5. RESULTS

5.1. Klf4 over-expression in bone marrow progenitors results in increased percent of macrophages

It was described by Holtschke and al. that null mutation for Icsbp leads to changed differentiation program of myeloid progenitors reflected by disproportional high production of granulocytes and low production of macrophages (Holtschke et al., 1996). In order to study the underlying mechanism of this switch, bipotent granulocyte-macrophage progenitor (GMP) population was isolated and gene expression profile of $Icsbp^{-/-}$ GMPs was compared with that of $Icsbp^{+/+}$ GMPs by Affymetrix technology. This analysis identified the transcription factor Klf4 as strongly down-regulated (10 fold) in $Icsbp^{-/-}$ progenitors (unpublished data). In order to test whether Klf4 down-regulation contributes to the impaired macrophage differentiation, we chose to determine if Klf4 reintroduction reverts the observed phenotype.

5.1.1. Constitutive Klf4 over-expression

Bone marrow cells from $Icsbp^{+/+}$ and $Icsbp^{-/-}$ mice were taken 72 hours after 5-fluorouracil (5-FU) treatment. 5-FU is a thymidine analogue which has a cytostatic effect on all actively cycling cells. Since hematopoietic stem cells (HSC) were shown to be either quiescent (Ogawa et al., 1993, Morrison et al., 1994) or cycling very slowly (Bradford et al., 1997; Cheshier et al., 1999), they are not affected by the 5-FU treatment (Hodgson et al., 1979; Van Zant et al., 1984), while other progenitors, which have a high proliferation potential are rapidly killed. Depletion of a large number of progenitor cells stimulates the homeostatic mechanisms which in return activate the stem cell compartment to divide and produce new stem cells and primitive progenitors. Final result of short-term 5-FU treatment (72h) is an enrichment of the bone marrow population for hematopoietic stem cells and the most primitive progenitors (Yeager et al., 1983).

In this experiment, fibronectin enhanced retroviral gene transfer was used in order to introduce the Klf4 cDNA in progenitor cells, which were then differentiated *in vitro* in controlled growth factor conditions. The procedure is described in detail in Materials and Methods. Shortly, bone marrow cells from $Icsbp^{+/+}$ and $Icsbp^{-/-}$ mice, enriched for HSC by 5-FU treatment were stimulated for 48 hours in liquid medium with combination of cytokines (SCF, Il-3, Flt-3-Ligand and TPO). The purpose of the cytokine stimulation is to bring the cells in active divisions, since *Oncovirinae* derived retroviral vectors infect only cycling cells. The cytokine combination used promotes proliferation and survival of very immature progenitors. Transplantation of these progenitors into lethally irradiated mice gives complete reconstitution of the hematopoietic system (experiments performed in our laboratory, unpublished data), confirming that they retain their multipotency and clonogenic capacity.

The efficiency of the cell transfection with retroviral vector was enhanced by using RetroNectin® coated dishes for the infection procedure. RetroNectin™ is a recombinant protein containing three functional domains of human fibronectin: the central cell binding domain, the heparin

binding domain II and the connecting segment-1. When coated on Petri dishes, fibronectin (or RetroNectin™) enhances virus-mediated gene transfer in the cells by mediating colocalization of virus particles and target cells on fibronectin molecules. Virus particles bind mainly to the heparin binding domain II, while cells expressing surface integrins VLA4 or VLA5 bind to connecting segment-1 or central cell binding domain respectively (Moritz et al., 1994). The colocalization results in highly concentrated virus titer in immediate vicinity of target cells (Moritz et al., 1996) and in reducing the effect of inhibiting agents in the medium (Chono et al., 2001), which greatly enhances the efficiency of the retroviral transduction.

Using this system, progenitor enriched cells were infected two times with MIEG3 retroviral vector carrying full length murine *Klf4* cDNA (cloning of this construct is described in Materials and methods). Infected cells have been identified by the marker gene, enhanced green fluorescent protein (eGFP) which is expressed along with the *Klf4* cDNA as bicistronic transcript and than translated from the internal ribosome entry site (IRES) of the viral vector. $eGFP^+$ cells were sorted by fluorescence activated cell sorting (FACS) and plated in semisolid methylcellulose medium in order to allow single cells to form distinct colonies. Each colony in methylcellulose medium comes from a progenitor cell which has a capacity to proliferate. Approximately 50 cells are necessary to form a colony, which means that the progenitor cell has to divide at least 5 times (2^5=32). Therefore, only very immature progenitors can give rise to colonies. These immature progenitors are not yet definitively committed to certain lineage and growth factors added to the medium or manipulation of intrinsic cell factors are used to switch the differentiation program in one or the other direction. For example, deletion of Icsbp, as mentioned before, results in an incline of granulocytes on the expense of macrophages. The differentiation of progenitors can be assessed by examining the morphology of formed colonies. Each cell type forms colonies with typical macroscopic shape and cell distribution pattern (observed under stereo-microscope with side light) and can be classified as M-CFU (macrophage - colony forming unit), G-CFU (granulocyte – colony forming unit) or GM-CFU (mixed population of granulocyte– and macrophage – colony forming units). These morphologic criteria were described previously (Nakahata, PNAS, 1982) and are routinely used to identify colonies. Transparency of methyl cellulose medium allows observing the size and shape of the individual cells in colonies under higher magnification (40x), which is helpful in determining the type of cells in the colony (granulocytes are round, small cells, compared to larger monocytes and macrophages which often form spindle-shaped protrusions and adhere to the bottom of the plate). Ultimately, cells are isolated from methylcellulose and microscopically examined after May-Grünwald-Giemsa (MGG) staining.

In this experiment, methylcellulose medium was supplemented with rmGM-CSF, a growth factor promoting development of both granulocytes and macrophages. Transduction of empty MIEG3 vector was used as a negative control. Transduction with full length mouse *Icsbp* was used as positive control, since Icsbp over-expression is known to switch progenitor development toward macrophages (Tsujimura et al., 2002).

The colonies in methylcellulose medium were counted after 7 days and classified by their morphology as M-CFUs, G-CFUs or GM-CFUs. The data are shown in Table 7.

Starting cell population	Introduced gene	Number of G-CFU per 10^4 cells plated	Number of M-CFU per 10^4 cells plated	Number of GM-CFU per 10^4 cells plated	Total colony number
Icsbp$^{+/+}$	(-)	49.5+/-2.5	67+/-8	76.5+/-3.5	193+/-9
	Icsbp	1.5+/-0.5	17.5+/-0.5	3.5+/-0.5	22.5+/-0.5
	Klf4	0	2.5+/-0.5	0	2.5+/-0.5
Icsbp$^{-/-}$	(-)	141.5+/-1.5	22.5+/-0.5	51+/-5	215+/-8
	Icsbp	5+/-1	36.5+/-5.5	10.5+/-1.5	52+/-8
	Klf4	8+/-1	16+/-2	12.5+/-0.5	36.5+/-1.5

Table 7: Constitutive over-expression of Klf4 (CFU Assay)
Colonies were classified into macrophage (M-CFU), granulocyte (G-CFU) and granulocyte-macrophage (GM-CFU) according to the previously described morphological criteria (Nakahata, PNAS, 1982). The number of colonies (per 10^4 plated progenitor cells) represents the mean of three independent samples with standard deviation.

As shown in Table 7, Klf4 over-expression in both Icsbp$^{+/+}$ and $^{-/-}$ cells resulted in strongly reduced colony formation. This effect was in accordance with previously published reports, showing the Klf4 involvement in growth arrest (Shields et al., 1996; Geiman et al., 2000; Shie et al., 2000).

Additionally, this experiment shows that Klf4 effects the differentiation of myeloid cells. In Icsbp$^{+/+}$ sample, only colonies with the characteristic M-CFU morphology were observed. Moreover, in Icsbp$^{-/-}$ progenitors, which are known to differentiate preferentially to granulocytes (Scheller et al., 1999), Klf4 over-expression resulted in highly increased percentage of M-CFUs, as compared to the vector control. This effect is comparable to Icsbp reintroduction, used as positive control, indicating that Klf4 is indeed capable of reverting the Icsbp deletion caused bias toward granulocytes.

The size and form of macrophage colonies did not differ from those in vector control (data not shown).

This data indicates that the over-expression of Klf4 either promotes commitment and differentiation of progenitors along macrophage lineage (like Icsbp) or alternatively, it selects macrophages by supporting their survival, while inhibiting the survival of granulocytes.

Since retroviral transduction procedure followed by sorting of GFP$^+$ cells by FACS requires growing progenitor cells in liquid culture for 4 days before plating in methylcellulose (starting point for observing differentiation of cells), it is possible that introduced Klf4 preselects certain progenitor populations and therefore influences the result of the differentiation assay. In order to avoid this problem inducible chimeric constructs where *Klf4* or *Icsbp* are fused to modified estrogen receptor (ER^{T2}) were cloned (as described in Materials and Methods) and used in the same experimental setting.

5.1.2 Inducible Klf4 over-expression

The disadvantage of the constitutive over-expression system is the activity of introduced genes before the progenitor cells are plated for the colony forming assay. This causes preselection of the progenitors and complicates quantification of the observed effects. In order to avoid this problem, analyzed transcription factors (Klf4 and Icsbp) were fused with E and F domains of a modified estrogen receptor (ER^{T2}) at their C-terminus, creating thereby chimerical proteins with ligand dependant activity. The natural estrogen receptor has the ability to directly bind to the DNA through its N-terminal part and activate the transcription of target genes (Beato M., 1996). The activation of the estrogen receptor transcriptional activity is dependant on the binding of its ligand (estradiol) to the C-terminal part (including E and F domains) (Danielian et al., 1992). Domain-swap experiments have shown that exchanging the N-terminal DNA binding region of ER with other transcription factors (TF) results in the activation of TF-specific target genes upon ER-ligand binding. The modified estrogen receptor is a mutant specifically and highly responsive to synthetic estrogen antagonist 4-OH-tamoxifen (4-OHT), but unable to bind its natural ligand estradiol. This eliminates the activation "leakiness" caused by residual estradiol, always present in the fetal calf serum used for the culture media (Danielian et al., 1993; Littlewood et al., 1995; Feil et al., 1996; Feil et al., 1997). Without 4-OHT, the ER^{T2} part of chimerical protein interacts with chaperone proteins, which keeps the formed complex sequestered in the cytosol or "loosely" associated with the nucleus (Greene and Press, 1986). Addition of 4-OHT to the medium and its binding to the ER^{T2} causes dissociation from the chaperone proteins, "tight" association with the nucleus in a punctuate pattern (Htun et al., 1999) and ultimately binding to the target DNA regions. This system is often used for studying transcription factors.

The fusion of human KLF4 with ER^{TM} fragment (estrogen receptor mutant similar to ER^{T2}, Littlewood et al., 1995; Feil et al., 1997) was described in (Foster et al., 2005). They demonstrated by immunofluorescence the cytoplasmic localization of the fusion protein in the absence of 4-OHT and its translocation to the nucleus coupled with KLF4 biological function when 4-OHT is added.

In order to exclude the possibility that fusion of ER^{T2} to the transcription factor alters its specificity, all experiments described in this work were performed with both chimerical inducible constructs as well as with constitutive constructs. The effects of the two were compared and they were qualitatively equal. The quantitative differences were attributed to the preselection effect of the constitutive expressing constructs. Therefore, in the further work, inducible constructs were used for quantification.

Bone marrow progenitor cells from $Icsbp^{+/+}$ and $^{-/-}$ mice were transduced with *Icsbp-ER*^{T2} and *Klf4-ER*^{T2} constructs in the same way as with constitutive ones (Section 5.1.1), but activation of the genes in this system could be delayed until plating for the CFU-assay. Addition of 4-OHT to the methylcellulose medium ensured activation of introduced constructs at the moment of plating and kept them active throughout the cell differentiation and colony formation (7 days). The control culture of vector-transduced cells were grown with and without 4-OHT. No changes in the cell differentiation or

proliferation were observed (data not shown), indicating that 4-OHT by itself does not change the differentiation or proliferation properties of the cells.

In addition to GM-CSF supplemented cultures, two more growth factors were used in this experiment: M-CSF, promoting primarily macrophage differentiation and G-CSF, promoting primarily granulocytes. In the case of G-CSF, two additional cytokines were added (SCF and TPO) in order to achieve optimal conditions for granulocyte differentiation (Filippi et al., 2004). Results from three independent experiments are shown in Table 8.

Starting cell population	Introduced gene (in MIEG3 vector)	Growth factor:	Number of G-CFU per 10^4 cells plated	Number of M-CFU per 10^4 cells plated	Number of GM-CFU per 10^4 cells plated	Total	Fold reduction in colony number	Significance of colony number reduction (t-test)
Icsbp$^{+/+}$	(-)	GM-CSF	37.2+/-10.6	77.5 +/- 9.7	20.3 +/- 5.8	135+/-22.7		
	Icsbp- ER^{T2}		12.3 +/- 1.8	81.3 +/- 13.1	6.7 +/- 3.1	100.3+/-16.4	1.3	0.214
	Klf4- ER^{T2}		3.5 +/- 0.4	29.2 +/- 4.4	3 +/- 1.1	35.5+/-5.6	3.8	0.003
	(-)	M-CSF	13.2 +/- 4.2	133 +/- 36.9	11.4 +/- 3.6	158+/-33.5		
	Icsbp- ER^{T2}		6.5 +/- 0.3	128 +/- 23.7	7.5 +/- 0.3	142+/-42.4	1.1	0.802
	Klf4- ER^{T2}		3.4 +/- 0.6	41 +/- 6.9	2.1 +/- 0.2	46.8+/-6.1	3.4	0.019
	(-)	G-CSF (SCF, TPO)	65.8+/-18.1	44.8 +/- 19.2	19.9 +/- 6.4	130.5+/-39.7		
	Icsbp- ER^{T2}		12.0 +/- 5.2	64.0 +/-19.3	8 +/- 3.6	84.0+/-25.7	1.6	0.4
	Klf4- ER^{T2}		1.9+/- 0.6	11.2 +/- 3.3	1.0 +/- 0.4	14.1+/-4.2	9.2	0.014
Icsbp$^{-/-}$	(-)	GM-CSF	91.9 +/- 7.4	24.4 +/- 5.7	18.9 +/- 9.6	135+/-9.1		
	Icsbp- ER^{T2}		16.3 +/- 2.5	69.5 +/- 10.2	10.0 +/- 7.8	95.8+/-5.3	1.4	0,001
	Klf4- ER^{T2}		7.0 +/- 3.3	19.8 +/- 2.7	2.7 +/- 1.2	29.5+/-3.1	4.6	7.9E-5
	(-)	M-CSF	92.1+/-18.9	48.8 +/- 6.7	12.2 +/- 9.5	153.1+/-21.7		
	Icsbp- ER^{T2}		11.8 +/- 2.4	73.7 +/- 7.4	11.7 +/-2.9	97.2+/-12.5	1.6	0,003
	Klf4- ER^{T2}		3.3 +/- 0.8	27.7 +/- 4.9	4.5 +/- 2.8	35.5+/-6.5	4.3	3.7E-5
	(-)	G-CSF (SCF, TPO)	138+/- 28.2	15.9 +/- 4.1	16.6+/-11.0	170.4+/-38.0		
	Icsbp- ER^{T2}		17.3 +/- 4.4	37.7 +/- 2.5	5.8 +/- 2.4	60.8+/-7.2	2.3	0.005
	Klf4- ER^{T2}		6.3 +/- 2.1	11.7 +/- 3.3	1.7 +/- 0.7	19.7+/-4.2	7.0	0.001

Table 8: Inducible over-expression of Klf4-ER^{T2} (CFU Assay)
Colonies were classified into macrophage (M-CFU), granulocyte (G-CFU) and granulocyte-macrophage (GM-CFU) according to the previously described morphological criteria (Nakahata et al., 1982). Mean values with standard deviation from three independent experiments (each performed in duplicate) are shown. The number of colonies is calculated per 10^4 plated progenitor cells.

Two features of colony formation were significantly changed when Klf4 was over-expressed: first, total number of colonies in each case was reduced and second, the percentage of colonies with macrophage morphology was increased in all three cytokine combinations tested.

5.1.2.1. Klf4 over-expression reduces proliferative capacity of cells

Klf4 over-expression in bone marrow progenitors, in all three cytokine conditions tested resulted in strong reduction of total colony numbers (Table 8). This finding is in accordance with previous reports showing that enforced Klf4 expression inhibits DNA synthesis and results in

decreased cell proliferation (Shields et al.,1996; Geiman et al., 2000; Shie et al., 2000). In vivo, Klf4 expression is limited to postmitotic, differentiated cells (Shields et al., 1996, Shie et al., 2000). Chen et al have shown that Klf4 over-expression in NIH 3T3 cells drives the expression of several cell cycle inhibitors like $p21^{Waf1}$ and $p57^{Kip2}$ and suppresses the expression of the cell cycle progression factors like cyclin D1 and cdc2 (Chen et al., 2003).

However, in comparison to 3.8-fold reduction in GM-CSF and 3.4 in M-CSF (media allowing macrophage growth), G-CSF containing media, which is restrictive for macrophages, gave in average 9.2-fold reduction in colony number (Table 8).

Colony reduction in case of Icsbp-ER^{T2} over-expression in wild type cells was statistically significant only when the cells were grown in G-CSF, SCF, TPO cytokine combination (Table 8).

5.1.2.2. Klf4 over-expression increases the percentage of M-CFUs

In the experiment described, Klf4 over-expression led to an increased percentage of M-CFUs in all three cytokine combinations tested (Figure 5). Development of macrophage colonies indicates that immature progenitors differentiated into monocyte/macrophage committed cells (designated also as macrophage-colony forming units, M-CFU) and finally into mature macrophages. Surprisingly, the relative increase of M-CFUs was most prominent with the G-CSF, SCF, TPO cytokine combination, which promotes granulocyte development and is restrictive for monocyte/macrophage survival. This indicates that the shift toward macrophages caused by Klf4 over-expression does not depend on the instructive extracellular signals, but is rather intrinsically determined by transcription factors.

The over-expression of either Klf4 or Icsbp in wild type cells had comparable effects, namely increasing percent of M-CFUs. $Icsbp^{-/-}$ cells have disproportional high development of G-CFUs and a reduced number of M-CFUs (Figure 5). Reintroduction of Icsbp reverts the balance towards monocytes, confirming the pivotal role of Icsbp in cell commitment (Holtschke, 1996; Tsujimura, J.Imm. 2002). Introduction of Klf4 in $Icsbp^{-/-}$ cells also increased the percentage of M-CFUs, suggesting that Klf4 itself, even without Icsbp is sufficient for macrophage maturation.

