6. DISCUSSION

Icsbp is a transcription factor with a pivotal role in myelopoiesis, responsible for directing development of myeloid progenitors toward macrophages on the expense of granulocytes (Holtschke et al., 1996; Scheller et al., 1999; Tsujimura et al., 2002). In order to study the underlying mechanism of this switch, global gene expression array of Icsbp$^{+/+}$ versus Icsbp$^{-/-}$ granulocyte-monocyte progenitors (GMPs) was performed in our laboratory. This analysis showed that the loss of Icsbp leads to deregulation of multiple transcription factors which have important roles in hematopoiesis (like *HoxA9, Meis1a, Gata2* or *Gata1*). Therefore, it was hypothesized that the phenotype observed in Icsbp$^{-/-}$ mice could be rather a cumulative consequence of multiple transcription factor deregulation, than the lack of Icsbp alone. One of the genes identified by this analysis as strongly down-regulated (10 fold) in the lack of Icsbp was Klf4. At the time this study began, expression of Klf4 in the hematopoietic system was not reported. Klf4 was described as epithelial transcription factor, indispensable for terminal cell maturation (Segre et al., 1999; Katz et al., 2002). However, other members of Krüppel-like family, closely related to Klf4, play important role in the hematopoietic system, for example Klf1 (Eklf) is critical for red blood cell maturation (Nuez et al., 1995) and Klf2 (Lklf) for quiescence of single positive T-lymphocytes (Kuo et al., 1997). 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.

Since previous reports connected Klf4 with cell cycle arrest (Schields et al., 1996; Chen et al., 2001; Geiman et al., 2000; Yoon et al., 2005) and with terminal cell differentiation (Segre et al., 1999; Katz et al., 2002; Jaubert et al., 2003; Katz et al., 2005), it was assumed that Klf4 down-regulation detected in Icsbp$^{-/-}$ GMPs could contribute to the excessive granulocyte proliferation and defective macrophage maturation observed in Icsbp$^{-/-}$ mice. In order to test this hypothesis, early bone marrow progenitors from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were stably transduced with a retroviral vector carrying 4-OHT inducible Klf4-ER^{T2} chimeric sequence and differentiation of such modified progenitors was followed.

Icsbp transduction of bone marrow progenitors in the similar experimental setting shows that reintroduction of Icsbp completely rescues granulocyte/macrophage misbalance of Icsbp$^{-/-}$ progenitors and moreover, that over-expression of Icsbp enhances differentiation along macrophage lineage (Tsujimura et al., 2002). These experiments complement the report of defective macrophage maturation in the absence of Icsbp (Holtschke et al., 1996; Scheller et al., 1999) and prove Icsbp to be a *bona fide* macrophage promoting factor.

Klf4 over-expression in bone marrow progenitors described in this work shows that Klf4 has macrophage promoting effect as well. Percentage of macrophage colonies developing from Klf4 over-expressing progenitors was comparable to that of Icsbp over-expressing progenitors (in both cases approximately twice as high as in the cells transduced with the vector control) (Figure 5, Figure 6, Figure 7). The relative increase of macrophages was also observed when the Klf4 over-expressing

progenitors were grown with the granulocyte-promoting cytokines (Figure 5, Figure 6, Figure 7), indicating that Klf4 overrides the instructive cytokine signals and intrinsically regulates the cell differentiation program. This experiment also demonstrates that Klf4 over-expression in myeloid progenitors does not simply lead to accelerated maturation and premature cell senescence, as it was described for epithelial cell progenitors (Jaubert et al., 2003). Instead, Klf4 promotes one cell type (macrophages) and reduces the other (granulocytes), even if the extracellular signals instruct the common progenitor cells otherwise.

Additionally, Klf4 over-expression in Icsbp$^{-/-}$ progenitors, which, as mentioned, give rise to disproportional high number of granulocyte colonies, completely reverted the maturation toward macrophages (Figure 5, Figure 6, Figure 7, Figure 8), indicating that the macrophage differentiation defect caused by the lack of Icsbp can be rescued by Klf4 alone. This finding, along with the down-regulation of Klf4 in Icsbp$^{-/-}$ GMPs (unpublished data), identical expression pattern of Icsbp and Klf4 in mature immune cells (Figure 9) and their responsiveness to the same stimuli (Figure 10) opened the question whether Klf4 is a downstream target of Icsbp, responsible (at least in part) for Icsbp effects in myelopoiesis. However, uncompromised Icsbp function in Klf4$^{-/-}$ progenitors (Figure 24, Figure 25), led us to the conclusion that Icsbp effects are not mediated through Klf4, but rather that these two factors function independently from one another during macrophage development.

