressed genes in myeloid (GMP) against erythroid (EP) precursors, indicating that it plays a role specifically in myeloid cells (subsequently published in Terszowski et al., 2005). The finding that Klf4 is present in myeloid progenitors was somewhat surprising since, at the time this study began, expression of Klf4 in the hematopoietic system was not reported. This protein was previously described as epithelial transcription factor, expressed in gut, skin, vascular endothelium (Schields et al., 1996; Ton-That et al., 1997; Yet et al., 1998; Segre et al., 1999), but also in embryonic

stem cells (Li et al., 2004). It belongs to the Krüppel-like family of transcription factors, characterized by three highly conserved zinc finger motives on the C-terminus (Schields et al., 1996). Proteins of the Klf family are shown to be involved in control of critical aspects of cell differentiation and activation. For example, the founding member of this group in mammals, Klf1 (Eklf, Erythroid Krüppel-like factor) was shown to be critical for red blood cell maturation (Nuez et al., 1995). Klf2 (Lklf, Lung Krüppel-like factor) is involved in quiescence of single positive T-lymphocytes (Kuo et al., 1997).

Klf4 has been studied most thoroughly in different epithelial cell systems with respect to its involvement in the cell cycle arrest and terminal cell differentiation. Initially, *Klf4* was discovered as a gene whose expression accompanies growth arrest (Shields et al., 1996). Analysis of the Klf4 *in vivo* expression pattern showed that it is primarily found in terminally differentiated and mitotically inert epithelial cells of the intestine (Shields et al., 1996) and epidermis (Segre et al., 1999). Conversely, the expression of Klf4 is reduced at conditions that involve increased proliferation such as neoplasm of the intestinal tract (Dang et al., 2000; Ton-That et al., 1997) and in cancer cell lines (Dang et al., 2001). Constitutive expression of Klf4 results in the inhibition of DNA synthesis (Shields et al., 1996; Geiman et al., 2000), suppression of cyclin D expression (Shie et al., 2000), cyclin B1 expression (Yoon et al., 2004) and in a cell cycle arrest between G1 and S phase, which is accompanied by the activation of cell cycle inhibitor $p21^{Waf1/Cip1}$ (Dang et al., 2000; Chen et al., 2001; Yoon et al., 2003; Yoon et al., 2004).

Targeted gene deletion of *Klf4* was independently performed by two research groups and in both cases it resulted in severe defects of the terminal differentiation of epithelial and endothelial cells. Segre et al. reported that Klf4-null mice die shortly after birth due to deficient development of epithelial cells of skin and a consequent loss of its barrier function (Segre et al., 1999). Katz et al. showed the strong reduction of goblet cells in the colon, which again indicates selective perturbation of late stages of cell differentiation (Katz et al., 2000). Other reports show the involvement of Klf4 in regulating the expression of differentiation dependant genes in epithelial tissues, such as Cyp1A1 (Zhang et al., 1998), laminin 3A (Miller et al., 2001), laminin γ1 (Higaky et al., 2002), smooth muscle cell differentiation marker SM22alpha (Adam et al., 2000), ALPI, VIL2, DSG2 (desmoglein 2), and numerous genes encoding keratins (Okano et al., 2000; Brembeck et al., 2000).

The importance of Klf4 in epithelial cell differentiation and importance of its analogues in erythrocyte and T-lymphocyte function prompted us to analyze the role of Klf4 in the myelopoiesis. In the course of work on this thesis, several reports have been published describing the involvement of Klf4 in regulation of Cd11d, macrophage activation marker expression (Noti et al., 2005) and in inflammatory responses of macrophages (Feinberg et al., 2005).

The comparative analysis of gene expression profiles in $Icsbp^{-/-}$ versus $Icsbp^{+/+}$ GMPs showed strong reduction of *Klf4* expression (10 fold) in the absence of Icsbp. Since previous reports connected Klf4 with cell cycle arrest and with terminal cell differentiation, we assumed that *Klf4* down-regulation detected in $Icsbp^{-/-}$ GMPs could contribute to the excessive granulocyte proliferation and

defective macrophage maturation observed in Icsbp$^{-/-}$ mice. For this reason we have chosen to analyze whether *Klf4* represents a target gene of Icsbp and whether it could be responsible (at least in part) for Icsbp role in the myeloid development.

## 1.6. The expression of eosinophil specific genes in the Icsbp$^{-/-}$ GMP

Another finding of the comparative gene expression analysis of Icsbp$^{-/-}$ and Icsbp$^{+/+}$ GMPs was the strong down-regulation of several eosinophil specific genes in the absence of Icsbp, like eosinophil major basic protein (*Mbp*), eosinophil peroxidase (*Epx*) or eosinophil ribonucelase (*Ear1* and *Ear2*) (unpublished data). It is already documented that Icsbp$^{-/-}$ mice have decreased production of monocytes and increased production of neutrophilic granulocytes. However, there are no studies analyzing whether this misbalance in the development of myeloid cells also includes defective development of eosinophilic granulocytes. As discussed before, the knock-out models of transcription factors often reveal only the first block in the developmental pathway and additional roles of the analyzed factor could be omitted. In order to broaden the description of the Icsbp$^{-/-}$ mice phenotype and to investigate the role of Icsbp in the regulation of eosinophil development and function, we decided to analyze whether this minor cell population is deregulated in Icsbp$^{-/-}$ mice.