This result suggests two possible mechanisms of Klf4 activity in macrophage differentiation. First, Klf4 could be placed downstream from Icsbp in the signaling cascade which leads to macrophage development, therefore Klf4 alone would be sufficient to bypass lack of Icsbp and trigger downstream signaling events. Alternatively, Klf4 could function independently of Icsbp and induce the macrophage differentiation by triggering a distinct set of genes.

The relation between these two factors in granulocyte/monocyte development was further investigated.

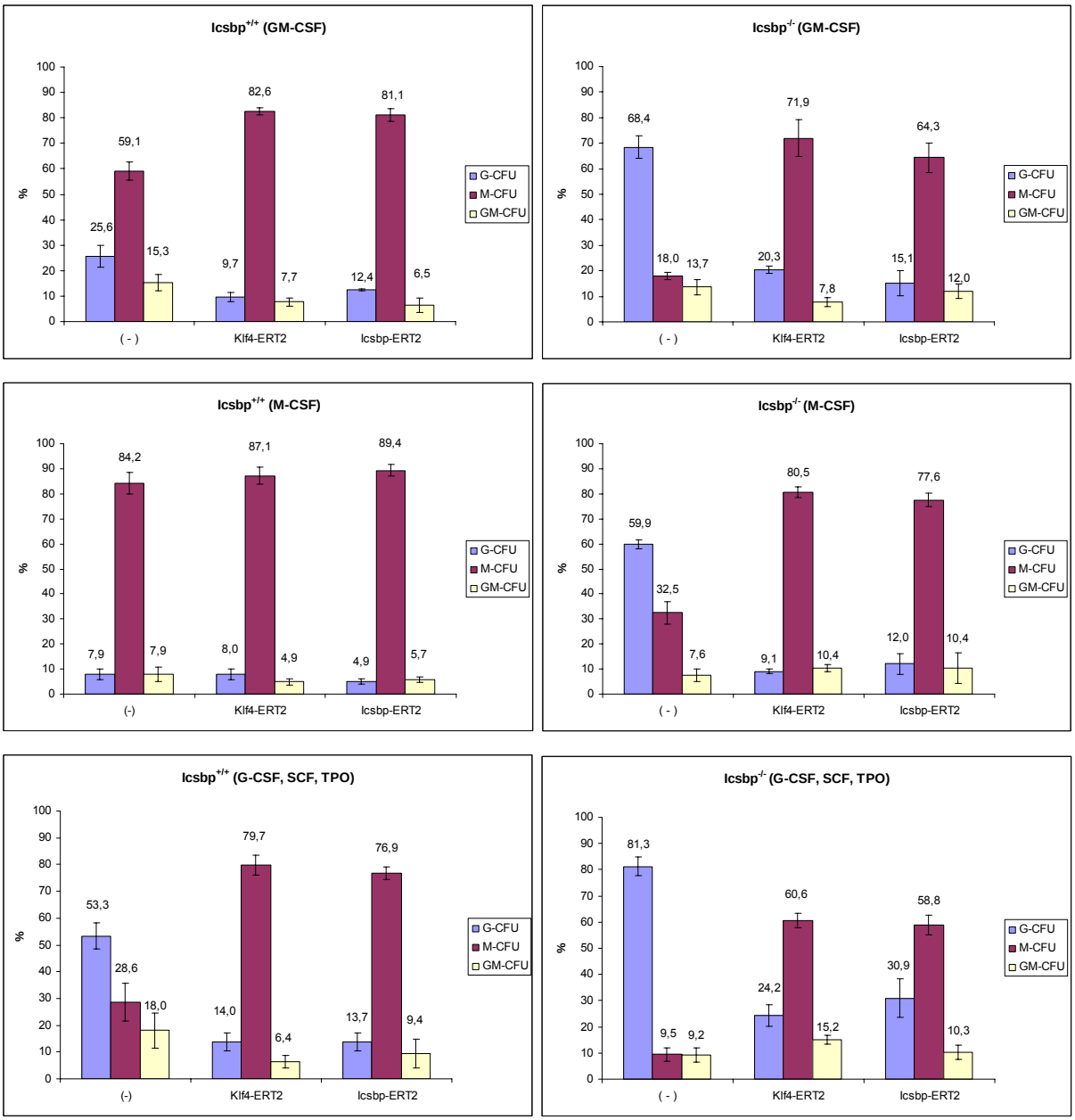


Figure 5*:* Klf4-ER^{T2} over-expression increases percentage of macrophage colonies (relative distribution of CFUs)

Percentages of each colony type were calculated from the mean colony numbers of three independent experiments (data shown in the Table 8).

The identity of observed colonies and distribution of differentiated cells between monocytic and granulocytic lineage were confirmed by two additional independent methods: light microscopy and flow cytometry. For microscopic examination, cells were isolated from methylcellulose medium and stained (as described in Materials and Methods) (Figure 6).

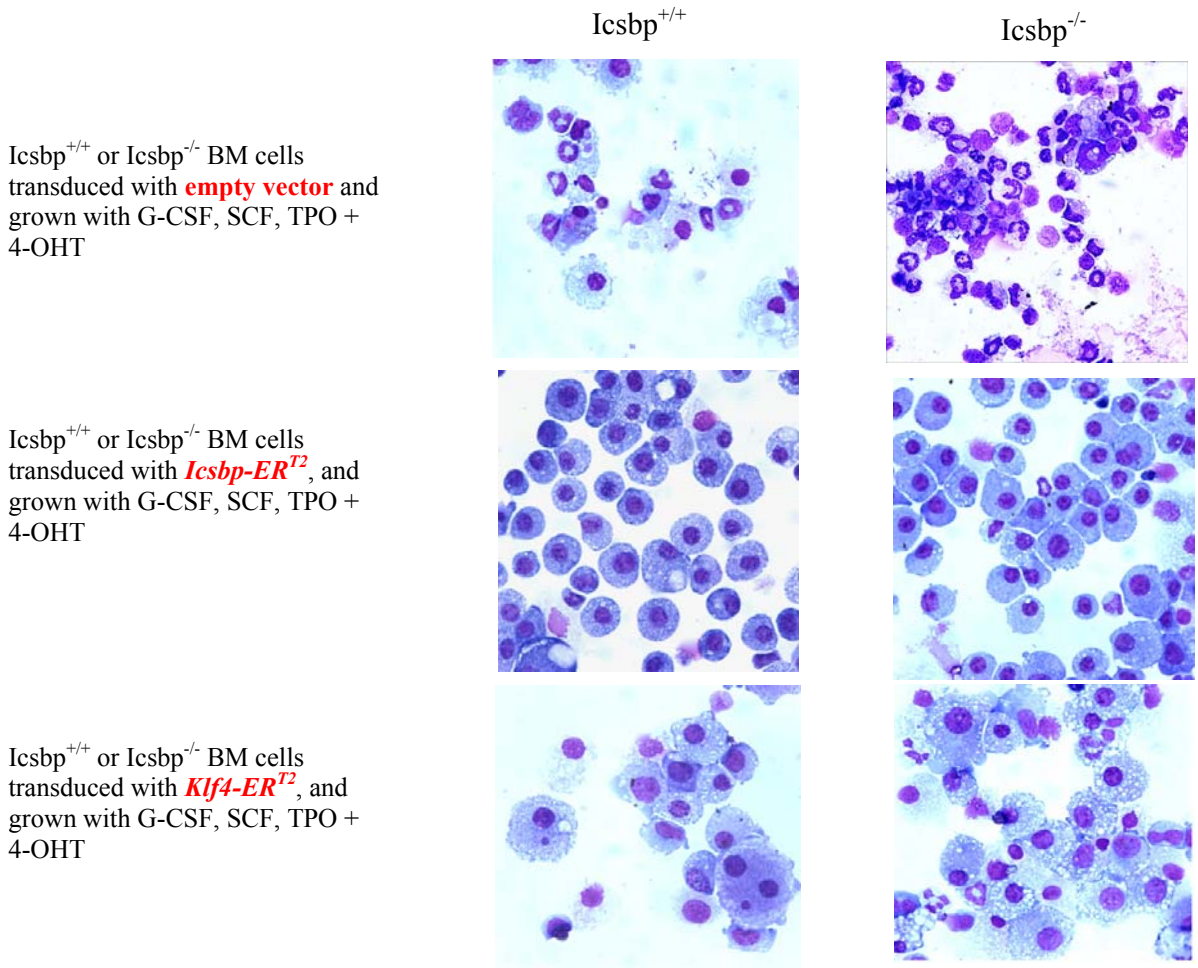


Figure 6: Klf4-ER^{T2} over-expression increases percentage of macrophages (microscope analysis)
After counting the colonies, cells were isolated from methyl cellulose, spun onto microscope slides and stained according to May-Grünwald-Giesa. The figures from one representative out of three independent experiments (Table 8) are shown.

FACS analysis was used to determine the expression of lineage specific markers on the surface of cells isolated from methylcellulose. Cells were stained for F4/80 antigen, marker of differentiated macrophages and Cd11b, an integrin expressed on both macrophages and granulocytes, but the level of expression is higher on macrophages. Results are shown in Figure 7. Wild type progenitor cells transduced with *Klf4-ER^{T2}* gave 72.3% of F4/80^{+}Cd11b^{high} cells, almost the same as progenitors transduced with *Icsbp-ER^{T2}* (80.3%), in comparison to 34.6% in the vector control population. Icsbp$^{-/-}$ cells had only 25.3% of F4/80^{+}Cd11b^{high} cells in the vector control sample, but reintroduction of Icsbp as well as Klf4 was able to rescue this phenotype and give 71.7% and 80.7% of F4/80^{+}Cd11b^{high} cells respectively.

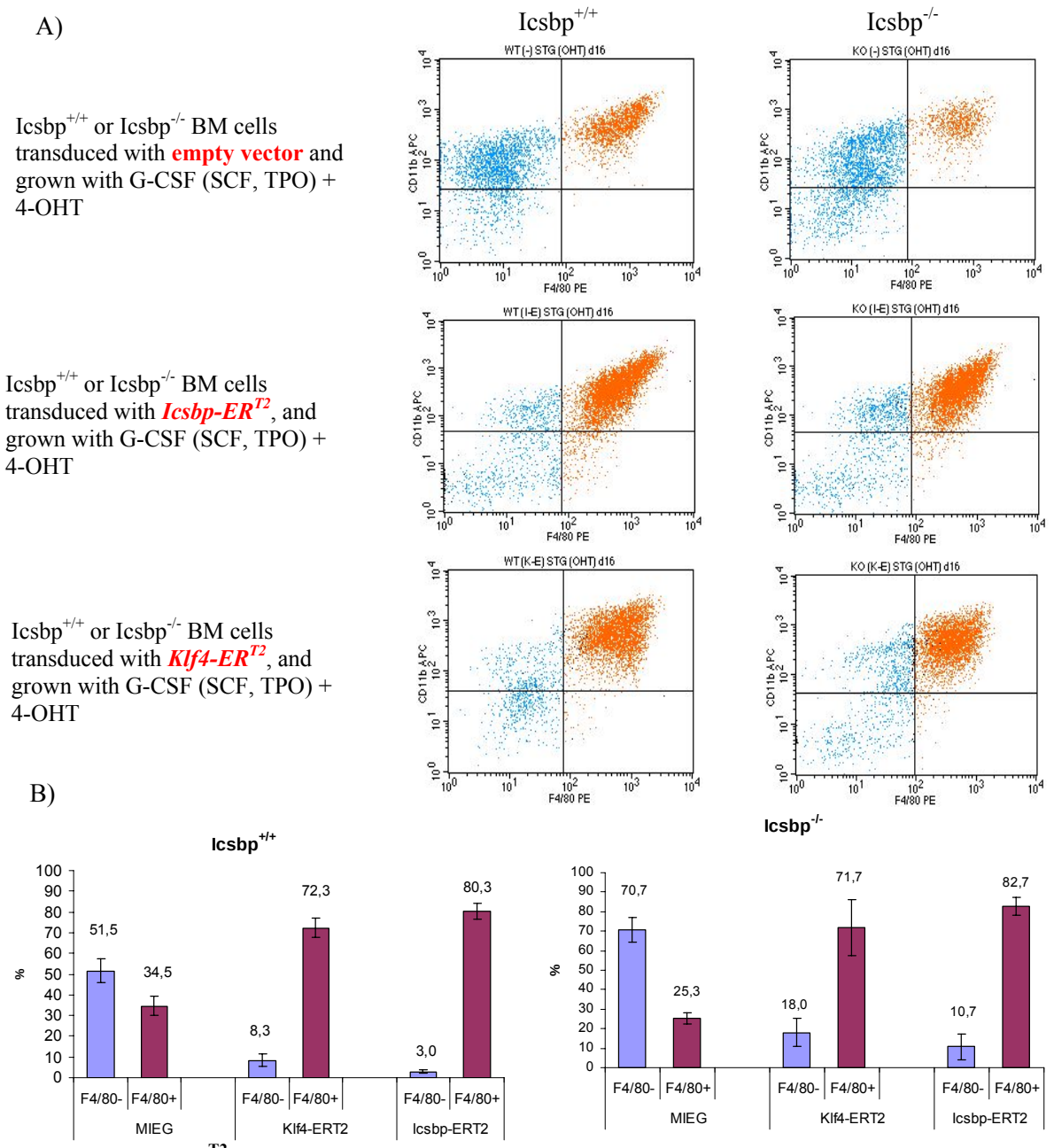


Figure 7: Klf4-ER^{T2} over-expression increases percentage of macrophages (analysis of cell surface markers)

After counting the colonies, cells were isolated from methyl cellulose, stained with anti-Cd11b and anti-F4/80 antibodies and analyzed by FACS. A) Cd11b:F4/80 dot plots from one representative experiment are shown. B) The mean values with standard deviations from three independent experiments are shown. Only Cd11b^{+} cells were analysed (out of these, F4/80^{+} cells are regarded as macrophages and F4/80^{-} as granulocytes).

In parallel to cultures in methylcellulose, progenitor cells from Icsbp$^{+/+}$ and $^{-/-}$ mice were transduced in the same way as for the CFU assay and grown in liquid media containing G-CSF, SCF, TPO cytokine combination and 4-OHT. This cytokine combination was chosen since it gave the most prominent difference in the colony forming assay. In this experimental setting, macrophage development was determined by the appearance of adherent cells in the culture. Granulocytes are, conversely, smaller and non-adherent. After allowing the cells to differentiate for 7 days, most Klf4 over-expressing cells adhered to the bottom of the culture plate, while very few negative control cells adhered (Figure 8). Comparable effects were observed in both Icsbp$^{+/+}$ and Icsbp$^{-/-}$ populations.

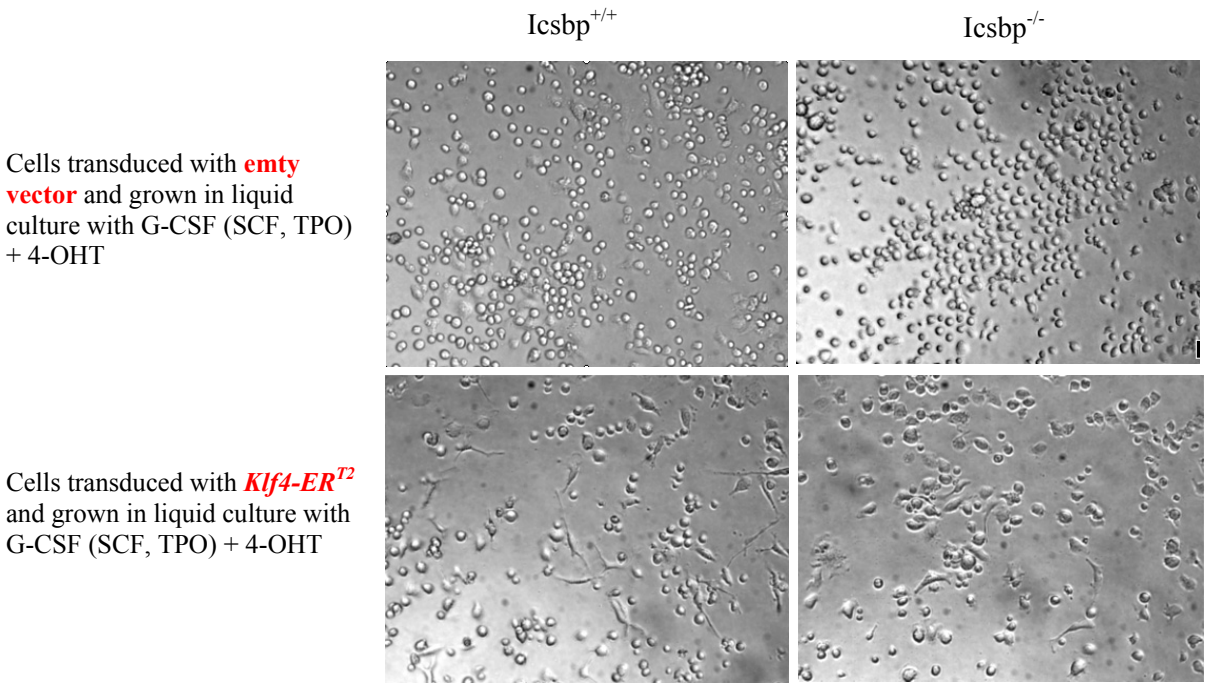


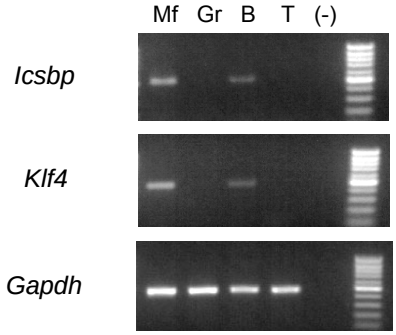
Figure 8: Liquid cultures of Klf4-ER^{T2} transduced myeloid progeniotrs show appearance of adherent cells

Bone marrow cells from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice transduced with either Klf4-ER^{T2} or vector control were grown in liquid media supplemented with G-CSF, SCF, TPO and 4-OHT. After 7 days, appearance of adherent cells was monitored.

This experiment suggests that the forced expression of Klf4 shifts the balance between granulocyte and macrophage production in favor of macrophages, even when external stimuli instruct the differentiation program otherwise (G-CSF containing media). This finding can be explained by two possible scenarios: 1) Klf4 intrinsically changes the progenitor cell differentiation program which is then conducted irrespectively of the growth factor instructions or 2) Klf4 over-expression is not compatible with granulocyte development, therefore monocytic progenitors are positively selected and resulting cell population is macrophage-enriched.