Even though both Icsbp and Klf4 promote macrophage differentiation, the experiments described in this work revealed an important difference in their functions.

1) Icsbp over-expression results in the absolute increase of macrophage colonies and decrease of granulocyte colonies, indicating that the role of this factor in myelopoiesis is instructive: it actively switches bipotent granulocyte-macrophage progenitors toward macrophage lineage. This was confirmed by the reciprocal phenotype of Icsbp$^{-/-}$ mice.

2) Klf4 over-expression, on the other side, does not lead to the increase in absolute number of macrophage colonies, but rather to decrease of granulocyte colonies. The interpretation of the Klf4 influence in the myeloid development is complicated by the strong cell cycle inhibitory effect of forced Klf4 expression. In general, there are two possibilities. Klf4 could either actively take part in the commitment process, inducing the macrophage differentiation on the expense of granulocytes, like Icsbp. But unlike Icsbp, Klf4 strongly inhibits the proliferation of cells which explains the decreased colony numbers. Alternatively, the role of Klf4 in myelopoiesis might not be inductive, but instead permissive, meaning that Klf4 does not affect the commitment process in the progenitor cells, but the development and/or survival of already committed cells, restricting the ones committed to the granulocyte lineage, while allowing development of monocytes and macrophages. The analysis of the gene expression changes activated early after inducing the chimerical Klf4-ER^{T2} protein in progenitor cells showed increased transcription of several macrophage specific genes and reduced transcription of granulocyte specific genes (Figure 27), indicating that Klf4 actively shifts the cell gene expression signature toward macrophage lineage. As discussed before (Introduction), phenotypical changes in the

differentiation process are preceded by the expression of a set of lineage affiliated genes. Importantly, up-regulation of the genes of the chosen fate is followed by active suppression of alternative developmental choices. In this context, it could be concluded that Klf4 actively takes part in orchestrating the expression of genes which finally results in macrophage maturation. Therefore, this study identifies Klf4 as novel macrophage promoting factor.

This result is in accordance with previously reported up-regulation of differentiation specific genes by Klf4 (Table 9). In addition to this, several studies showed that Klf4 simultaneously down-regulates several cell cycle progressing factors and up-regulates cell cycle inhibitors (Table 9), indicating that Klf4 acts as general proliferation/differentiation switch. This is confirmed by observations of defective skin (Segre et al., 1999) and colon goblet cell differentiation (Katz et al., 2002) when Klf4 is deleted, and accelerated epidermal cell maturation when Klf4 is ectopically expressed (Jaubert et al., 2003).

Klf4 activation targets		
$p21^{Waf1}$	Cell Cycle	Zhang et al., 2000; Chen et al., 2003; Rowland et al., 2005
$p57^{Kip2}$	Cell Cycle	Chen et al., 2003
Urokinase-type plasminogen activator receptor	Adhesion	Wang et al., 2004
Laminin α3a	Adhesion	Miller et al., 2001
Laminin γ1	Adhesion	Higaki et al., 2002
Intestinal alkaline phosphatase	Metabolism, Differentiation marker	Hinnebusch et al., 2004
Keratin 19	Structural, Differentiation marker	Brembeck et al., 2000
Keratin 4	Structural, Differentiation marker	Jenkins et al., 1998
Bradykinin B2 receptor	Signaling	Saifudeen et al., 2005
Epstein-Barr virus ED-L2	Viral	Jenkins et al., 1998

Klf4 repression targets		
Cyclin D1	Cell cycle	Shie et al., 2000
Cyclin B1	Cell cycle	Yoon et al., 2004
Cyclin E	Cell cycle	Yoon et al., 2005
Cdc2/CDK1	Cell Cycle	Chen et al., 2003
Wee1	Cell Cycle	Chen et al., 2003
Cd11d	Adhesion	Noti et al., 2005
Laminin α1	Adhesion	Piccinni et al., 2004
Ornithine decarboxylase	Metabolism	Chen et al., 2002
Histidine decarboxylase	Metabolism	Ai et al., 2004
Cytochrome p450 A1	Metabolism	Zhang et al., 1998
Smooth muscle protein 22α	Structural	Adam et al., 2000
α-Smooth muscle actin	Structural	Adam et al., 2000

Table 9: Target genes regulated by Klf4

Experiments described here show that Klf4 over-expression in myeloid progenitors has similar effect to that in epithelial and endothelial cells - reduced cell proliferation and consequent reduction of total colony formation. However, the reduction of granulocyte colonies was much stronger than macrophage colonies (Table 7), showing that Klf4 over-expression has different effects in these two cell types. Based on this, we can speculate that other cellular processes, in particular apoptosis, could be differentially regulated by Klf4 in granulocytes and macrophages. If Klf4 has apoptosis protective effect in macrophages and proapoptotic in granulocytes, myeloid progenitors with forced Klf4

expression would undergo apoptosis when committed to granulocyte lineage, which would result in the selection and enrichment of the monocytic progenitors and relative increase in macrophage colonies. To test this hypothesis, early phases of differentiation process had to be analyzed. However, a technical problem of analyzing early stages of development is in sorting purified cell populations due to the low expression of the lineage specific markers. Also, *in situ* staining techniques are complicated by the fact that morphology of the early progenitors of both lineages is undistinguishable. Further study of Klf4 over-expression effects, possibly in a different cell system (i.e. multipotent hematopoietic cell lines) is needed in order to completely explain the mechanism of Klf4 action in early progenitors of granulocyte and monocytes lineage.

In addition to the analysis of the Klf4 over-expression effects, the myeloid development was analyzed in Klf4$^{-/-}$ mice. Mice with a conditional deletion of Klf4 in all interferon α/β responsive tissues (100% deletion in the bone marrow progenitor cells, Figure 12), had no overt deregulation in the immune cell distribution in the hematopoietic tissues 2 months after Klf4 deletion was induced (Figure 13). The functional assay showed that the differentiation pattern of bone marrow progenitors in response to cytokines was not changed in the absence of Klf4 (Figure 14). This result, together with previous reports, suggests that the requirement for Klf4 in the cell maturation varies in different cell types. While Klf4 deletion in epithelial tissues resulted in hyperplasia (Jaubert et al., 2003) and defective terminal differentiation (Segre et al., 1999; Katz et al., 2002; Jaubert et al., 2003), myeloid cell development was not affected by the lack of Klf4. Therefore, the role of Klf4 in normal myelopoiesis is redundant and its absence can be compensated.

Alternatively, Klf4 could be employed not so much in the normal myelopoiesis, but in the activation of macrophages or in protection from stress-induced conditions which lead to carcinogenesis. Looking closer on the macrophage marker genes induced by Klf4 (Figure 27), Klf4 rapidly increased the expression of two types of macrophage scavenger receptors: *Cd36*, major internalization receptor for oxidised LDL, connected to the formation of macrophage foam cells and atherogenic lesions (Kunjathoor et al., 2002; Tsukamoto et al., 2002) and *Marco* (Macrophage receptor with collagenos structure), which is involved in bacterial clearance by macrophage mediated phagocytosis (Arredouani et al., 2004). Additionally, Klf4 increased the expression of *Ceruloplasmin*, an oxidase which is shown to be important in processing the senescent erythrocytes (Sarkar et al., 2003). Recently, Feinberg et al. reported the involvement of Klf4 in proinflammatory signaling in macrophages (Feinberg et al., 2005). Therefore, Klf4$^{-/-}$ mice could have impaired response in inflammatory conditions or atherogenesis, but these areas require further study.

One more possibility is that Klf4 is involved in the protection from the carcinogenic processes. A number of reports demonstrate the tumor-suppressive activity of Klf4. It is shown to be up-regulated in response to DNA damage in p53 dependant fashion (Zhang et al., 2000) and this induction of Klf4 is essential for mediating G1/S checkpoint function (Yoon et al., 2003). Consistent with the DNA-damage checkpoint function, the expression of KLF4 is down-regulated in different dysplastic

conditions of the intestines (Dang et al., 2000), colon (Shie et al., 2000) and stomach (Wei et al., 2005; Katz et al., 2005). Loss of heterozygosity of the KLF4 locus and hypermethylation of the KLF4 promoter were identified in human colorectal cancers (Zhao et al., 2004). Conversely, over-expression of KLF4 in a colon cancer cell line reduced the colony formation, cell migration, invasion and *in vivo* tumor formation, confirming that KLF4 acts as a tumor suppressor (Dang et al., 2003).