5.1.2.3. *Klf4* is expressed in primary macrophages, but not in granulocytes

At the time this study began, the expression of Klf4 in hematopoietic cells has not yet been reported. The global gene profiling of the granulocyte-macrophage progenitors (GMP) performed in our laboratory, showed *Klf4* to be highly expressed in this population. In order to analyze the expression of *Klf4* in the mature cells descending from GMP, primary murine granulocytes (from the bone marrow) and macrophages (from the peritoneal exudate) were stained with lineage specific markers (Gr1 and F4/80 respectively) and sorted by FACS (as described in Materials and Methods). Total RNA was isolated from the purified populations, converted to cDNA and analyzed by PCR with *Klf4* specific primers. As shown in Figure 9, *Klf4* transcript was detected in macrophages, but not in granulocytes. This is another indication that *Klf4* expression is sustained by macrophages, but has to be suppressed in order to allow granulocyte development.



Primary cells isolated by FACS from wild type mice:

Macrophages: from peritoneal cavity, F4/80^{+}Cd11b^{+}
Granulocytes: from bone marrow, Gr1^{+}, Cd11b^{+}
B-Lymphocytes: from spleen, B220^{+}
T-Lymphocytes: from spleen, Cd3^{+}

Figure 9: *Klf4* expression pattern in primary murine immune cells
Primary cells were isolated from wild type mice as indicated, total RNA was isolated from the purified cell populations and RT-PCR was performed. One representatove out of two independent experiments is shown.

Additionally, *Klf4* expression in the primary murine B- and T- lymphocytes was analyzed. Total spleen cells were labeled with B- and T-lymphocyte specific markers (B220 and Cd3 respectively) and sorted by FACS. Total RNA was isolated and processed in the same way as for myeloid cells. As shown in Figure 9, *Klf4* signal was detected in B-lymphocytes, but not in T-lymphocytes.

The same samples were used for amplification of the *Icsbp* transcript. As shown in Figure 9, *Klf4* expression profile in immune cells matches *Icsbp* expression profile, further suggesting that these two proteins are, independently or in cooperation, involved in the same cellular processes.

Moreover, in mature bone marrow macrophages both Icsbp and Klf4 can be up-regulated by interferon γ, important macrophage activation factor (Figure 10).

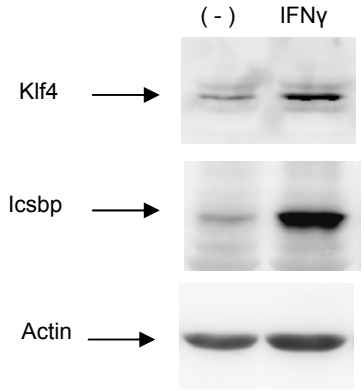


Figure 10: Icsbp and Klf4 are up-regulated by interferon γ
Primary bone marrow macrophages were grown from wild type mice (as described in Materials and methods) and treated 16 hours with 100U/ml interferon γ (IFNγ). Total protein extracts from treated and control cells were separated by SDS-PAGE and the blots were probed with specific anti-Klf4, anti-Icsbp and anti-Actin antibodies. One representative out of three independent experiments is shown.

5.2. Analysis of the hematopoietic system in Klf4$^{-/-}$ mice

Since Klf4 over-expression in the bone marrow progenitors enhances macrophage maturation, the question was raised whether loss of Klf4 has opposite effects.

Klf4$^{-/-}$ mice were independently created and described by two groups (Segre et al., 1999 and Katz et al., 2002). Both groups reported that these mice are born at normal Mandelian ratio, but die shortly after birth. Segre and al. attribute the death of the mice to defective keratinocyte terminal differentiation and consequent loss of the skin barrier function (Segre et al., 1999). Katz and al. reported defective differentiation of colon goblet cells in one day old mice (Katz et al., 2002). In both Klf4$^{-/-}$ models, terminal cell differentiation was disturbed, but further investigation was disabled by the early death of mutant mice. Conditional Klf4 knock-out model, with deletion induced specifically in the gastric compartment confirmed that Klf4 plays a role in cell differentiation – these mice had precancerous changes in their stomachs caused by altered cell proliferation and differentiation (Katz et al., 2005).

The mice with *loxP* flanked *Klf4* allele (created as described in Katz et al., 2002) were a kind donation from the laboratory of Klaus Kästner (University of Pennsylvania School of Medicine, Philadelphia, USA) and were used for our study of the Klf4 function in the hematopoietic system.

In the course of work on this thesis, several reports were published connecting Klf4 with the macrophage function. Klf4 expression was observed in J774a macrophage cell line, where it was implied to mediate proinflammatory signaling (Feinberg et al., 2005). In THP1 (monocytic cell line) and HL60 promyelocytic cell line) Klf4 was shown to regulate the expression of macrophage-specific integrin Cd11d (Noti and al., 2004). Chen et al. show Klf4 to be the target gene of interferon γ, important macrophage activation factor (Chen et al., 2002). The gene expression profiling of isolated hematopoietic progenitor populations shows high *Klf4* expression exclusively in the progenitors of the myeloid lineage (Terszowski et al., 2005), suggesting that this protein has a specific role in the myeloid development. However, the role of Klf4 in myelopoiesis has not been studied yet.

5.2.1. Conditional Klf4 deletion in *Klf4*$^{fl/fl}$ mice

In order to analyze the effect of Klf4 deletion in hematopoiesis, mice with floxed *Klf4* allele (containing *loxP* sites in introns 1 and 3) were cross-bred with heterozygous mice transgenic for *Cre*-recombinase under the control of interferon α/β responsive *Mx* promoter. Deletion of *loxP* flanked region was induced in 4-6 weeks old mice by injecting intraperitoneally 250μg of synthetic double stranded RNA compound poly(I)poly(C). Injections were repeated 3 times in 48h intervals. Double stranded RNA molecules like poly(I)poly(C) provoke antiviral immune response in the host. As part of an invoked immune reaction, endogenous interferon α/β is produced, which activates *Mx* promoter, the expression of Cre recombinase and subsequent deletion of *loxP* flanked region in all tissues responsive to interferon α/β. Reportedly, *Mx* promoter driven Cre-recombinase gives most rapid deletion in the liver, followed by bone marrow, spleen, lungs and kidneys (Radtke et al., 1999). Apart from these organs, deletion was shown in lymph nodes, thymus, heart and brain. Partial deletion also takes place in skin and intestines, organs reported to have the strongest defects in constitutive Klf4 knock-out mice. However, since Klf4$^{+/-}$ mice were histologically and phenotypically normal, it was

assumed that partial deletion of Klf4 in skin and intestines after poly(I)poly(C) injection would not cause defects.

Wild type MxCre$^{+/-}$ animals were treated with poly(I)poly(C) in the same way as Klf4$^{fl/fl}$ littermates and used as controls in experiments.

In order to test the efficiency of deletion in hematopoietic tissues, liver, spleen, bone marrow, lymph nodes and thymus were isolated from the mice 7 days after the last poly(I)poly(C) injection. Genomic DNA was isolated and analyzed by PCR with described genotyping primers (Katz et al., 2002). As shown in Figure 11, complete deletion in liver and bone marrow is observed, detected as the 425 bp fragment after PCR amplification. In spleen, thymus and lymph node, apart from the 425bp deletion band, the floxed allele 296 bp band is also present, meaning that *Klf4* deletion in these two organs is only partial.

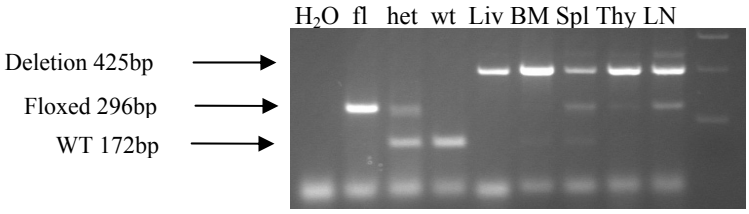


Figure 11: Deletion of *Klf4* in hematopoietic tissues 7 days after poly(I)poly(C) injection (on a genomic DNA level)
The organs were isolated 7 days after injecting the third dose of poly(I)poly(C); genomic DNA was isolated from the organs and PCR typing was performed with described primers (Katz et al., 2002). One representative analysis from six mice analyzed is shown.

For this study, it was of particular importance to determine whether Klf4 deletion efficiently takes place in bone marrow progenitor cells. In order to test this, total bone marrow cells were harvested 2 months after the last poly(I)poly(C) injection and lineage committed cells (determined by the expression of surface lineage markers) were depleted by MACS, as described in Materials and Methods. Primitive progenitors, negative for any lineage marker expression (Lin$^-$) were used for total RNA and genomic DNA isolation. As shown in Figure 12, PCR from genomic DNA gave 425 bp product, showing the deletion of the floxed region, while RT-PCR gave no signal in the same cells, confirming that *Klf4* transcription in primitive bone marrow progenitors is abolished.

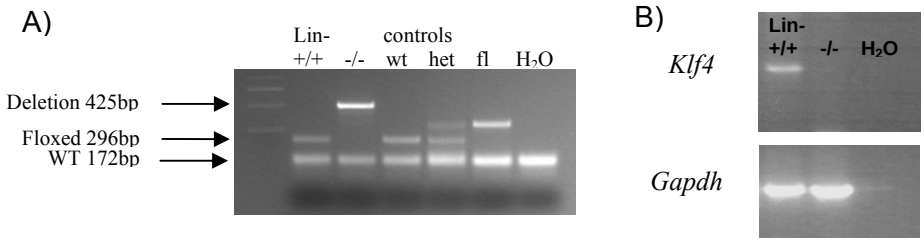


Figure 12: Efficient *Klf4* deletion in Lin$^-$ bone marrow progenitors
Lineage negative cells were selected by MACS. Genomic DNA and total RNA were isolated from purified lineage negative cells. A) PCR typing of genomic DNA; B) RT-PCR with *Klf4* and *Gapdh* specific primers. One representative out of three independent experiments is shown.

5.2.2. Analysis of the immune cell distribution in hematopoietic tissues shows no overt deregulation in hematopoiesis of Klf4$^{-/-}$ mice

In order to analyze the distribution of immune cells in hematopoietic tissues, cell suspensions from bone marrow, spleen, thymus and lymph node of Klf4$^{fl/fl}$MxCre$^{+/-}$ mice (2 months after poly(I)poly(C) injection) were prepared as described in Materials and Methods. Peripheral blood samples were taken from the tail vein, and peritoneal cells were obtained by rinsing peritoneal cavity with cold PBS. Samples from age and sex matched wild type mice injected in the same way with poly(I)poly(C) were used as control.

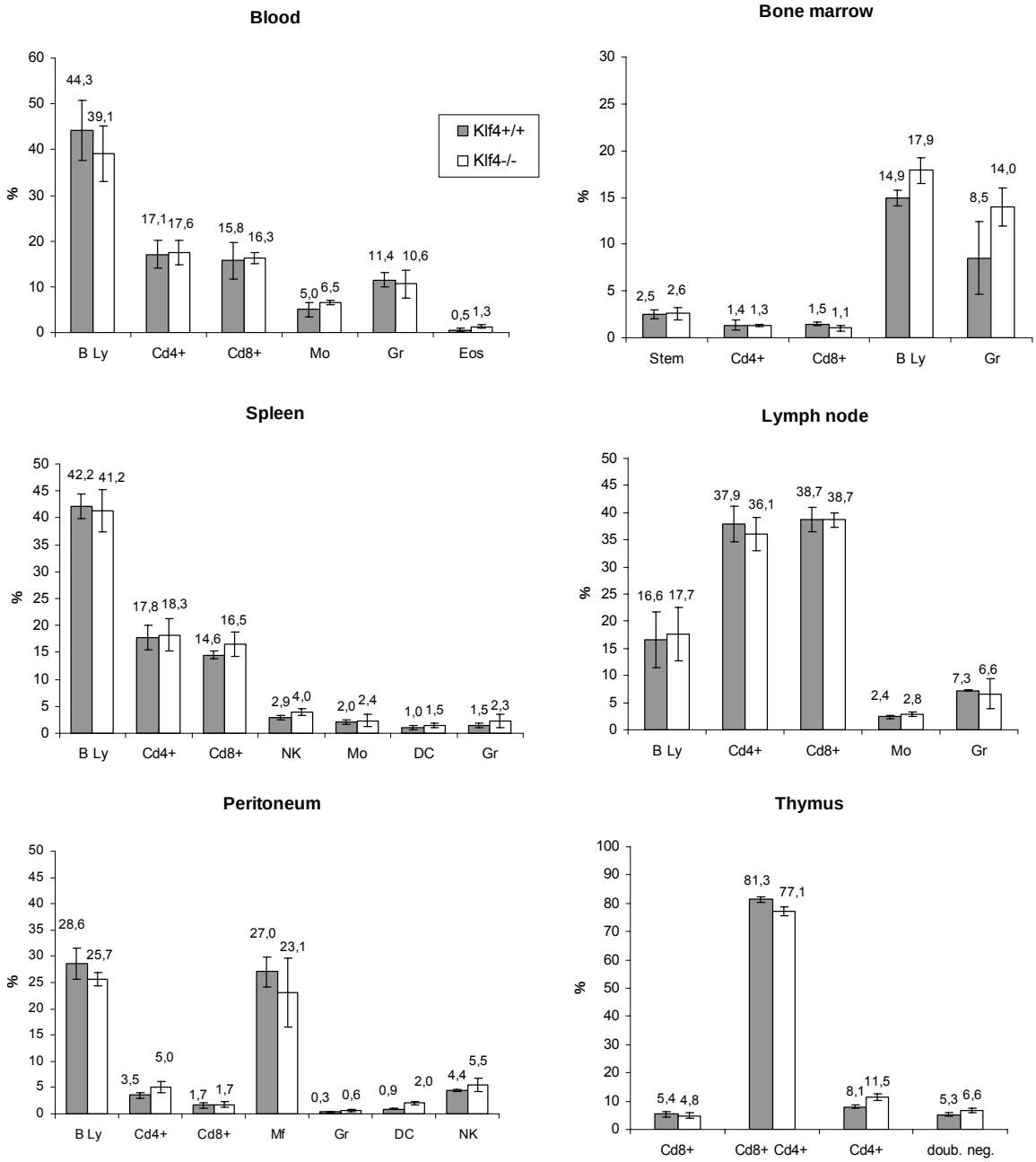


Figure 13: Immune cell distribution in hematopoietic tissues of Klf4$^{-/-}$ mice: a) peripheral blood; b) bone marrow; c) spleen; d) lymphocyte activation markers in spleen lymphocytes; e) lymph node; f) peritoneal lavage; g) thymus

Single cell suspensions were isolated from the organs of Klf4$^{-/-}$ and $^{+/+}$ mice, labeled with the fluorescent antibodies (as described in Materials and Methods) and analyzed by FACS. Six mice per group were analyzed.

Composition of prepared cell suspensions was analyzed by FACS (antibodies used are listed in Materials and Methods), and confirmed by microscopic examination of smears and cytospins stained according to May- Gruenwald-Giemsa (not shown). As shown in Figure 13, distribution of immune cells in all hematopoietic tissues tested was not perturbed by Klf4 deletion.

5.2.3. Clonogenic capacity and differentiation pattern of bone marrow progenitors is not altered by Klf4 deletion

As an additional test of Klf4 function in hematopoiesis, in particular in myeloid development, colony forming unit assay was performed with total bone marrow cells from Klf4$^{-/-}$ and Klf4$^{+/+}$ mice. Bone marrow cells were seeded (10^4 cells/ml) in methylcellulose medium supplemented with GM-CSF, M-CSF or G-CSF, SCF, TPO cytokine combination. The colonies were counted after 7 days and classified by standard morphologic criteria. As evident from Figure 14, Klf4 deletion changed neither the number of colonies formed, nor their differentiation pattern. These data suggest that the role of Klf4 in hematopoiesis is redundant and that the lack of Klf4 can be compensated.

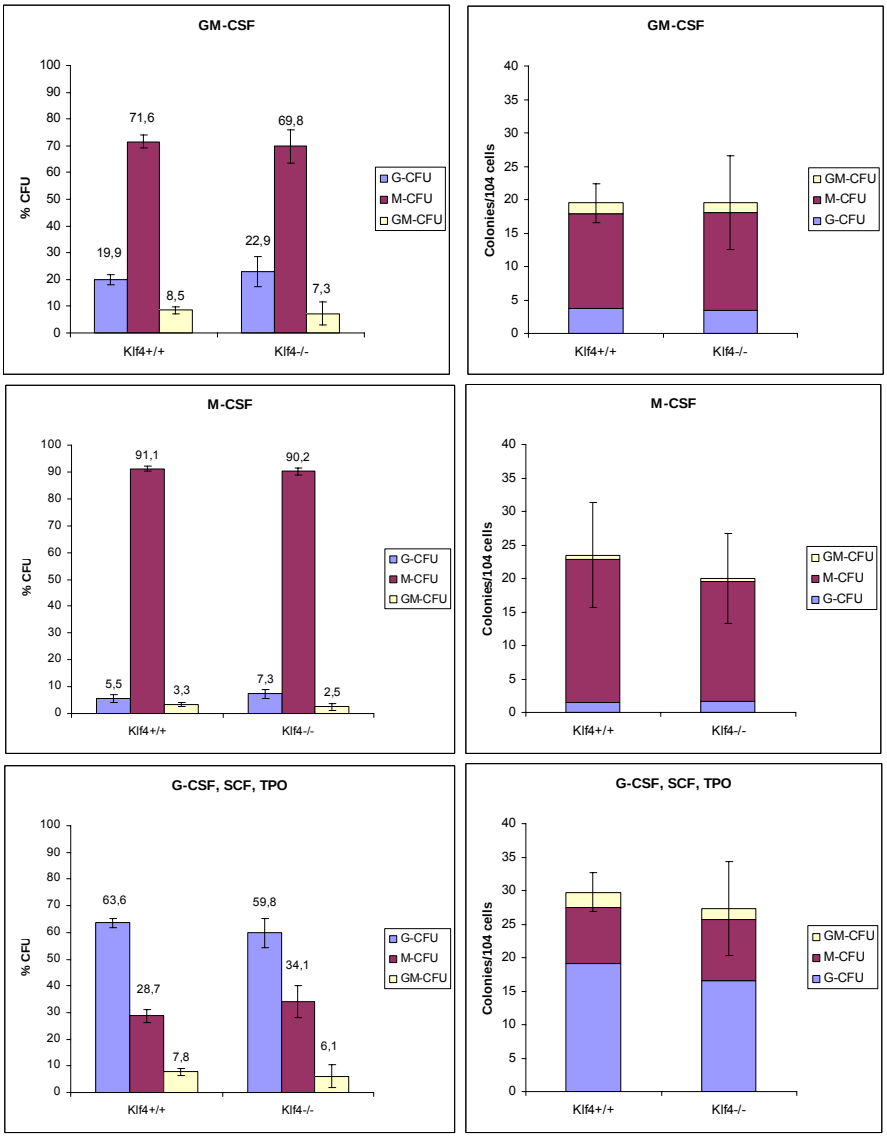


Figure 14: Differentiation potential of myeloid progenitors is not affected by Klf4 deletion
Bone marrow cells were isolated from Klf4$^{-/-}$ and $^{+/+}$ mice and plated in methylcellulose medium with indicated cytokines. Colonies were scored after 7 days, according to established morphological criteria

5.3. $p21^{Waf1}$ is a downstream target gene of Klf4

In order to analyze in more detail the mechanism of Klf4 action in myeloid development, we searched for the target genes that could explain the reduced colony formation and increased percent of cells differentiating along the macrophage lineage. Transcriptional profiling of NIH 3T3 cells over-expressing Klf4 was described previously (Chen et al., 2003). One of the genes up-regulated by Klf4 was $p21^{Waf1}$. Independently, Klf4 was shown to bind directly on $p21^{Waf1}$ promoter and up-regulate its expression (Zhang et al., 2000).