Seemingly in contrast with KLF4 tumor suppressing features, oncogenic activity of this protein was also reported. KLF4 is frequently over-expressed in mammary (Foster et al., 2000; Pandya et al., 2004) and squamous cell carcinoma (Foster et al., 1999; Foster et al., 2005). In the hematopoietic system, *Klf4* locus has been identified as a site of retroviral integration in B-cell lymphomas occuring in AKXD and NFS.V+ mice (Suzuki et al., 2002), which identifies this protein as oncogene rather than tumor suppressor. This switching from tumor suppressor to oncogene function (reviewed in Rowland et al., 2005) is not understood and is attributed to the "cell type specificity" (Foster et al., 1999).

To the best of our knowledge, studies about KLF4 involvement in the hematological neoplasms have not yet been performed. The experiments described in this work show that in myeloid progenitor cells full length Klf4 has a cytostatic activity. However, truncated Klf4 construct lacking its DNA binding region shows the ability to block the differentiation of myeloid progenitors (Figure 30, Figure 31, Figure 32), thus revealing the Klf4 transforming potential. It is not known whether Klf4 mutations resulting in deletion or disabling of its DNA binding region can be found in hematological diseases. However, point mutations in the Klf4 open reading frame and subsequent decrease in transcriptional activity were reported in colon cancer patients (Zhao et al., 2004). Based on the literature data and on Klf4 effects discussed above, we could speculate that functional Klf4 protein has tumor suppressive effects in myeloid cells, while its mutations lead to leukemogenesis. Further research in this direction is needed in order to elucidate whether Klf4 mutations play a role in leukemogenesis or conversely, whether functional Klf4 protein has a protective and tumor-suppressive activity in the hematological diseases.

In order to analyze the mechanism leading to the reduced colony formation and macrophage maturation in Klf4 over-expressing myeloid progenitors, we searched for the Klf4 downstream target genes that could explain observed effects. Up-regulation of $p21^{Waf1}$ by Klf4 was previously described (Chen et al., 2003; Zhang et al., 2000). Correspondingly, gastric epithelial cells from $Klf4^{-/-}$ mice express reduced levels of $p21^{Waf1}$ (Katz et al., 2005). In this work, it was confirmed that $p21^{Waf1}$ expression in primary murine macrophages is up-regulated by Klf4 over-expression and down-regulated by Klf4 deletion (Figure 17). According to the literature data, $p21^{Waf1}$ was a good candidate target gene which could explain both Klf4 effects in the macrophage differentiation and in the cell cycle inhibition.

There is growing evidence that the role of $p21^{Waf1}$ in the cell growth and differentiation is far more complex than just arresting the cell cycle. Findings that $p21^{Waf1}$ can actually be up-regulated by

mitogens in lymphocytes (Nourse et al., 1994), fibroblasts (Michieli et al., 1994) or myeloid cells (Schepers et al., 2003) opened a different perspective on its involvement in differentiation. Moreover, silencing of $p21^{Waf1}$ expression seems to be necessary for the terminal differentiation in some cells: forced $p21^{Waf1}$ expression in late keratinocyte differentiation stages inhibited the expression of the terminal differentiation markers (di Cunto et al., 1998). In myelopoiesis, $p21^{Waf1}$ expression coincides with most highly proliferative stages (Steinman et al., 1998), indicating that it plays additional roles, not related to its cell cycle arrest activity.

Interestingly, in mature myeloid cells $p21^{Waf1}$ expression is differentially regulated: while monocytes/macrophages show strong $p21^{Waf1}$ signal (Figure 9; Asada et al., 1999), the expression is undetectable in granulocytes (Figure 9; Steinman et al., 1998).

Published data further suggests a differential role of $p21^{Waf1}$ in these two cell types. In monocytes $p21^{Waf1}$ expression enhances differentiation and survival (Asada et al., 1999). The proteasome cleavage of $p21^{Waf1}$ in U937 monocytic cell line inhibited monocyte differentiation (Dublet et al., 2005). The expression of $p21^{Waf1}$ in monocytes is shifted from nucleus to the cytosol, where $p21^{Waf1}$ was shown to physically interact with ASK1 (Apoptosis Signal-regulating Kinase1) and inhibit stress-activated MAP kinase cascade resulting in apoptosis-resistant phenotype (Asada et al., 1999; Asada et al., 2004a; Asada et al., 2004b). Additional mechanisms of $p21^{Waf1}$ involvement in the protection from apoptosis include inhibiting caspase-3 activation by binding to procaspase-3 (Suzuki et al., 1999) and stabilization of antiapoptotic protein c-IAIP (Steinman et al., 1999). Apoptosis protective effect of interferon γ treatment of bone marrow macrophages was attributed to the increase in $p21^{Waf1}$ expression (Xaus et al., 1999).

On the other side, forced expression of $p21^{Waf1}$ in granulocytes developing from a murine 32Dcl3 myeloblast cell line (Ghanem et al., 2005) showed a proapoptotic effect.

It is interesting to mention that $p21^{Waf1-/-}$ mice, just like $Klf4^{-/-}$ mice, don't show any overt deregulation of hematopoiesis (Deng et al., 1995).

In order to test whether up-regulation of $p21^{Waf1}$ is responsible for the Klf4 effect on the macrophage development, the full length $p21^{Waf1}$ construct was over-expressed in myeloid progenitors in the same way as Klf4-ER^{T2}. Since subcellular localization of $p21^{Waf1}$ was reported to play a role in the monocyte differentiation (Asada et al., 1999; Yaroslavskiy et al., 1999; Schepers et al., 2003), chimerical p21-ER^{T2} construct was cloned and the ability of the ER^{T2} fragment to translocate into the nucleus in the presence of its ligand and conversely to stay in the cytosol in the ligand absence was used to manipulate the localization of p21-ER^{T2}. Comparison of the constitutive $p21^{Waf1}$ over-expression effect (Figure 18, Figure 19) and p21-ER^{T2} over-expression (Figure 20, Figure 21, Figure 22) showed that fusion of $p21^{Waf1}$ with ER^{T2} fragment did not compromise its function.

The effect of $p21^{Waf1}$ over-expression on the myeloid progenitors, assessed by CFU assay and FACS analysis of macrophage differentiation markers, showed that it largely reproduced the effect of the Klf4-ER^{T2} over-expression in respect of relative increase of macrophages (Figure 5, Figure 6,

Figure 7 compared to Figure 20, Figure 21, Figure 22). The same effect was observed in wild type as well as in Klf4$^{-/-}$ cells, indicating that p21^{Waf1} itself, without Klf4 is sufficient to cause macrophage differentiation. This result is in accordance with previously described monocyte differentiation induction by ectopic p21^{Waf1} expression in U937 cells (Asada et al., 1999). Additionally, p21-ER^{T2} over-expression activated the same macrophage specific genes as Klf4-ER^{T2} and conversely repressed the same granulocyte specific genes (Figure 27), indicating that Klf4 macrophage inducing effects could be mediated through p21^{Waf1} up-regulation.

In respect of proliferation inhibition, p21^{Waf1} over-expression showed reduction of the total colony formation only in the case of nuclear p21 expression: p21-ER^{T2} over-expression with 4-OHT stimulation or constitutive p21^{Waf1} expression (native p21^{Waf1} has strong nuclear localization signal). The cytosolic retention of the p21-ER^{T2} protein did not significantly effect the proliferative capacity of cells. Together with the differentiation induction effects of these constructs, these observations suggest that the roles of p21^{Waf1} in differentiation and cell cycle inhibition are not linked. Moreover, they seem to be determined by the p21^{Waf1} subcellular localization: nuclear p21^{Waf1} localization is necessary for the cell cycle inhibition, while differentiation promoting effect is not strictly compartmentalized.

It should also be mentioned that the increase in macrophage maturation and the reduction of the colony formation caused by (nuclear) p21^{Waf1} over-expression, although qualitatively equal to Klf4 over-expression, show differences in the magnitude of changes. Namely, p21^{Waf1} effects are milder than that of Klf4 in colony formation reduction (Figure 18), in increase of the macrophage differentiation (Figure 19, Figure 20, Figure 21, Figure 22) and in activation of macrophage specific genes (Figure 27). This indicates that the function of Klf4 in these processes is only partially mediated by p21^{Waf1} and that there are other, p21^{Waf1} independent factors involved in the Klf4 induced macrophage maturation and cell cycle arrest.

In order to show that transcriptional activity of Klf4 is necessary for its function, two transcriptionally incompetent Klf4 constructs were designed: Klf4$^{\Delta Zn}$-ER^{T2}, which lacks the zinc finger region and therefore cannot bind to the DNA, and Klf4$^{\Delta N}$-ER^{T2}, which contains the zinc finger region only. According to previous reports (Yet et al., 1998; Geiman et al., 2000), zinc finger region alone has no effect on the transcription.