$p21^{Waf1}$ is best known for its ability to regulate cell cycle by interacting with cyclin/cdk complexes and PCNA. However, there are numerous reports of $p21^{Waf1}$ cell cycle independent functions, preferentially in regulating apoptosis and differentiation of cells. In the normal myelopoiesis, $p21^{Waf1}$ expression was shown to coincide with most highly proliferative stages (Steinman et al., 1998; Yaroslavskiy et al., 1999), indicating that its role in the cell development is more complex than mere cell cycle inhibition. Moreover, silencing of $p21^{Waf1}$ expression seems to be necessary for the terminal differentiation in some systems: forced $p21^{Waf1}$ expression in late keratinocyte differentiation inhibited the expression of the terminal differentiation markers (Di Cunto et al., 1998). Ghanem et al. showed that forced expression of $p21^{Waf1}$ has a proapoptotic effect on granulocytes developing from a murine 32Dcl3 myeloblast cell line (Ghanem et al., 2005).

On the other side, $p21^{Waf1}$ expression in monocytes seems to enhance their survival and differentiation. This is based on several observations. First, $p21^{Waf1}$ expression level does not reside in terminally differentiatiated macrophages as it does in granulocytes. High levels can still be detected, but this expression is shifted from nucleus to the cytosol (Asada et al., 1999). The role of cytoplasmic $p21^{Waf1}$ expression was suggested by the same author: $p21^{Waf1}$ was shown to physically interact with ASK1 (Apoptosis Signal-regulating Kinase1) in the cytosol of U937 cells differentiated into monocytes and inhibit stress-activated MAP kinase cascade resulting in apoptosis-resistant phenotype (Asada et al., 2004).

Taken together, previously published data suggest that $p21^{Waf1}$ plays an important role in the initial differentiation stages of all myeloid cells. It should be noted that at these stages, cells are not yet committed to granulocyte or macrophage lineage. Terminal differentiation of these two lineages (and possibly the commitment event itself) seems to have opposing requirements: full differentiation of monocytes and macrophages is assisted by high $p21^{Waf1}$ expression, while granulocyte development requires $p21^{Waf1}$ silencing.

Based on this it was hypothesized that sustained expression of Klf4 in common myeloid progenitors affects the differentiation program by up-modulating $p21^{Waf1}$ which would then antagonize terminal differentiation of granulocytes, while enhancing differentiation and survival of monocytes. The suggested mechanism is shown in Figure 15.

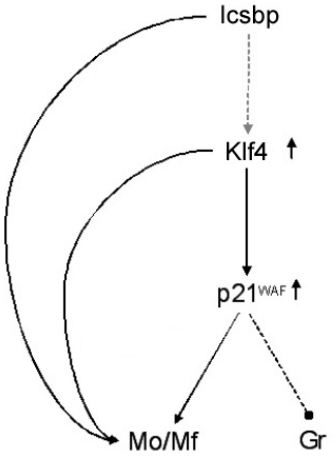


Figure 15: Suggested mechanism of Klf4 role in myelopoiesis
Abbreviations: Mo/Mf = monocytes/macrophages; Gr = granulocytes

In order to test this hypothesis, several experiments were performed:

- analyzing the $p21^{Waf1}$ expression in mature myeloid cells
- testing if $p21^{Waf1}$ expression is positively regulated by Klf4 in myeloid cells
- testing if $p21^{Waf1}$ over-expression results in switch towards macrophages (like Klf4 over-expression)
- testing if $p21^{Waf1}$ by itself, in the absence of Klf4 is capable of switching the differentiation towards macrophages

5.3.1. Expression of $p21^{Waf1}$ in mature immune cells is limited to monocytes/macrophage lineage

Involvement of $p21^{Waf1}$ in macrophage differentiation described in publications from Asada (Asada et al., 1999) and Ghanem (Ghanem et al., 2005) suggest that $p21^{Waf1}$ expression antagonizes terminal differentiation of granulocytes, while enhancing differentiation and survival of monocytes.

The first step in testing this hypothesis was to analyse the $p21^{Waf1}$ expression profile in mature immune cells. Primary macrophages, granulocytes, B- and T-lymphocytes were isolated by FACS (as described in Materials and Methods) and $p21^{Waf1}$ expression was analyzed by RT-PCR. Indeed, $p21^{Waf1}$ transcript was only detected in macrophages (Figure 16).

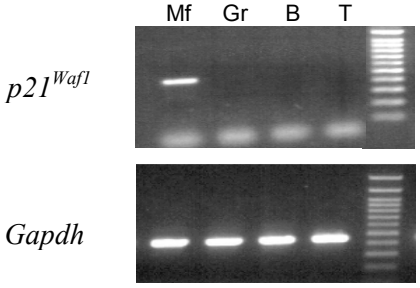


Figure 16: $p21^{WAF}$ expression in the immune cells
Mature primary immune cells were isolated by FACS as indicated in Figure 5. Total RNA was isolated and RT-PCR was performed. Abbreviations: Mf=macrophages; Gr=granulocytes; B=B-lymphocytes; T=T-lymphocytes. One representative out of two independent experiments done is shown.

5.3.2. Klf4 up regulates p21^{Waf1} expression in bone marrow macrophages

Klf4 up regulates p21^{Waf1} expression in different cell systems (Chen et al., 2003; Zhang et al., 2000; Rowland et al., 2005). In order to test if this is also the case in the primary bone marrow macrophages, p21^{Waf1} expression was compared in macrophages obtained from the wild type mice, Klf4$^{-/-}$ mice and macrophages transduced with *Klf4-ER^{T2}* construct. Bone marrow progenitors from wild type and Klf4$^{fl/fl}$ mice were transduced with empty vector, *Cre-ER^{T2}* or *Klf4-ER^{T2}* constructs, sorted for eGFP expression and differentiated into macrophages for 7 days in M-CSF conditioned medium (without 4-OHT addition). Mature macrophages were treated for 48 hours with 4-OHT in order to induce Cre-recombinase i.e. Klf4-ER^{T2} activity. After 48 hours, total RNA was isolated from the cells and analyzed by Northern blotting. As shown in Figure 17, induction of Cre-ER^{T2} function resulted in down-regulation of *Klf4* signal (lane 3), while Klf4-ER^{T2} over-expression gave a strong signal of the chimerical transcript (higher than native *Klf4* transcript). The down-regulation of *Klf4* was accompanied by down-regulation of *p21^{Waf1}*, while Klf4-ER^{T2} over-expression resulted in up-regulation of *p21^{Waf1}* mRNA. In order to show that Cre-recombinase did not influence the expression of analyzed genes, wild type cells transduced with Cre-ER^{T2} (lane 2) were used as a control.

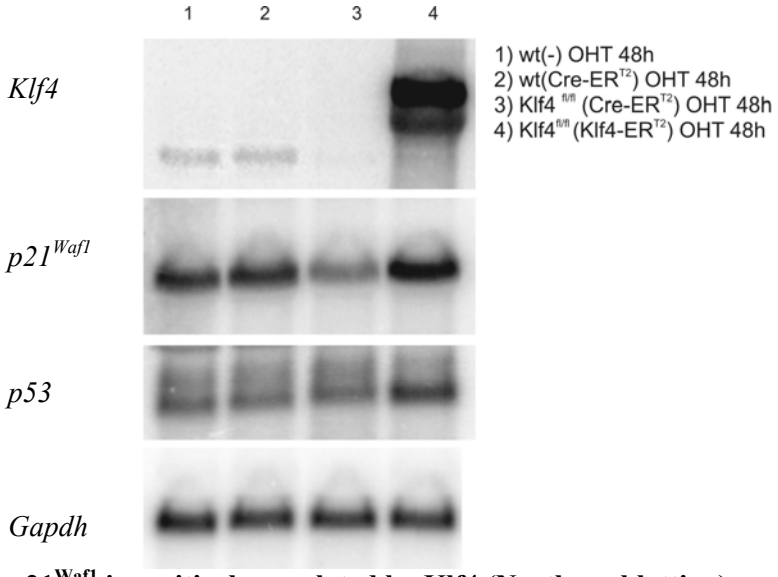


Figure 17: p21^{Waf1} is positively regulated by Klf4 (Northern blotting)
Bone marrow cells from wild type and Klf4$^{-/-}$ mice were transduced with empty vector, Cre-ER^{T2} or Klf4-ER^{T2} as indicated and differentiated into macrophages *in vitro*. After 7 days, the cells (mature macrophages) were treated with 4-OHT for 48h. The cells were harvested, total RNA was isolated and 10µg was used for Northern blotting. One representative out of three independent experiments is shown.

Regulation of p21^{Waf1} expression in cells is affected by many factors (review Gartel et al., 1999). One of the most important regulators is the tumor suppressor p53, responsible for maintaining G1/S cell cycle check point function and maintaining DNA integrity. According to the earlier reports, p53 directly binds to p21^{Waf1} promoter and up-modulates *p21^{Waf1}* expression (el-Deiry et al., 1993). The cooperation of p53 and Klf4 in regulating *p21^{Waf1}* expression was demonstrated by Zhang et al. (Zhang et al., 2000). They showed that these two proteins physically interact and synergistically

activate $p21^{Waf1}$ transcription in an embryonic fibroblast cell line. They also showed that Klf4 can be up-regulated by p53.

In order to test whether Klf4 effects the expression of p53, *p53* mRNA level was compared by Northern blotting in macrophages with normal (wild type) Klf4 expression level, macrophages over-expressing Klf4-ER^{T2} and macrophages with Klf4 deleted. As shown in Figure 17, *p53* is up-regulated in cells over expressing Klf4. The observed up regulation of $p21^{Waf1}$ is, therefore, most likely the result of both p53 and Klf4 effect on $p21^{Waf1}$ promoter. To further test the effect of $p21^{Waf1}$ in myeloid development, this protein was over-expressed in progenitor cells from the wild type mice.

5.3.3. Over-expression of $p21^{Waf1}$ in the bone marrow progenitors enhances macrophage differentiation

5.3.3.1 Constitutive $p21^{Waf1}$ over-expression

The hypothesis that $p21^{Waf1}$ is the target gene triggered by Klf4 and responsible (at least in part) for cell cycle inhibition and macrophage maturation observed when Klf4 is over-expressed, implicates that over-expression of $p21^{Waf1}$ by itself should cause the same changes in myeloid development. To test this, full length murine $p21^{Waf1}$ was cloned in a retroviral vector (as described in Materials and Methods) and transduced in bone marrow progenitors in the same way as Klf4 in previously described experiment (Section 5.1.1). Cells infected with an empty vector were used as a negative control. Transduced cells were plated in methylcellulose medium with G-CSF, SCF, TPO cytokine combination and the differentiation pattern was assessed by the CFU-assay. As shown in Figure 18, the percentage of macrophage-colony forming units (M-CFUs) was significantly increased in $p21^{Waf1}$ over-expressing cells, compared to the vector control cells. Additionally, the surface markers of cells isolated from the methylcellulose show an incline of the $F4/80^{+}$ fraction in $p21^{Waf1}$ over-expressing samples (Figure 19), indicating that constitutive high $p21^{Waf1}$ expression during myeloid development prompts the progenitor cells to differentiate along the macrophage lineage.

However, in comparison to 79.7% of M-CFUs formed when Klf4 is over-expressed (and 72.3% $F4/80^{+}$ cells according to the FACS analysis), p21 over-expression gave 62.2% M-CFUs (59.8% $F4/80^{+}$ cells).

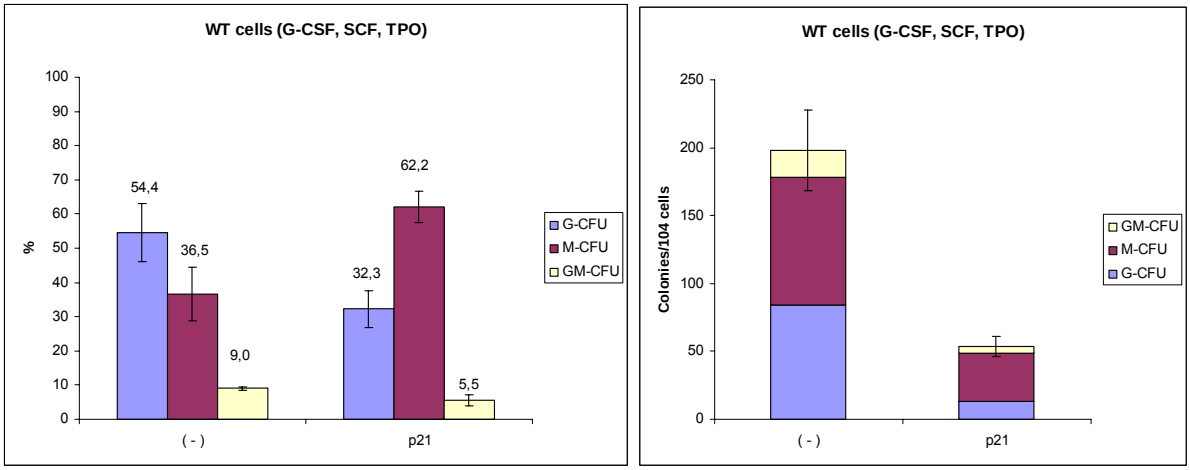


Figure 18: Over-expression of p21^{Waf1} increases percentage of macrophage colonies and reduces total colony formation
Bone marrow cells transduced with *p21^{Waf1}* or vector control were plated in methylcellulose medium with G-CSF, SCF and TPO. Colonies were scored after 7 days according to the established morphological criteria. A) Relative distribution of CFUs is shown. B) Absolute colony numbers are shown. Mean values with standard deviations from three independent experiments are shown. Statistical significance of the difference in total colony numbers, determined by two-tailed t-test is 0.019.

Bone marrow cells from wt mice transduced with **empty vector** or ***p21^{Waf1}*** and grown with G-CSF (SCF, TPO)

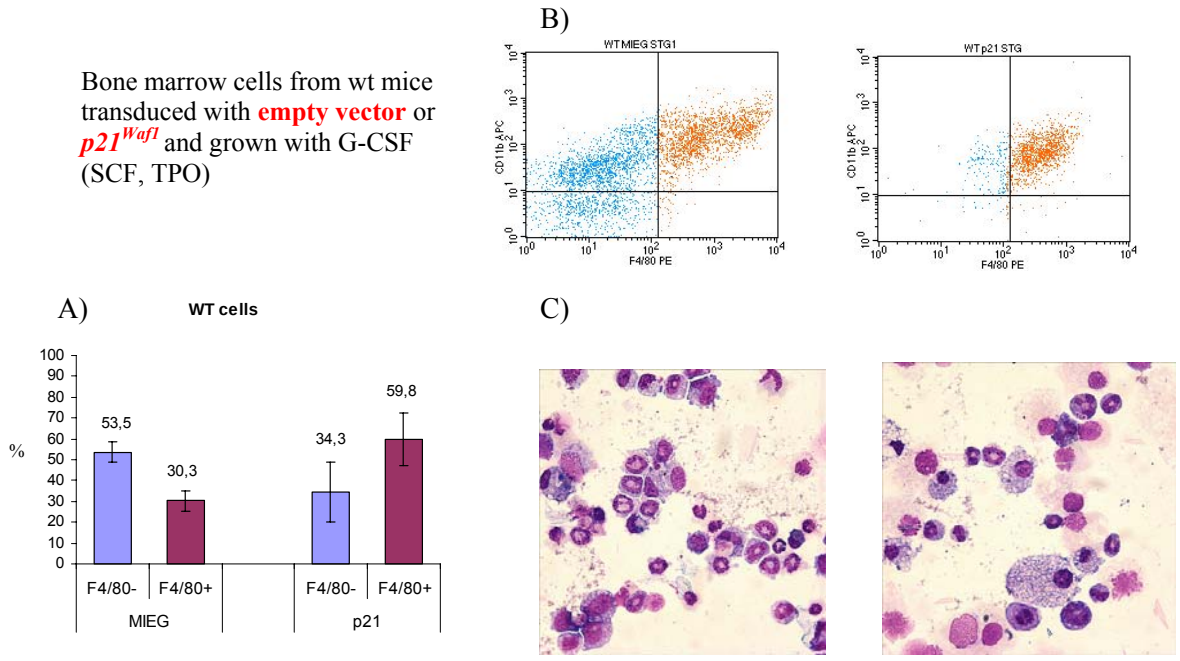


Figure 19: Over-expression of p21^{Waf1} increases macrophage maturation
Bone marrow cells from wild type mice were transduced with p21^{Waf1} or vector control and differentiated in methylcellulose medium supplemented with G-CSF, SCF, TPO. After 7 days, cells were isolated from methylcellulose, stained for Cd11b and F4/80 and analyzed by FACS. A) The mean values of Cd11b^{+}F4/80^{-} and Cd11b^{+}F4/80^{+} cells with standard deviations from three independent experiments are shown. B) Cd11b:F4/80 dot plots and C) corresponding microscope preparations from one representative experiment are shown.

The contribution of p21^{Waf1} to the reduced colony formation observed when Klf4 is over-expressed remains questionable. There are reports showing that p21^{Waf1} expression during normal myeloid development coincides with most highly proliferative states (Steinman et al., 1998), indicating that the p21^{Waf1} level and the cell quiescence do not necessarily correlate. Even though the deletion of a cell cycle inhibitor was expected to give increased cell proliferation, the bone marrow

progenitors from $p21^{Waf1}$ knock-out mice show reduced colony forming potential, probably because $p21^{Waf1}$ is necessary for maintaining the pool of hematopoietic stem cells (Cheng et al., 2000). Conversely, over-expression of $p21^{Waf1}$ was reported to increase colony formation of bone marrow cells (Braun et al., 1998).