Experiments described here showed that over-expression of Klf4$^{\Delta N}$-ER^{T2} construct in the myeloid progenitors had no effect on the differentiation and proliferation of cells (Figure 29, Figure 30, Figure 31, Figure 32), confirming that Klf4 transactivsting activity is necessary for its function.

Surprisingly, the over-expression of the Klf4$^{\Delta Zn}$-ER^{T2} construct showed dramatic effect on the differentiation of cells, blocking their proliferation and maintaining their proliferative capacity (Figure 29, Figure 30, Figure 31, Figure 32, Figure 33). This construct seems to act as dominant negative, since in the wild type cells native Klf4 was not able to rescue its effect. Klf4$^{\Delta Zn}$-ER^{T2} construct cannot bind the DNA and activate the transcription of Klf4 direct targets. However, the normal myeloid development in the Klf4$^{-/-}$ mice suggests that it is not the mere absence of Klf4 transcriptional activity

that is responsible for this differentiation block. The possible mechanism of the Klf4$^{\Delta Zn}$-ER^{T2} transforming activity could be a sequestration of Klf4 interaction partners and the deregulation of their transcriptional activities. One possible mechanism was recently suggested, showing the direct interaction of Klf4 and β-catenin, which results in the repression of β-catenin mediated gene expression, cell cycle arrest and differentiation. The zinc-finger deletion mutant of Klf4 was still able to bind β-catenin, but the transcriptional inhibition of the complex was abolished, moreover, this mutant acted as dominant negative, increasing the β-catenin signaling pathway (Zhang et al., 2006). Together with previous reports, this supports the idea that Klf4 function is strictly context-determined and dependant on other transcription factors present in the nucleus. Identification of Klf4 interaction partners will be necessary to fully explain the function of this protein. The dominant negative Klf4 mutant can serve as valuable tool for further studies of Klf4 protein-protein interactions.

Apart from studying the roles of Icsbp and Klf4 in the regulation of granulocyte and monocyte differentiation, the involvement of Icsbp in another aspect of myelopoiesis is analysed in the course of work on this thesis – the generation of eosinophilic granulocytes.

The analyses of knock-out models of hematopoietic transcription factors (as discussed in the Introduction) provide valuable information about their significance in the process of generating blood cells. However, the null-mutations often reveal only the earliest or most striking deregulation points, masking the additional roles of the studied factor. The description of the Icsbp$^{-/-}$ mice phenotype based on a number of studies published in previous years outlined two major points of Icsbp involvement in the immune system function. First, Icsbp-null mice are immunodeficient, due to the defective production of Il-12 and subsequent deregulation of interferon γ mediated immune responses (Holtschke et al., 1996; Giese et al., 1997; Hein et al., 2000; Masumi et al., 2002). Second, the lack of Icsbp leads to the misbalance in the production of myeloid cells, reflected in decrease of macrophages and high production of neutrophilic granulocytes (Holtschke et al., 1996; Schmidt et al., 1998; Scheller et al., 1999; Tsujimura et al., 2002). The data presented in this work demonstrate an additional and novel role of Icsbp involvement in myelopoiesis – the regulation of eosinophilic granulocyte generation.

Minor cell populations like eosinophils, which comprise only 1-2% of all peripheral blood leukocytes, are often studied in models which bring up the analyzed population. In the case of eosinophils, the established models increasing their number include mice transgenic for the main eosinophil growth factor Il-5 (Dent et al., 1990) or inflammations caused by agents which lead to the endogenous Il-5 production (Finkelman et al., 1997). In the work presented here, two of such inflammatory models were used in order to study the eosinophil representation and function in the Icsbp$^{-/-}$ mice: sterile peritoneal inflammation caused by thioglycolate injection and systemic parasite infection with the intestinal nematode *Nippostrongylus brasiliensis*. In both models, the immune response of the organism involves the activation of helper T-cells and secretion of Th-2 type cytokines, Il-4, Il-5, Il-9 and Il-13 which activates the specialized effector cells – eosinophils in the

first line (reviewed in Behm et al., 2000). The production of eosinophils in the bone marrow is thereby stimulated and their accumulation in the inflamed tissues is increased (Rennick et al., 1990). The thioglycolate injection leads to the strong increase of eosinophils in the peritoneal cavity of wild type mice (Louahed et al., 2001) and systemic parasite infection leads to peripheral blood eosinophilia (Rennick et al., 1990). In the case of Icsbp$^{-/-}$ mice, vigorous T-cell activation and production of high quantities of Th2 cytokines was observed (Figure 37). However, the expected eosinophil increase in both inflammatory models was significantly hampered (Figure 35, Figure 36). In accordance with the in vivo data, the culturing of Icsbp$^{-/-}$ bone marrow cells in Il-5 supplemented media resulted in significantly lower percentage of eosinophils in comparison to the Icsbp$^{+/+}$ cells (Figure 38, Figure 39 A,B). The inadequate eosinophil production in the Icsbp$^{-/-}$ mice in spite of high concentrations of their main growth factor Il-5, indicated a proliferation and/or differentiation defect of Icsbp$^{-/-}$ progenitors based either on the inability of the committed eosinophil precursors to respond to the Il-5 or by deviated differentiation potential of uncommitted progenitors which have the possibility to chose the eosinophil lineage fate. In order to reconcile the two, the differentiation of Icsbp$^{-/-}$ eosinophils was further studied *in vitro*.

The first prerequisite for the eosinophils to respond to Il-5 stimulation is the adequate expression of the Il-5 receptor. Il-5 receptor is, like Il-3 and GM-CSF receptor, a heterodimer consisting of the cytokine-specific α-subunit and common signal-transducing β_c-subunit. The α-chain is specific for each receptor and its role is to bind the corresponding ligand. Since short cytoplasmic domains of α -chains cannot by themselves activate intracellular mediators (JAK-STAT proteins), the signal is transduced through the long cytoplasmic tails of the β_c-subunit, shared by all three receptors (reviewed in Woodcock et al., 1999). The fact that any α-chain:ligand complex formed utilizes the same β_c-chain to transduce the signal in the cell was used to estimate the functionality of this unit in the Il-5 signaling. As shown in Figure 39D, the responsiveness of Icsbp$^{-/-}$ bone marrow cells to GM-CSF stimulation was not reduced (and was even increased) in comparison to wild type cells, suggesting the functional signal transduction through the receptor β_c-subunit. The expression of the α-subunit, on the other side, showed moderate reduction in Icsbp$^{-/-}$ eosinophils and their precursors (Figure 41). Together, these data indicate that the Il-5 receptor dimers on Icsbp$^{-/-}$ eosinophils are functional, but reduced in number, which results in reduced, but not completely abolished proliferation and survival in response to Il-5 stimulation. This was confirmed by testing the starvation induced apoptosis of Icsbp$^{-/-}$ and $^{+/+}$ eosinophils; they both responded to the Il-5 “rescue”, but Icsbp$^{-/-}$ cells required higher cytokine doses (Figure 42).

However, it is questionable how much this reduced proliferation and survival of Icsbp$^{-/-}$ eosinophils found in vitro contribute to the inability of Icsbp null mice to produce and sustain high number of eosinophils in vivo. Icsbp$^{-/-}$ mice secrete very high amounts of the Il-4 and Il-5 in response to the parasite infection. In vitro culturing conditions include even higher cytokine concentrations (50ng/ml in comparison to 3-7 ng/ml measured in vivo, Figure 37), which should compensate for

lower receptor expression. Still, this is not the case - high Il-5 doses do not rescue the compromised eosinophil differentiation in Icsbp$^{-/-}$ mice, indicating additional, cytokine independent defects in the eosinophil differentiation.

Unlike other myeloid cells, the stages in the eosinophil differentiation are not yet fully defined. At the time this study began, it was not known which progenitor population forbears the eosinophil lineage. Different studies demonstrated that eosinophils could be grown from GMP, MEP or CMP populations (Graf et al., 1992; Akashi et al., 2000; McNagny et al., 2002). However, neither one of this populations has exclusive eosinophil differentiation potential. The elusive eosinophil committed progenitors were only recently identified by Iwasaki et al., who used the activation of the Gata1 transcription, the feature unique for eosinophils among other myeloid cells, to identify the small fraction of GMP descending cells which give rise exclusively to eosinophils (Iwasaki et al., 2005). This progenitor population was phenotypically identified as Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} and currently represents the earliest eosinophil committed progenitor defined. The analysis of Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} cells in the bone marrow of Icsbp$^{-/-}$ mice showed a significant reduction of this population in absence of Icsbp (Figure 40). Together with the reduced colony formation in functional assays (Figure 39 A and C), these data suggest that Icsbp$^{-/-}$ mice have a reduced pool of eosinophil committed progenitors.