In the experiment performed here, over-expression of $p21^{Waf1}$ led to average 3.5 fold reduction of the colony formation (Figure 18), which is milder than the Klf4 effect (10 fold reduction, Table 2). This suggests that $p21^{Waf1}$ is not solely responsible for the reduced clonogenicity of Klf4 over-expressing progenitors and that Klf4 has other, $p21^{Waf1}$ independent mechanisms to arrest cell proliferation. Previous publications show that Klf4 up-regulates the expression of other cell cycle inhibitors, like $p57^{Kip2}$, and down-regulates the expression of several cell cycle progression factors like cyclin D1, cdc2 and WEE1 (Chen et al., 2003). Additional reports shows that Klf4 directly binds to cyclin D1 promoter (Shie et al., 2000), cyclin B1 promoter (Yoon et al., 2004) and cyclin E promoter (Yoon et al., 2005) suppressing their transcription.

Taken together, the results of this experiment show that $p21^{Waf1}$ over-expression in progenitor cells leads to relative increase of macrophages and reduced overall colony formation comparable to the Klf4 over-expression, suggesting that Klf4 effect on differentiation and proliferation of myeloid cells could be mediated through $p21^{Waf1}$. However, although qualitatively in the same direction, the effects of Klf4 and $p21^{Waf1}$ differ quantitatively, with $p21^{Waf1}$ showing milder changes than Klf4. This indicates that Klf4 effects are not relying solely on $p21^{Waf1}$ up-regulation and that there are other, $p21^{Waf1}$ independent mechanisms triggered by Klf4.

5.3.3.2. $p21^{Waf1}$-ER^{T2} over-expression

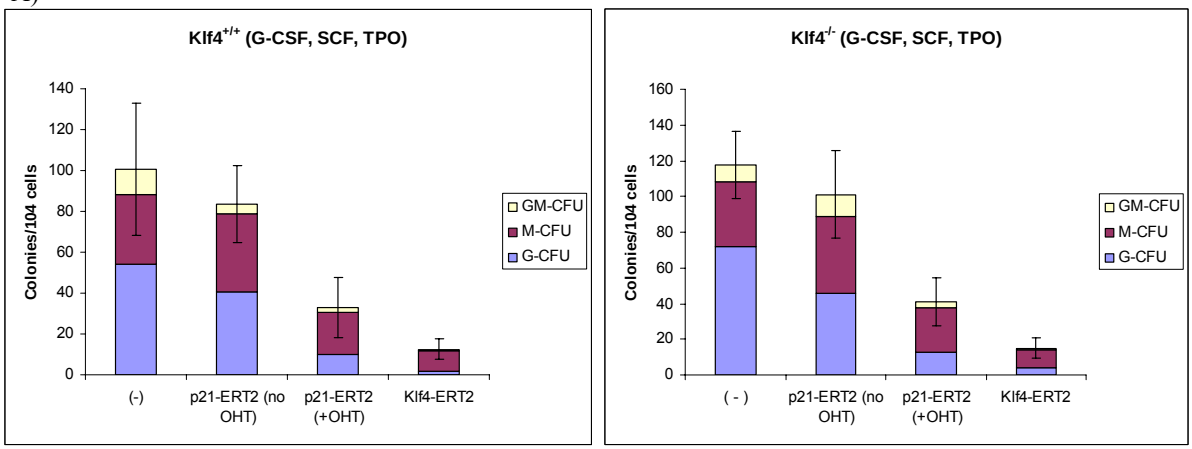
Since intracellular compartmentalization of $p21^{Waf1}$ plays a role in certain phases of myeloid cell development (Asada et al., 1999; Yaroslavskiy et al., 1999; Schepers et al., 2003), the feature of ER^{T2} chimerical proteins to localize in cytosol or nucleus depending on the presence of the ligand was used. Fusion *p21-ER^{T2}* construct was cloned in the MIEG3 retroviral vector (as described in Materials and Methods). Differentiation of cells transduced with this construct was analyzed both in the presence of 4-OHT in the medium, which leads to translocation of p21-ER^{T2} into the nucleus, or without 4-OHT, in which case p21- ER^{T2} remains in the cytosol.

Bone marrow cells were isolated from $Klf4^{fl/fl}$ and $Klf4^{+/+}$ mice 2 weeks after the last poly(I)poly(C) injection. Lineage committed cells were depleted from the total bone marrow by MACS (as described in Materials and Methods). Immature (lineage marker negative) progenitors were infected with retroviral construct carrying p21-ER^{T2} or vector-control in the same way as in the previous experiments and differentiated in methylcellulose medium with G-CSF, SCF, TPO cytokine combination with or without 4-OHT.

The analysis of formed colonies (Figure 20), as well as FACS analysis and microscope examination of cells differentiated in methylcellulose medium (Figure 21, Figure 22) show that both cytosolic and nuclear expression of p21-ER^{T2} led to an increase of macrophages in the culture.

However, the reduction of total colony numbers was observed only in the case of nuclear p21-ER^{T2} expression (Figure 20A).

A)



B)

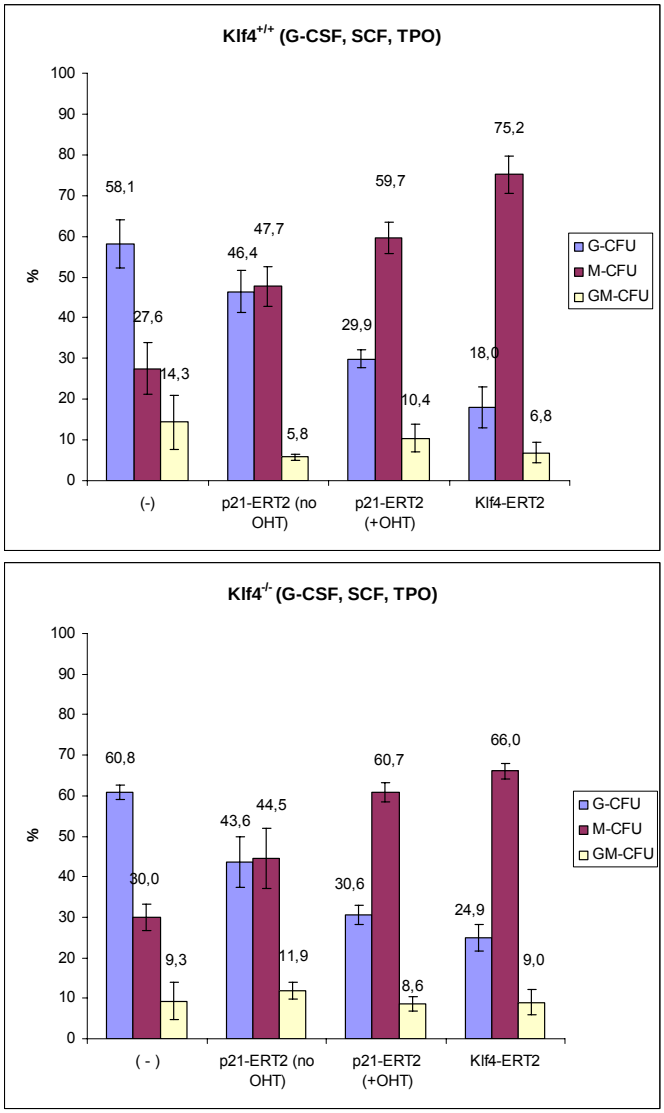


Figure 20: p21- ER^{T2} over-expression induces macrophage differentiation in Klf4$^{+/+}$ and Klf4$^{-/-}$ progenitor cells (CFU assay)

Purified lineage negative cells were transduced with p21-ER^{T2} construct or vector control and differentiated in methylcellulose medium with G-CSF, SCF and TPO, with or without 4-OHT (as indicated). Grown colonies were counted after 7 days. The mean values with standard deviations from three independent experiments are shown. A) Absolute colony numbers B) Relative distribution of CFUs

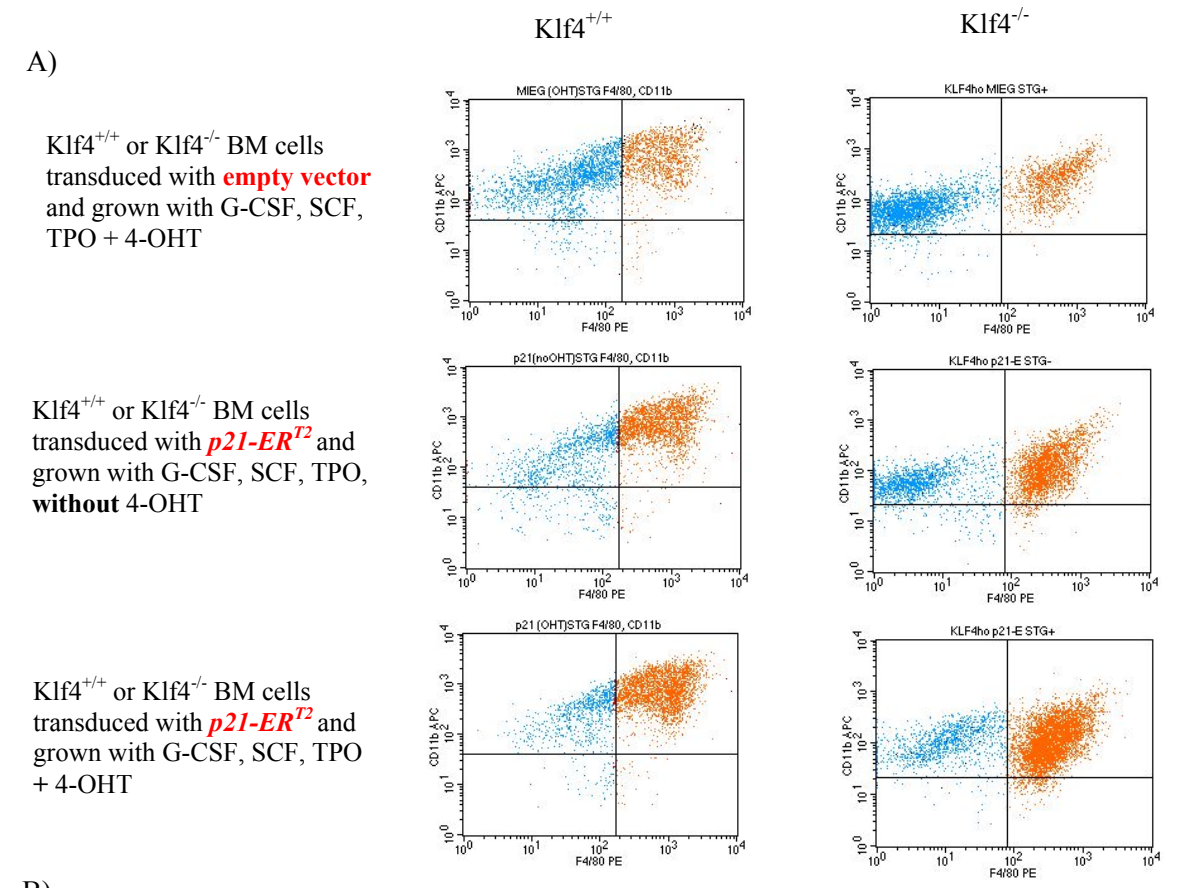


Figure 21: p21- ER^{T2} over-expression induces macrophage differentiation in Klf4$^{+/+}$ and Klf4$^{-/-}$ progenitor cells (cell surface marker analysis)

Progenitor cells transduced either with p21-ER^{T2} or vector control and differentiated in methylcellulose medium (as indicated on Figure 20) were isolated, stained for Cd11b and F4/80 and analyzed by FACS. A) Cd11b:F4/80 dot plots from one representative experiment are shown. B) The mean values of Cd11b^{+}F4/80^{-} and Cd11b^{+}F4/80^{+} cells with standard deviations from three independent experiments are shown.

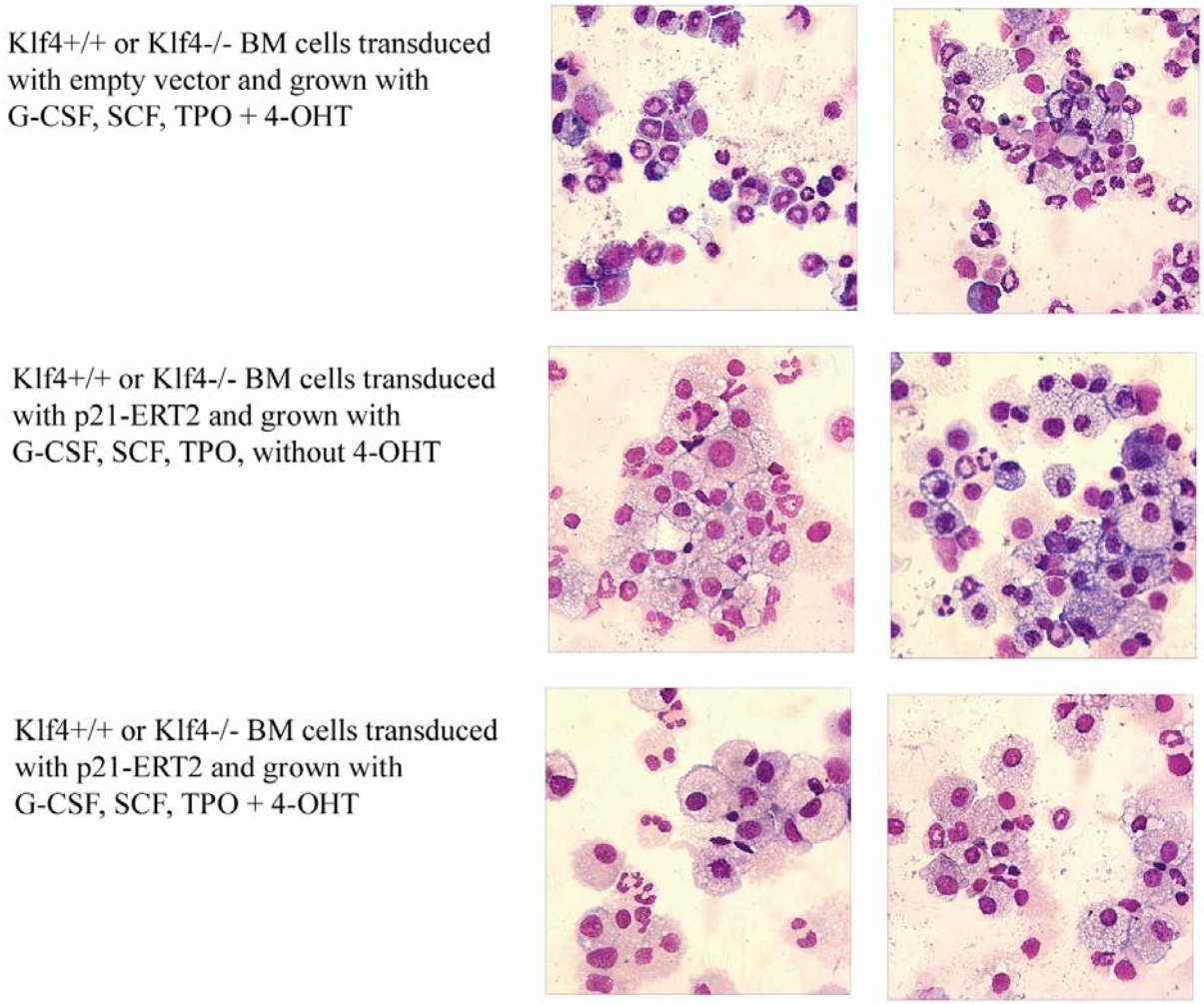


Figure 22: p21- ER^{T2} over-expression induces macrophage differentiation in $Klf4^{+/+}$ and $Klf4^{-/-}$ progenitor cells (microscope analysis)
Progenitor cells transduced either with p21-ER^{T2} or vector control and differentiated in methylcellulose medium with G-CSF, SCF, TPO were isolated, stained according to May-Grünwald-Giemsa and analyzed. The samples from one representative experiment are shown.

Together with the experiment described in the previous section, these results suggest that the roles of $p21^{Waf1}$ in cell proliferation and in cell differentiation might not be linked. For the proliferation control, nuclear localization of $p21^{Waf1}$ is necessary, while differentiation control processes are not strictly compartmentalized. In both cases it should be mentioned that compartmentalization of $p21^{Waf1}$ in this system is not absolute, since native $p21^{Waf1}$ present in both wild type and $Klf4^{-/-}$ cells (although reduced in the latter case), can freely change its location and compensate the lack of chimerical protein in the "depleted" compartment.

The concept of different $p21^{Waf1}$ functions determined by its subcellular localization was described by (Besson et al., 2004) where nuclear $p21^{Waf1}$ halts cell cycle progression, while cytoplasmic $p21^{Waf1}$ has an opposite function, contributing to oncogenesis.

5.4. Icsbp and Klf4 induce macrophage differentiation through independent pathways

The Affymetrix array analysis of granulocyte-monocyte precursor cells in Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice performed in our laboratory showed 10 fold down-modulation of *Klf4* transcript in the absence of Icsbp (unpublished data). There are other observations indicating that these two proteins are involved in the same processes in macrophages: 1) both Icsbp and Klf4 are up-regulated in response to interferon γ stimulation (Tsujimura et al., 2002; Chen et al., 2002) which reinforces development of the macrophage lineage; 2) expression pattern of *Klf4* and *Icsbp* in mature immune cells is the same (Figure 9); 3) over-expression of either Icsbp or Klf4 in progenitor cells results in increased macrophage differentiation.

However, luciferase reporter constructs under the control of 2 kb region upstream of the *Klf4* transcription start did not show increased activity when Icsbp was co-expressed (data not shown). Additionally, no difference in Klf4 expression was observed in macrophages from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice (Figure 23), indicating that Klf4 expression is not directly regulated by Icsbp. Therefore, it remains unclear whether these two factors act in the same signaling pathway in the myeloid development or independent from each other.

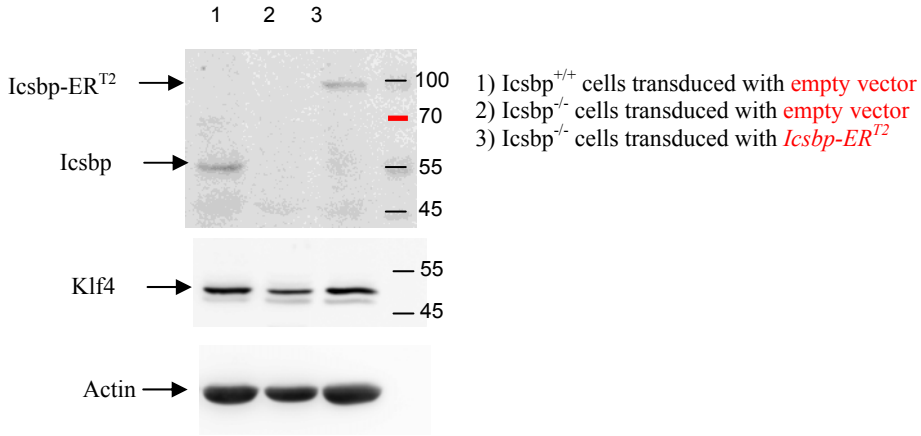


Figure 23: Klf4 expression is not affected by Icsbp in mature macrophages (Western blot)
Icsbp$^{+/+}$ or Icsbp$^{-/-}$ bone marrow progenitor cells were transduced with empty vector or Icsbp-ER^{T2} as indicated and differentiated into macrophages in 4-OHT-free medium (as described in Materials and Methods). Mature macrophages were stimulated with 4-OHT for 48 hours, harvested and total protein lysates were separated by SDS-PAGE on 5-15% gradient gel. Icsbp, Klf4 and Actin were detected using commercial antibodies.

5.4.1. Icsbp over-expression leads to macrophage development in Klf4$^{-/-}$ progenitors

Since Klf4 over-expression leads to a macrophage shift in both Icsbp$^{+/+}$ and Icsbp$^{-/-}$ cells (Figure 5), it could be assumed that Klf4 either acts downstream of Icsbp or through an independent pathway. In order to distinguish between these two possibilities, Icsbp was forcedly expressed in Klf4$^{-/-}$ progenitor cells and differentiation pattern was determined by CFU-assay.

Bone marrow cells were isolated from Klf4$^{fl/fl}$ mice 2 weeks after the last poly(I)poly(C) injection. Lineage negative cells were selected by MACS and transduced with retroviral constructs carrying *Icsbp-ER^{T2}* in the same way as in previous experiments. As negative control, Klf4$^{-/-}$ cells transduced with vector backbone were used. Transduced cells were plated in methylecellulose medium

with G-CSF (SCF, TPO) and 4-OHT. After 7 days, differentiation of cells was evaluated by the morphology of colonies (Figure 24), FACS analysis of cell surface markers and morphological microscope analysis (Figure 25).

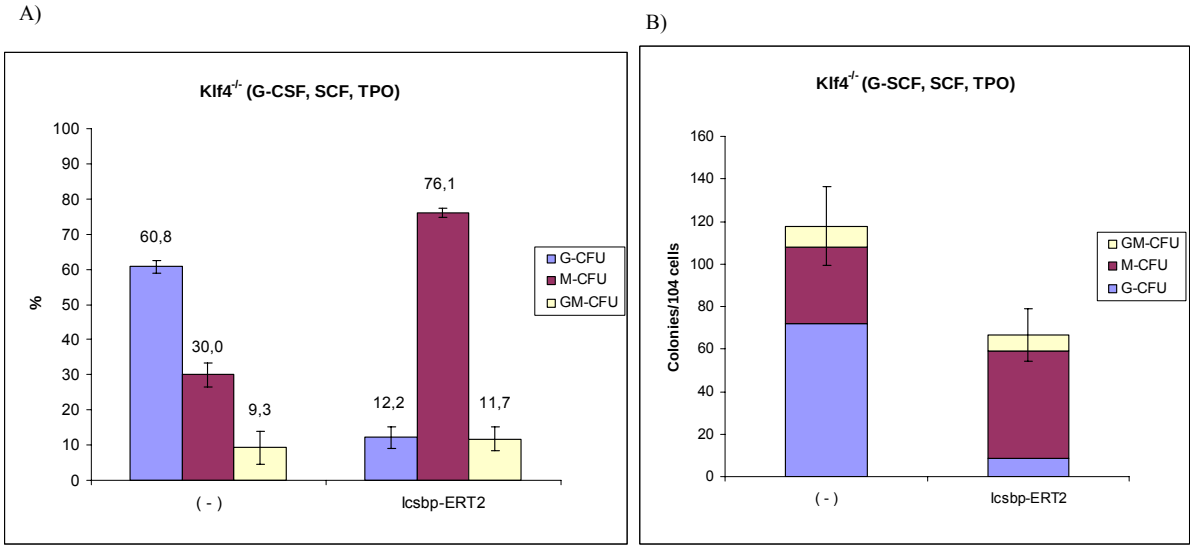


Figure 24: Icsbp over-expression leads to macrophage development in the absence of Klf4 (CFU assay)

Klf4$^{-/-}$ progenitor cells were transduced with *Icsbp-ER^{T2}* or vector control and differentiated in methylcellulose medium with G-CSF, SCF, TPO and 4-OHT. Colonies were counted after 7 days. A) Relative distribution of colonies. B) Absolute colony numbers.
The mean values with standard deviations from three independent experiments are shown.

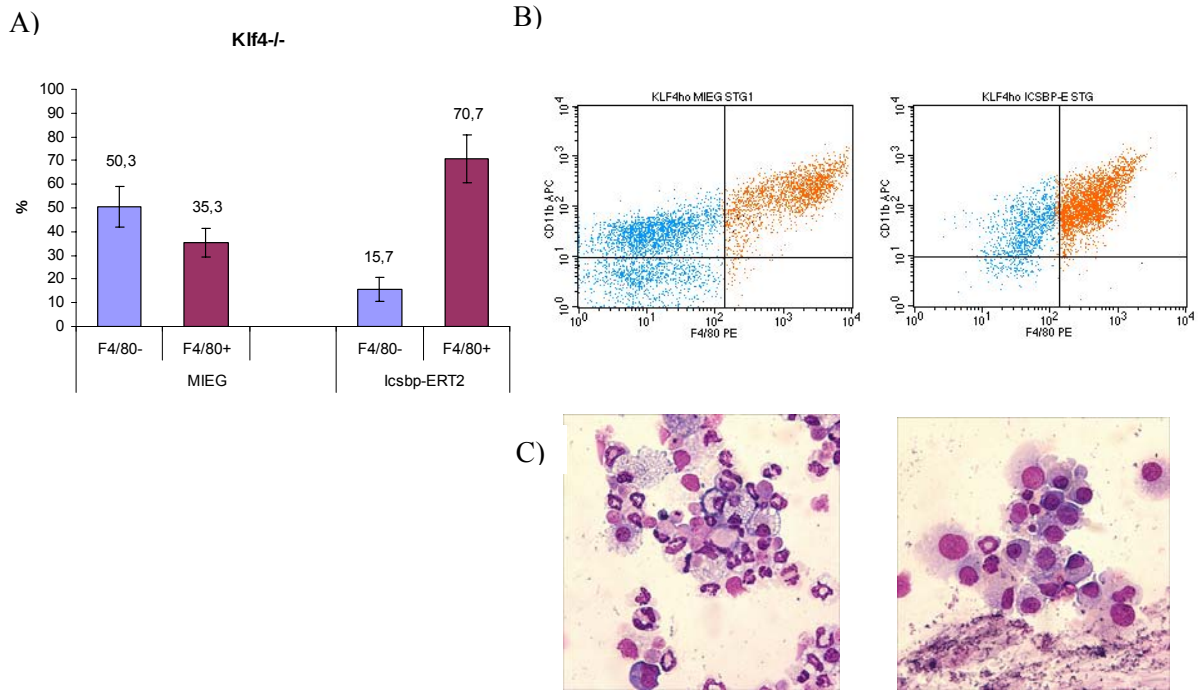


Figure 25: Icsbp over-expression leads to macrophage development in the absence of Klf4 (FACS analysis, microscope analysis)

Progenitor cells transduced either with *Icsbp-ER^{T2}* or vector control and differentiated in methylcellulose medium with G-CSF, SCF, TPO and 4-OHT were isolated, stained for Cd11b and F4/80 and analyzed by FACS. A) The mean values of Cd11b^{+}F4/80^{-} and Cd11b^{+}F4/80^{+} cell percentages with standard deviations from three independent experiments are shown. B) Cd11b:F4/80 dot plots and C) corresponding microscope preparations from one representative experiment are shown.

Icsbp over-expression in Klf4$^{-/-}$ cells resulted in increased percent of M-CFUs (76.1% in Icsbp-ER^{T2} sample and 30.0% in vector control sample), which is comparable with the effects observed in wild type cells (76.9% in Icsbp-ER^{T2} sample and 28.6% in vector control, Figure 5), indicating that Icsbp functions independently from Klf4 in myeloid differentiation. The results were confirmed by the analysis of surface cell markers by FACS (Figure 25).

Based on the experiments described, the model of Icasb and Klf4 involvement in myeloid differentiation is emerging. Initial assumption that Klf4 lies downstream of Icsbp in signaling events leading to macrophage development (Figure 15) was not experimentally supported. It is more likely that Icsbp and Klf4 function independently, possibly compensating each other in cells with Icsbp or Klf4 deletion. Alternatively, Icsbp and Klf4 signaling pathways could converge on the same down-stream effectors.

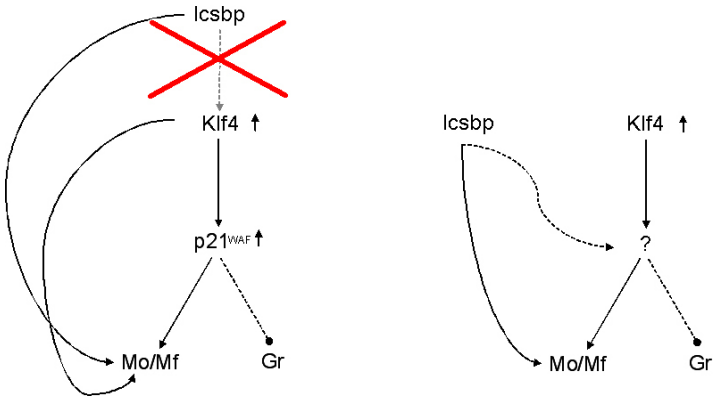


Figure 26: The scheme of suggested Klf4 role in myelopoiesis
Abbreviations: Mo/Mf = monocytes/macrophages; Gr = granulocytes

5.5. Analysis of the molecular mechanism of Klf4 activity in hematopoietic cells

5.5.1. The over-expression of Klf4-ER^{T2} actively induces macrophage differentiation program in uncommitted myeloid progenitors

As suggested in Section 5.1.2.2., the role of Klf4 in shifting the differentiation of myeloid cells toward macrophages could be explained by two potential mechanisms: 1) Klf4 could either function actively in the commitment process, inducing the macrophage differentiation program in bipotent progenitors or 2) Klf4 could act permissive, meaning that it does not affect the progenitor commitment, but acts after this stage by restricting the development and/or survival of cells committed to the granulocyte lineage, while allowing the survival of monocytes and macrophages. In case of Icsbp, the absolute number of macrophage colony forming units is increased when Icsbp is over-expressed and decreased when Icsbp is deleted, indicating that the role of Icsbp is inductive. In case of Klf4, the decision between two possible mechanisms mentioned above is complicated by the strong cytostatic effect of Klf4 over-expression: since it causes strong reduction in the proliferative capacity of cells, the resulting numbers of all three types of myeloid colony-forming units (G-CFUs, M-CFUs and GM-CFUs) are reduced. Additionally, Klf4$^{-/-}$ mice have unperturbed macrophage differentiation.

In order to reconcile the two possibilities, the differentiation of Klf4-ER^{T2} over-expressing cells was analyzed at the early stage (16 hours after inducing Klf4 function by 4-OHT). This time frame allows the added 4-OHT to diffuse through the cell membrane and initiate transcriptional activity of the chimerical Klf4-ER^{T2} protein, but does not allow the cell population to duplicate (determined by cell counting, data not shown). This means that the gene expression changes at this point are caused by Klf4 transcriptional activation and not by change of the cell type representation in the culture.

The bone marrow cells from wild type mice transduced with Klf4-ER^{T2} or vector controls were grown in liquid medium supplemented with G-CSF, SCF and TPO (without 4-OHT) for 72 hours. Subsequently, 4-OHT was added to the medium and the cells were grown for another 16 hours. After 16 hours, the cells were harvested, total RNA was isolated and applied on Affymetrix chips (analysis performed by Dr. Lars Bullinger, University of Ulm, Germany). In analyzing the data from the chip, particular attention was paid on lineage-specific genes. As a result, several macrophage specific transcripts (along with p21^{Waf1}) were increased more than 2 fold in the Klf4 over-expressing cells: scavenger receptors *Cd36, Marco* (Murphy et al., 2005) and oxidase important in processing phagocytosed senescent erythrocytes, *Ceruloplasmin* (Sarkar et al., 2003). On the other side, the expression of two granulocyte proliferation markers, *Cd177* (Stroncek et al., 2004) and *Mxd1* (Poortinga et al., 2004) was reduced (see Section 5.5.2, Figure 27).

This result suggests that the role of Klf4 in the differentiation is not just permissive, instead it actively taking part in the orchestration of the macrophage differentiation program by up-regulating the transcription of macrophage-specific genes and down-regulating the transcription of granulocyte-specific genes.

It should also be noted that pre-culturing the cells with G-CSF, SCF and TPO before 4-OHT stimulation primes the differentiation toward granulocyte lineage. The up-regulation of macrophage genes in thus treated cells further reinforces the conclusion that Klf4 plays active role in the commitment event of progenitor cells.

5.5.2. The p21-ER^{T2} over-expression leads to the same gene expression changes as Klf4-ER^{T2} over-expression

In order to further analyze the involvement of Klf4 downstream target gene p21^{Waf1} in the differentiation of myeloid cells, the gene expression profiling of p21-ER^{T2} over-expressing cells was performed by Affymetrix technology in the same way as described in the Section 5.5.1.

The bone marrow cells from wild type mice transduced with p21-ER^{T2} were grown in liquid medium supplemented with G-CSF, SCF and TPO (without 4-OHT). After 72 hours, the sample was divided into two fractions and one half was incubated with 4-OHT and the other without it. After 16 hours, the cells were harvested, total RNA was isolated and applied on Affymetrix chips. The gene expression pattern of p21-ER^{T2} transduced cells was compared to that of Klf-ER^{T2} transduced cells, using vector transduced cells were as a negative control. This analysis showed that both nuclear and

cytosolic p21^{Waf1} expression led to the same transcriptional changes (regarding myeloid-specific genes) as Klf4 over-expression, namely up-regulation of *Cd36*, *Marco* and *Ceruloplasmin* transcripts, and reduction of *Cd177* and *Mxd1*. The data from Affymetrix chips were additionally confirmed by quantification of named transcripts by real-time PCR (Figure 27).

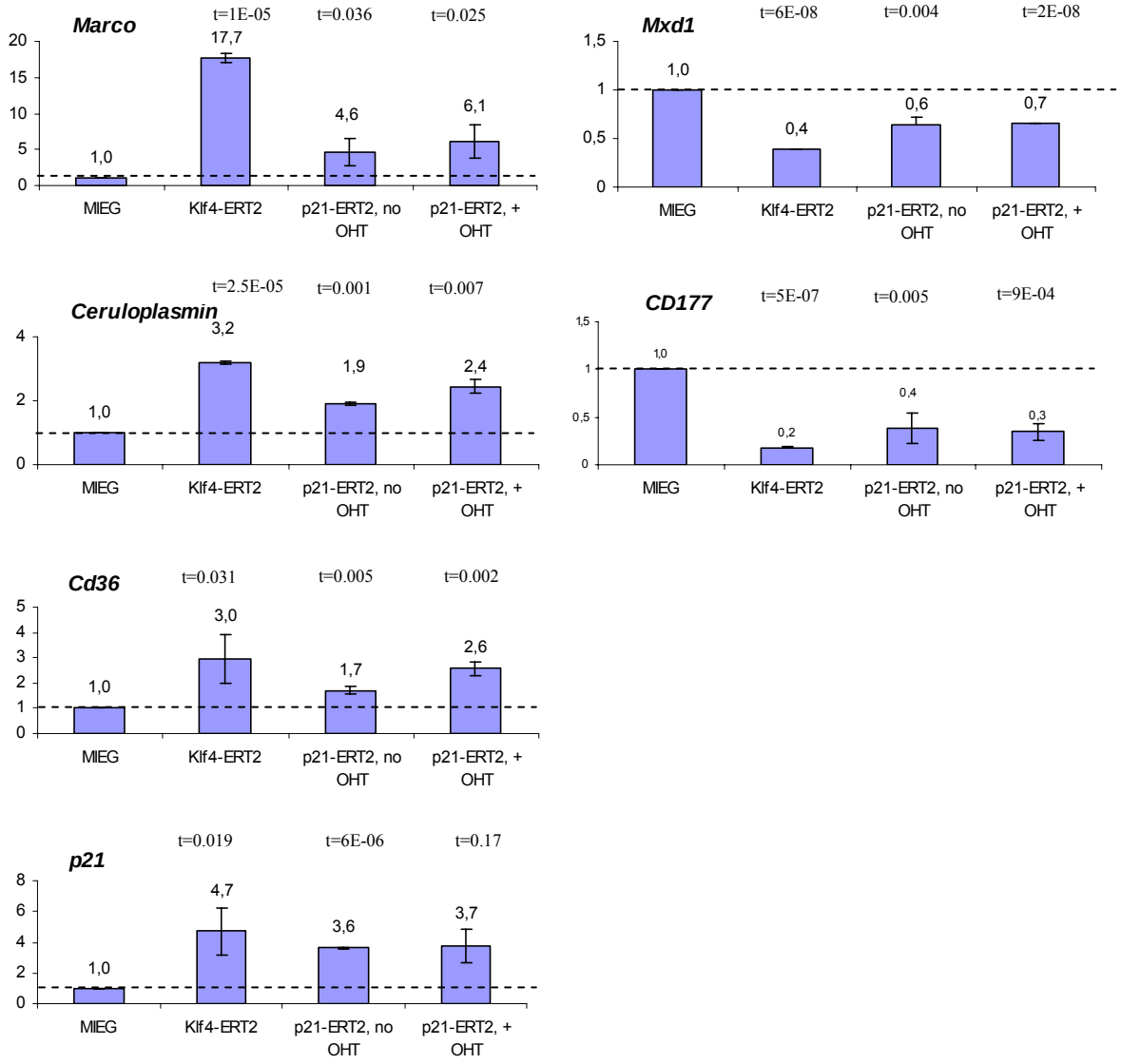


Figure 27: Quantification of lineage-specific transcripts in Klf4-ER^{T2} or p21-ER^{T2} over-expressing myeloid progenitors (real-time quantitative PCR)
The progenitor cells from wild type mice were transduced with *Klf4-ER^{T2}*, *p21-ER^{T2}* or vector controls and grown in liquid medium with G-CSF, SCF, TPO for 72 hours. Subsequently, 4-OHT was added and cells were stimulated for 16 hours (in case of p21-ER^{T2} over-expressing cells one fraction was further incubated without 4-OHT). The cells were harvested, total RNA was isolated, converted to cDNA and specific transcripts were analysed by quantitative real-time PCR.