Furthermore, the differentiation of Icsbp-/- eosinophil progenitors (estimated by analyzing the colony growth in medium supplemented with a combination of general myeloid- and eosinophil-promoting cytokines) showed aberrant pattern, reflected in very low eosinophil representation (25% in comparison to 75% in wild type samples) and high monocyte representation (found in more than 90% of Icsbp$^{-/-}$ colonies and 20% of wild type colonies; Figure 43). Considering the already established role of Icsbp as a monocytes/macrophage promoting factor (Holtschke et al., 1996; Scheller et al., 1999; Tsujimura et al., 2002), it is quite unexpected that any progenitor cell population from Icsbp null mice shows increased macrophage differentiation potential. We could speculate that the over-responsiveness of Icsbp$^{-/-}$ progenitors to GM-CSF (Scheller et al, 1999; Figure 39D) and reduced responsiveness to Il-5 (Figure 39A, B and C) could redirect the eosinophil-instructive cytokine signals toward monocyte/neutrophil-instructive signals. However, the similar monocyte/macrophage overgrow in Icsbp$^{-/-}$ samples was observed when the cells were cultured in media supplemented with Il-5 only (Figure 38 and Figure 39A, B), suggesting an aberrant differentiation potential of Icsbp$^{-/-}$ progenitors. Additionally, numerous experiments show (as discussed in the Introduction) that cytokine signalling does not affect the commitment itself and that the cytokine receptor expression follows as a consequence of the lineage determination. Mice with null mutation for Il-5Rα are not completely devoid of eosinophils (Yoshida et al., 1996; Nishinakamura et al., 1996). Conversely, forced expression of Il-5Rα in GMP cells (direct precursors of EoP) does not significantly increase the frequency of eosinophil progenitors, suggesting the permissive, but not instructive role of Il-5 in the eosinophil lineage determination (Iwasaki et al., 2005). Therefore, the deviated differentiation

potential of Icsbp$^{-/-}$ eosinophil progenitors most likely results from the misbalance of intrinsic factors involved in the lineage commitment. Indeed, the expression of one crucial factors in eosinophil development, *Gata1* is significantly reduced in Icsbp$^{-/-}$ eosinophils and their progenitors (Figure 44). Eosinophils are only myeloid cells which "tolerate" the expression of this typical erythroid transcription and moreover, they depend on it for normal development. The studies on mice with targeted deletions of different Gata1 promoter regions show that loss of Gata1 leads to the loss of eosinophil production (Hirasawa et al., 2002; Yu et al., 2002). Therefore, the reduction of Gata1 expression in the context of other common myeloid transcription factors, like Pu.1 and C/ebp changes the characteristic eosinophil molecular profile to profile of other myeloid cells and favors their development. This model of myeloid cell generation through subtle changes in transcription factor expression was first suggested by McNagny (McNagny et al., 2002) and applied on our experimental system, it explains the molecular background for the aberrant differentiation pattern of Icsbp$^{-/-}$ eosinophil progenitors.

At this point it is not clear how Icsbp affects Gata1 expression. The reduction of Gata1 in Icsbp$^{-/-}$ mice is observed selectively in the eosinophil lineage (Figure 44), which implies the involvement of the specific regulatory DNA sequence in the Gata1 proximal (IE) promoter (DNAse I hypersensitive region II, HSII). Namely, Yu et al. demonstrated that the targeted deletion of this promoter region results in a complete loss of eosinophils in knock-out mice, without affecting other hematopoietic lineages (Yu et al., 2002). The HSII region contains high affinity binding sequence for Gata1 itself (Tsai et al., 1991), which underlines the importance of maintaining Gata1 expression for the proper eosinophil development. The highly homologous regulatory DNA sequence was found in the Il-5Rα and MBP promoter (Rothenberg et al., 2006), indicating that the same mechanism which regulates the expression of these *bona fide* eosinophil genes, regulates the eosinophil-specific expression of Gata1. The global gene expression analysis in the Icsbp$^{+/+}$ and $^{-/-}$ GMPs performed in our laboratory showed the strong reduction of *Mbp* expression, along with several other eosinophil specific genes, like *Epx* or *Ear1/2*, in the absence of Icsbp (unpublished data). Therefore, the expression of eosinophil specific genes is affected by the Icsbp deletion even before the expression of Gata1 is invoked in the GMP population and before these cell get committed to the eosinophil lineage. This, along with the Gata1 down-regulation in committed eosinophil progenitors and subsequent reduction of the eosinophil differentiation in Icsbp$^{-/-}$ mice, implies Icsbp as an important factor in regulating the expression of eosinophil specific genes and in determining the eosinophil lineage generation.