This result shows that p21-ER^{T2}, apart from its cell cycle inhibitor function, plays a role in the myeloid cell differentiation by enhancing the differentiation of macrophages. This is in accordance with the results from the functional differentiation assay (Section 5.3.3) and further suggests that Klf4 effects in the differentiation could be mediated through p21-ER^{T2} up-regulation.

Similar to the data from functional CFU assay (Section 5.3.3.2), the effects of p21-ER^{T2} and Klf4-ER^{T2} over-expression are qualitatively in the "same direction", but they differ quantitatively. Namely, the intensity of p21-ER^{T2} induced changes in the myeloid gene transcription is reduced in comparison to Klf4-ER^{T2} induced changes. For example, Klf4-ER^{T2} over-expressing myeloid progenitors show 17.7 fold increase of the *Marco* mRNA, while progenitors with cytosolic and nuclear p21-ER^{T2} over-expression show 4.6 and 6.1 fold *Marco* increase respectively (Figure 27). We could speculate that this difference relies on the level of *p21^{Waf1}* transcript, which is higher in Klf4-ER^{T2} transduced cells than in p21-ER^{T2} transduced cells (Figure 27, Note: the primers used for *p21^{Waf1}* amplification do not distinguish between the endogenous *p21^{Waf1}*and transduced *p21-ER^{T2}*). This observation confirms that Klf4 acts as a very strong inducer of p21^{Waf1} expression and argues in favor of the p21^{Waf1} as a downstream mediator of Klf4-induced effects in the myeloid differentiation. Alternatively, the observed quantitative differences could be based on additional, p21^{Waf1} independent mechanisms that Klf4 employs in regulating gene transcription.

It should be mentioned that chimerical p21-ER^{T2} protein is expressed in the cytosol throughout the 72 hours pre-incubation period in G-SCF, SCF, TPO supplemented media. The data from the CFU-assay show that cytosolic p21-ER^{T2} expression under these conditions affects the differentiation process in the sense of enhancing macrophage maturation. Therefore, it cannot be excluded that higher macrophage and lower granulocyte transcript levels come from higher percentage of macrophages (or their committed precursors) in the analyzed cell population.

5.5.3. The analysis of the molecular mechanism of Klf4 activity by using Klf4 mutants

In described experiments it was hypothesized that macrophage differentiation and proliferative arrest observed when Klf4 is over-expressed are based on its ability to modulate the transcription of the target genes. In order to prove that Klf4 transcriptional activity is necessary for its role in macrophage differentiation and proliferation arrest, two deletion mutants of Klf4 were designed: Klf4$^{\Delta Zn}$ mutant, containing the N-terminal part of the protein while the zinc finger region was removed (amino acids 1-401) and Klf4$^{\Delta N}$ mutant, containing only the zinc finger region (amino acids 402-483).

Analysis of the Klf4 regions important for the transcriptional activation was described by Yet et al. (Yet et al., 1998) and Geiman et al. (Geiman et al., 2000). Both authors reported the presence of the strong transactivating domain in the N-terminus, while zinc finger region itself did not show any transcriptional activity.

Based on these reports, it was expected that neither mutant (Klf4$^{\Delta N}$ or Klf4$^{\Delta Zn}$) effects the transcription of its target genes and subsequent differentiation and proliferation of cells. Klf4$^{\Delta N}$ mutant (zinc finger region alone) is capable of DNA binding, but according to the previous publications, this is not sufficient for transcriptional activation. However, the blocking of the DNA binding sites and dominant negative effect remain a possibility. Klf4$^{\Delta Zn}$ mutant has the zinc finger region removed, therefore it cannot bind DNA and activate the transcription of its direct target genes. However, it can still interact with other proteins in the transcriptional machinery and indirectly effect the transcription.

The Klf4 deletion mutants (designated Klf4$^{\Delta N}$ and Klf4$^{\Delta Zn}$) are schematically shown in Figure 28. Although both constructs reportedly have nuclear localization capability (Shields et al., 1997), ER^{T2} region was fused to the C-terminus of the mutated *Klf4* sequence (as described in Materials and Methods). Fusion constructs were subcloned into MIEG3 vector, virus-containing supernatants were made (as described in Materials and Methods) and used for retroviral transduction.

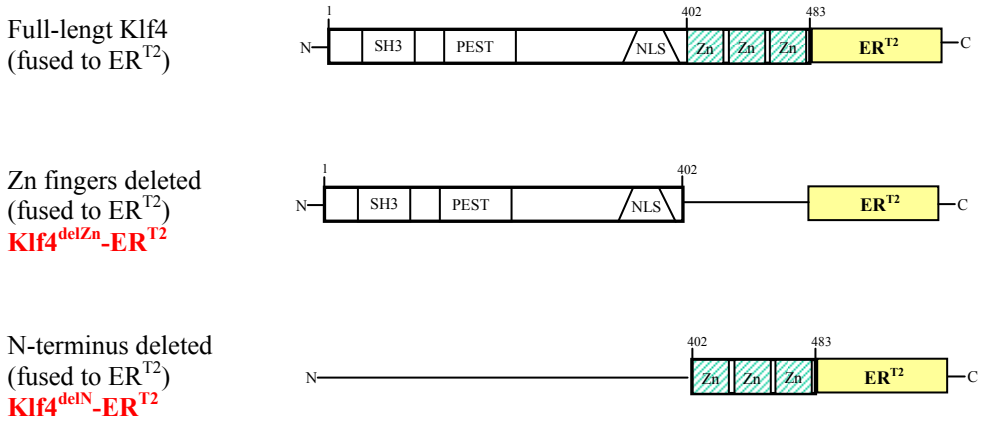


Figure 28: Scheme of the Klf4 deletion mutants
The mutant constructs were designed as described in Materials and Methods and subcloned into MIEG3 vector.

In order to test the effect of described Klf4 mutant constructs in myelopoiesis, bone marrow cells were taken from Klf4$^{+/+}$ and Klf4$^{-/-}$ mice, lineage negative cells were purified by MACS (as described in Materials and Methods) and infected with *Klf4*$^{\Delta N}$*-ER*^{T2} and *Klf4*$^{\Delta Zn}$*-ER*^{T2} carrying retrovirus. Empty virus backbone was used as negative control, and full length *Klf4-ER*^{T2} construct as positive control. Transduced cells (sorted for eGFP expression by FACS) were plated in methylcellulose medium containing 4-OHT (in order to ensure nuclear translocation of transduced constructs) and the G-CSF, SCF, TPO cytokine combination. After 7 days incubation, grown colonies were counted and morphologically analyzed. The results are shown in Figure 29 and Figure 30.

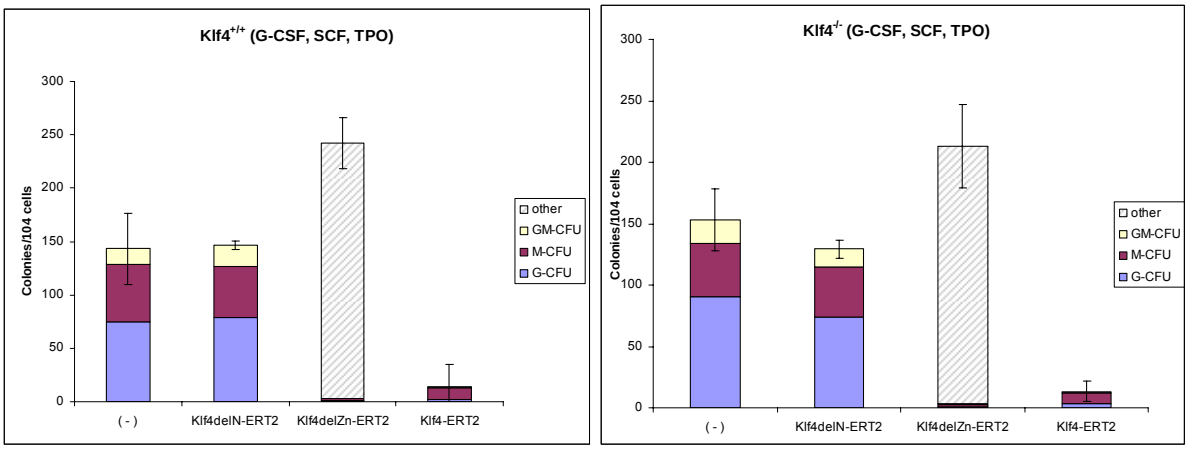


Figure 29: Analysis of the proliferative potential of myeloid progenitors expressing Klf4 deletion mutants, CFU-assay (total colony numbers)
Purified lineage negative cells were transduced with deletion mutants or vector control and differentiated in methylcellulose medium with G-CSF, SCF, TPO and 4- OHT. Grown colonies were counted after 7 days. The mean values with standard deviations from three independent experiments are shown.

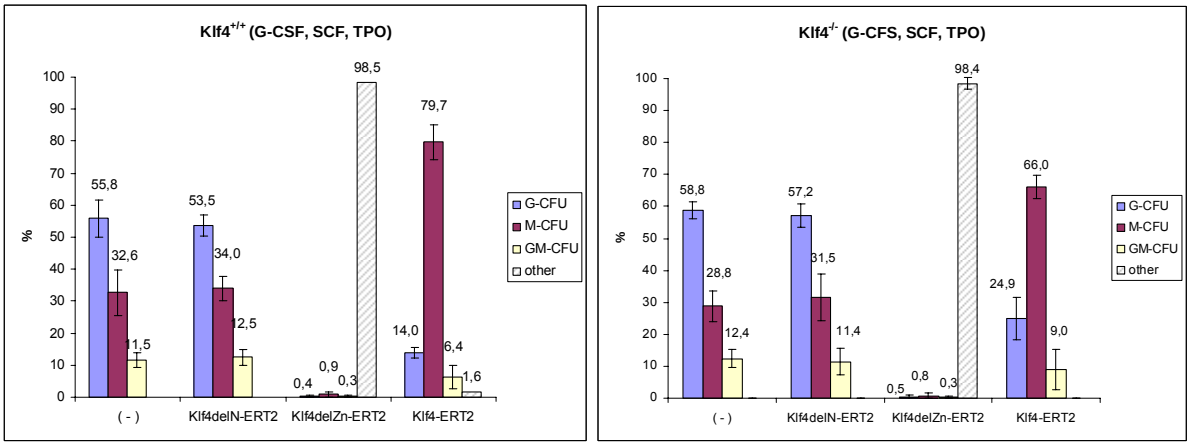


Figure 30: Analysis of the differentiation pattern of myeloid progenitors over-expressing Klf4 deletion mutants, CFU-assay (relative distribution of CFUs)
Purified lineage negative cells were transduced with deletion mutants or vector control and differentiated in methyl cellulose medium with G-CSF, SCF, TPO and 4-OHT. After 7 days the colonies were analyzed and identified according to the standard morphological criteria. The mean values with standard deviations from three independent experiments are shown.

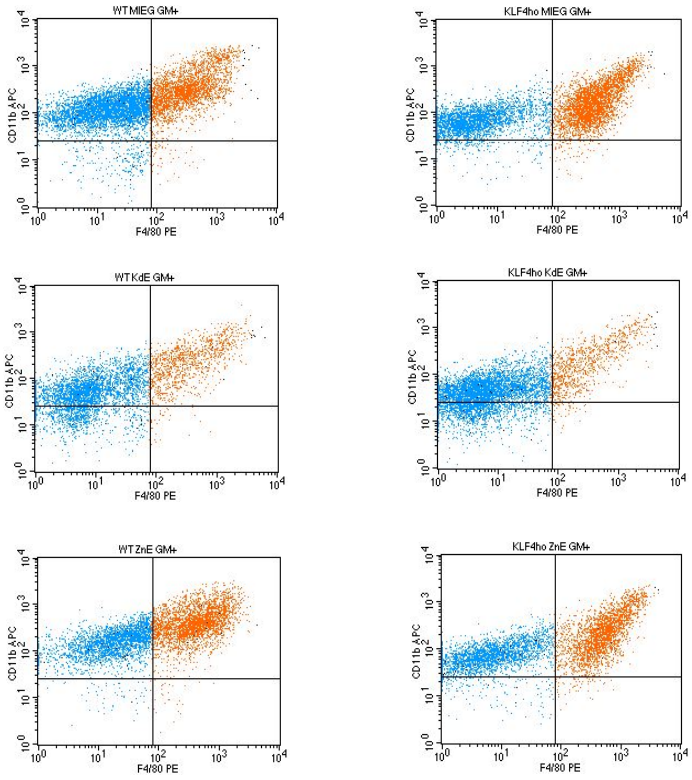
In case of Klf4$^{\Delta N}$-ER^{T2} over-expression, no difference in colony numbers or their morphology was detected in comparison to the vector control samples (Figure 29 and Figure 30) indicating that the Klf4 mutant with N-terminal part deletion does not effect the differentiation and proliferation of cells during myelopoiesis. FACS analysis of macrophage maturation markers (Figure 31) and microscopic examination of cells after May-Grünwald-Giemsa staining (Figure 32) confirmed this finding.

A)

$Klf4^{+/+}$ and $Klf4^{-/-}$ cells transduced with empty vector, grown with G-CSF, SCF, TPO + 4-OHT

$Klf4^{+/+}$ and $Klf4^{-/-}$ cells transduced with $Klf4^{delZn}$-ER^{T2}, grown with G-CSF, SCF, TPO + 4-OHT

$Klf4^{+/+}$ and $Klf4^{-/-}$ cells transduced with $Klf4^{delN}$-ER^{T2}, grown with G-CSF, SCF, TPO + 4-OHT



B)

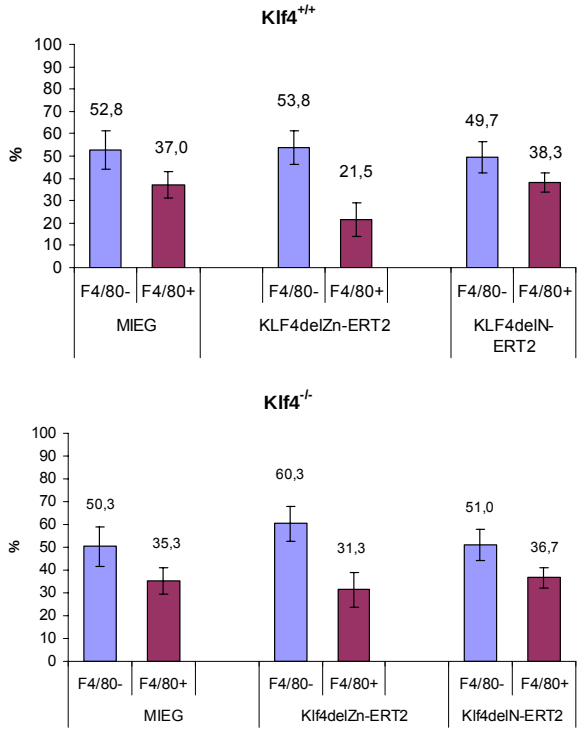


Figure 31: $Klf4^{\Delta Zn}$-ER^{T2} transduction reduces, while $Klf4^{\Delta N}$-ER^{T2} transduction does not effect the expression of macrophage maturation markers (FACS analysis)

Progenitor cells transduced with deletion mutants or vector control were differentiated in methylcellulose medium with G-CSF, SCF, TPO and 4-OHT. After 7 days the cells were isolated, stained for Cd11b and F4/80 and analyzed by FACS. A) Cd11b:F4/80 dot plots from one representative experiment are shown. B) The mean values of $Cd11b^{+}F4/80^{-}$ and $Cd11b^{+}F4/80^{+}$ cell percentages with standard deviations from three independent experiments are shown.

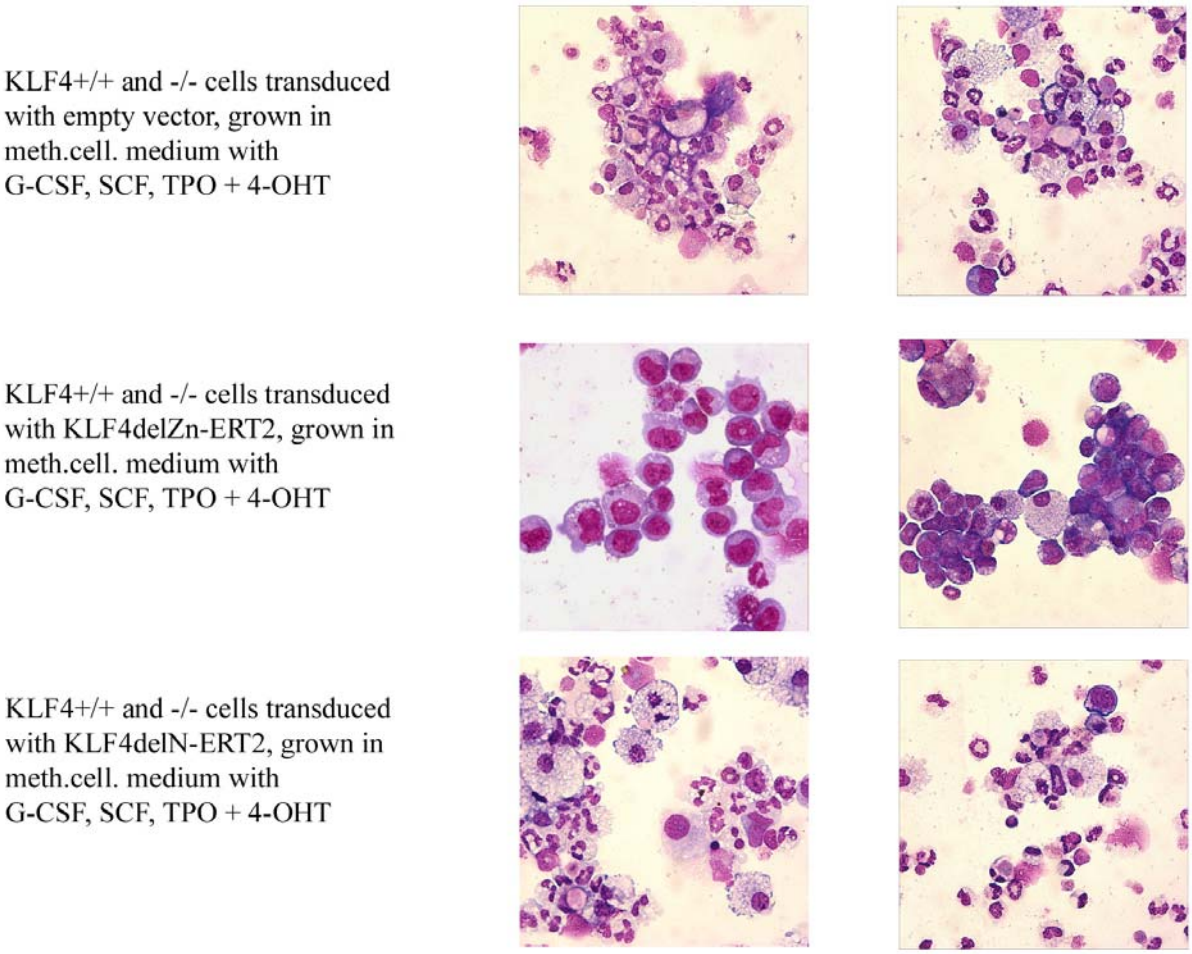


Figure 32: Klf4$^{\Delta Zn}$-ER^{T2} transduction reduces, while Klf4$^{\Delta N}$-ER^{T2} transduction does not effect macrophage maturation (microscope analysis)
Progenitor cells transduced with deletion mutants or vector control were differentiated in methylcellulose medium with G-CSF, SCF, TPO and 4-OHT. After 7 days the cells were isolated, stained according to May-Grünwald-Giemsa and analyzed. The microscope preparations from one representative experiment are shown.

Unexpectedly, transduction of cells with the Klf4 mutant with the zinc finger region removed resulted in deficient cell development. The morphology of the colonies grown from Klf4$^{\Delta Zn}$-ER^{T2} transduced progenitors with GM-CSF or G-CSF, SCF, TPO combination was grossly aberrant and could not be classified into any of the three defined categories (G-CFU, M-CFU or GM-CFU). These colonies (designated as “other” in Figure 29 and Figure 30) were large, compact and without any sign of cell migration through the medium (Figure 33B and Figure 33F).

The number of colonies was slightly increased when Klf4$^{\Delta Zn}$-ER^{T2} transduced cells were grown with G-CSF, SCF, TPO combination (average 1.7 fold) (Figure 29). M-CSF did not support the growth of these cells; very few colonies were detected with this cytokine and their size was significantly reduced (data not shown).

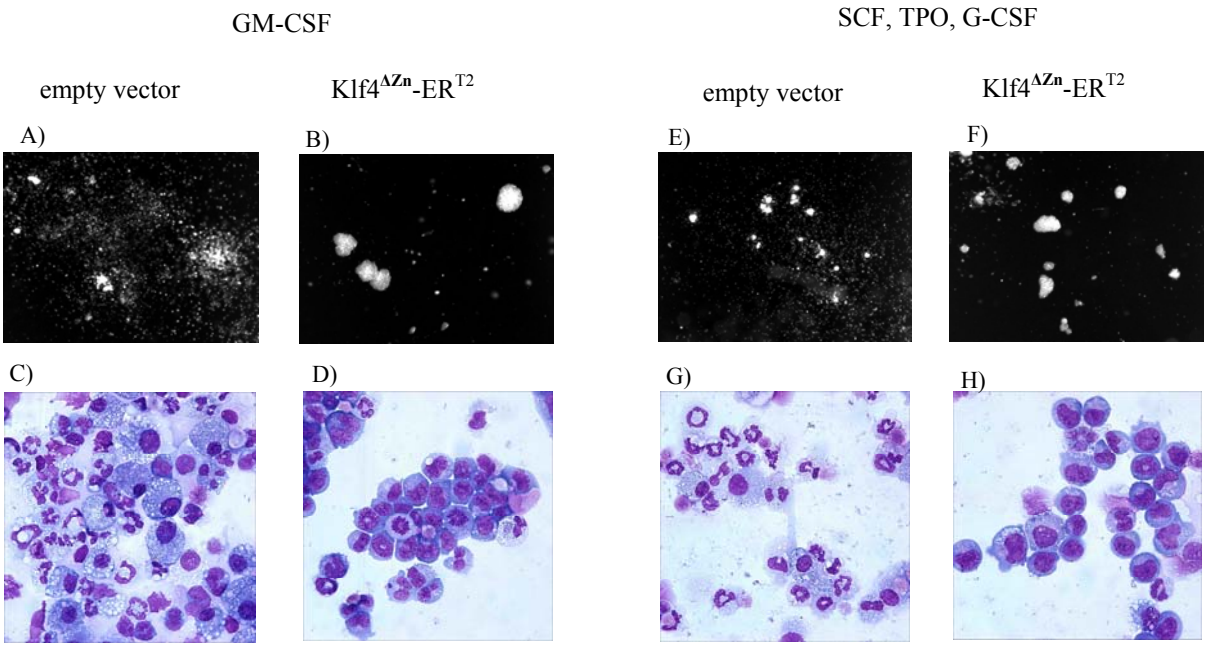


Figure 33: Klf4$^{\Delta Zn}$-ER^{T2} transduction causes aberrant colony formation and myeloid maturation block

A), B), E) and F) The colonies grown in methylcellulose medium with 4-OHT and indicated cytokines C), D), G) and H) Microscope slides of isolated cells are shown (from one representative out of three independent experiments performed).

FACS analysis of Klf4$^{\Delta Zn}$-ER^{T2} transduced cells grown in the methylcellulose medium with G-CSF, SCF, TPO combination (Figure 31), showed strong reduction of F4/80 expression, indicating reduced maturation of cells into macrophages. Additionally, the intensity of Cd11b expression was reduced (displayed as shift of the Cd11b positive cloud downwards), which is the characteristic of immature myeloid progenitors. Indeed, microscopic examination of the cell morphology after May-Grünwald-Giemsa staining (Figure 33D and Figure 33H) showed that Klf4$^{\Delta Zn}$-ER^{T2} transduced cells uniformly show the morphology of immature myeloid progenitors.

The same effect of Klf4$^{\Delta Zn}$-ER^{T2} transduction was observed in both Klf4$^{-/-}$ and Klf4$^{+/+}$ cells. Since native Klf4 in the cells was not able to rescue the effect of Klf4$^{\Delta Zn}$-ER^{T2} transduction it could be concluded that this construct acts as dominant negative.

In order to analyze the proliferative capacity of Klf4$^{\Delta Zn}$-ER^{T2} transduced cells, they were isolated from the G-CSF, SCF, TPO or GM-CSF (with 4-OHT) supplemented methylcellulose after 7 days of incubation, plated in fresh methylcellulose medium supplemented with the same growth factors and incubated another 7 days. Replating of vector controls in the same way does not give rise to any new colonies under these conditions (data not shown), since the cells already differentiated and lost the capability to proliferate within the first 7 day-incubation period. Klf4$^{\Delta Zn}$-ER^{T2} transduced cells, however, showed significant colony formation in the second incubation period with both cytokine conditions tested (>100 colonies/10^4 cells plated), confirming that the transduced cells retained their proliferative capacity, which is characteristic for immature progenitors. Nuclear localization of the Klf4$^{\Delta Zn}$-ER^{T2} construct was necessary for this effect, since replating the cells in the medium with the

same growth factors, but without 4-OHT did not show new colony formation. Instead, the cells differentiated (Figure 34). This indicates that Klf4$^{\Delta Zn}$-ER^{T2} induced maturation block is not irreversible and can be rescued by exporting Klf4$^{\Delta Zn}$-ER^{T2} out of the nucleus.

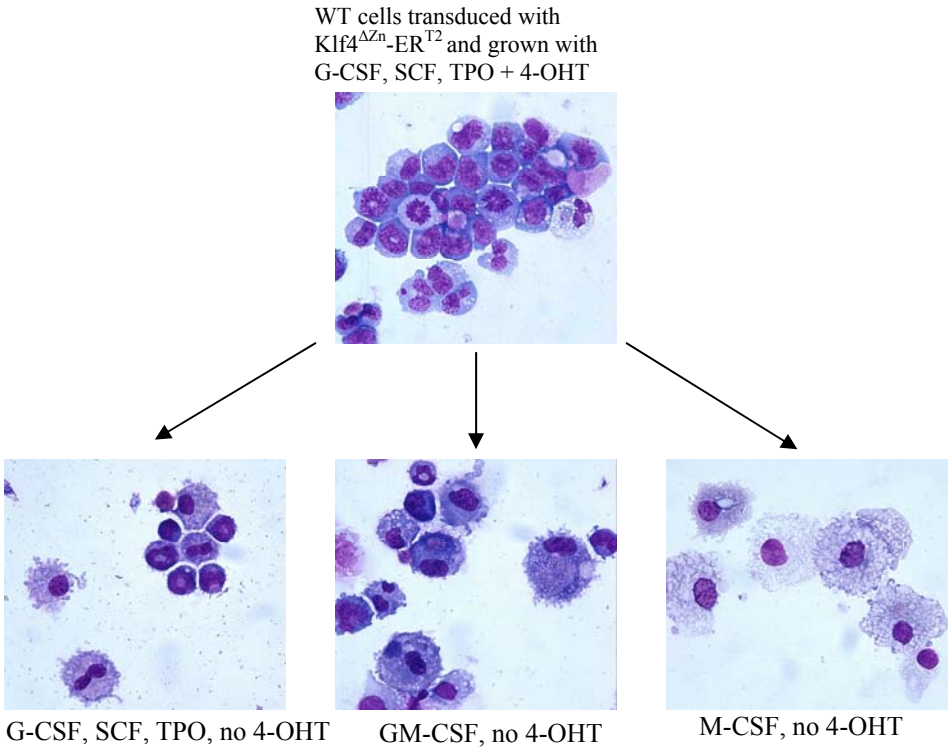


Figure 34: The maturation block caused by Klf4$^{\Delta Zn}$-ER^{T2} transduction is reversible
The wild type cells transduced with Klf4$^{\Delta Zn}$-ER^{T2} were grown in methylcellulose medium with 4-OHT and G-CSF, SCF, TPO cytokine combination. After 7 days, the cells were isolated (the morphology of cells was determined by microscopic analysis after MGG staining) and replated in 4-OHT-free methylcellulose medium supplemented with indicated cytokines. After 7 days incubation, cells were isolated and microscopically examined after MGG staining.

It follows from the described experiments that the Klf4 deletion mutant containing the zinc finger region alone has no activity in proliferation and differentiation of myeloid cells. On the other side, deletion of the zinc finger region from the full-length Klf4 sequence results in dominant negative activity which causes the myeloid differentiation block. Both findings indicate that Klf4 interacts with other proteins in the nucleus and that these interactions are necessary for its transcriptional activity. Moreover, we could speculate that the dominant negative effect of the Klf4$^{\Delta Zn}$-ER^{T2} construct relays on the sequestration of corresponding interaction partners.

5.6 Analysis of the Icsbp role in another aspect of myelopoiesis – the generation of eosinophils

It was described previously that Icsbp null mutation leads to deregulation of the myeloid differentiation program, reflected by disproportional high production of granulocytes and low production of macrophages (Holtschke et al., 1996; Scheller et al., 1999; Tsujimura et al., 2002). In an attempt to analyze this myelopoietic misbalance on the molecular level, gene expression pattern of

Icsbp$^{-/-}$ GMPs was compared to that of GMPs Icsbp$^{+/+}$. Apart from already documented switch between the production of granulocytes and macrophages, this analysis revealed several eosinophil-specific genes, like major basic protein (*Mbp*), eosinophil peroxidase (*Epx*) and eosinophil ribonuclease 1 and 2 (*Ear1/2*) to be significantly down-regulated in the absence of Icsbp (unpublished data). This finding prompted us to analyze another aspect of myelopoiesis in Icsbp$^{-/-}$ mice, namely the development and function of eosinophils.

5. 6.1. The number of eosinophils is reduced in Icsbp$^{-/-}$ mice

5. 6.1.1. The local eosinophilia in response to thioglycolate elicited inflammation is reduced in Icsbp$^{-/-}$ mice

As mentioned before (Introduction), eosinophils constitute only 1-2% of all circulating leukocytes. Analyzing changes (in particular reduction) of such minor population poses certain difficulty and in order to bring out the differences, experimental models of either local or systemic eosinophilia (increased number of eosinophils) are used, like using mice transgenic for major eosinophil growth factor Il-5, sterile inflammation models or parasite infections. One of the standard models used for more than 50 years to elicit the local inflammatory response in the peritoneal cavity is the injection of 3% thioglycolate solution (Galilly et al., 1967). Thioglycolate leads to the nonenzymatic modification of proteins with advanced-glycosylation-end-products (AGE), which in turn activate variety of cells harbouring AGE-receptors (Brett et al., 1993). Among others, AGE-proteins stimulate macrophages, which produce numerous mediators of inflammation (Vlassara et al., 1988), and endothelial cells, leading to increased transendothelial permeability (Kirstein et al., 1990). Louahed at al. demonstrated the production of cytokines that stimulate eosinophil development and mobilization (mainly Il-5, Il-4, Il-9 and eotaxin) in response to i.p. thioglycolate injection (Louahed et al., 2001). The final result of this treatment is, therefore, sterile inflammation of the peritoneal cavity, characterized by sequential infiltration of granulocytes, eosinophils and macrophages. Accessibility of peritoneal cells and recovery of large cell numbers make this system very useful in studying minor cell populations, like eosinophils.

The thioglycolate induced inflammation has previously been used in our laboratory to study the monocyte and neutrophil peritoneal infiltration in mice with the targeted deletion of Icsbp. This study revealed, as expected, increased number of granulocytes and reduced number of monocytes in the peritoneal cavity of Icsbp$^{-/-}$ mice (unpublished observations).

In order to analyze the eosinophilic response in the thioglycolate elicited inflammation, age and sex-matched Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were injected intra-peritoneally with 2ml of 3% thioglycolate solution in PBS. After 72 hours, the distribution of immune cells in the peritoneal exudate was analyzed by flow-cytometry and light microscopy.

For the flow-cytometric analysis of the myeloid cells the following combination of cell surface markers was used: Cd11b, integrin expressed on all myeloid cells, F4/80, macrophage surface marker

(also expressed on eosinophiles, but with lower intensity) and Gr1, granulocyte surface marker (expressed on eosinophils with very low intensity). The monocyte/macrophage population was identified as Cd11b^{high}F4/80^{high}Gr1^{-}, eosinophil population as Cd11b^{low}F4/80^{med}Gr1^{low} and granulocyte population as Cd11b^{+}F4/80^{-}Gr1^{high}. The sorting of cells in regions indicated in Figure 35A by FACS and subsequent microscope analysis of sorted fractions confirmed the cell identity (data not shown).

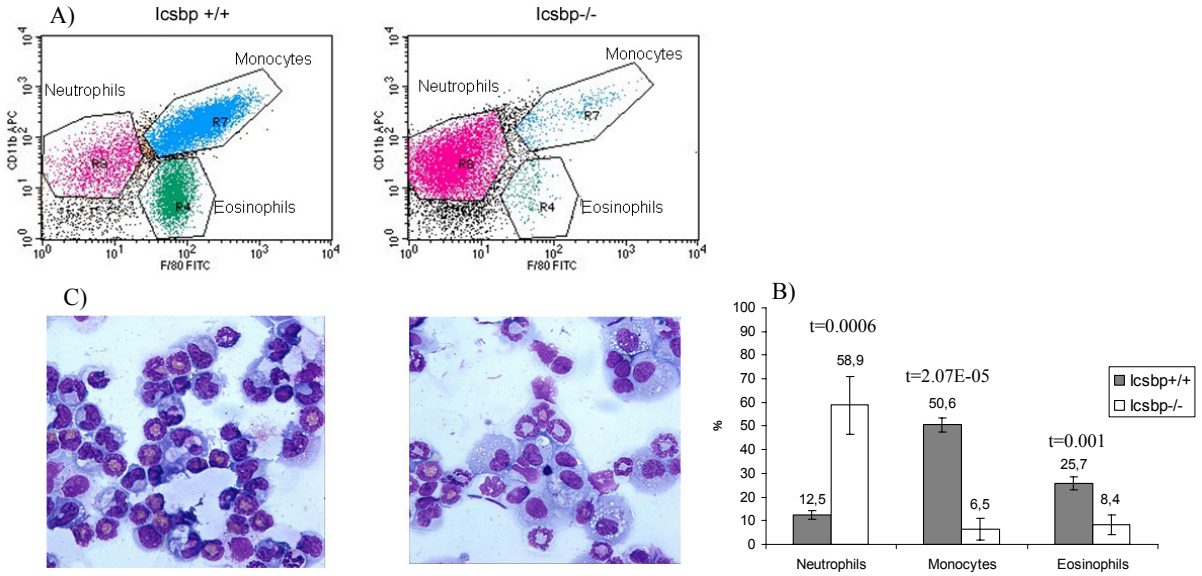


Figure 35: The distribution of myeloid cells in the peritoneal exudate of Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice in the thioglycolate elicited peritoneal inflammation

Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were injected i.p. with 2ml of 3% thioglycolate solution. 72 hours after the injection peritoneal cells were harvested and used for FACS or microscope analysis.

A) Peritoneal cells were stained with antibodies against Cd11b, F4/80 and Gr1 and analyzed by FACS. The Cd11b:F4/80 dot plots from representative samples are shown. B) The percentage of neutrophils (Cd11b^{+}Gr1^{+}F4/80^{-}), monocytes (Cd11b^{+}Gr1^{-}F4/80^{+}) and eosinophils (Cd11b^{low}Gr1^{low}F4/80^{low}) was determined by FACS. The mean values with standard deviations from three independent experiments shown. In each experiment 5 mice per group were used. The statistical significance was determined by student's t-test. C) The cell identity was confirmed by microscope analysis of peritoneal cells, stained according to May-Grünwald – Giemsa.

As shown in Figure 35A and B, peritoneal exudate of wild type mice consisted mostly of macrophages (50.6%) and eosinophils (25.7%) 72 hours after thioglycolate injection. In Icsbp$^{-/-}$ mice, both macrophages and eosinophils were strongly reduced (6.5% and 8.4% respectively), while the percentage of granulocytes was increased (58.9% in Icsbp$^{-/-}$ mice compared to 12.5% in wild type mice).

The result of the FACS analysis was confirmed by examining the cell morphology after May Grünwal-Giemsa staining (Figure 35C).

5.6.1.2. The systemic eosinophilia in response to parasite infection is reduced in Icsbp$^{-/-}$ mice

In order to further test the ability of Icsbp$^{-/-}$ mice to elicit eosinophilia, another inflammatory challenge was applied. Parasite infections are historically associated with an increased number of blood and tissue eosinophils, which are considered to be the end-stage cells of the host antiparasite defense (reviewed in Behm, Paras.Tod, 2000). The gastrointestinal nematode *Nippostrongylus*

brasiliensis is often used as a model of short-lived infection that has a systemic as well as gastrointestinal phase (Finkelman, Ann.Rev.Imm, 1997).

The infection of the Icsbp$^{-/-}$ and $^{+/+}$ mice was performed by sub-cutaneous injection of 750 stage-three larvae (L3) of the mouse adapted strain of *N.brasiliensis* (performed in the cooperation with D.Struck, Institute for Microbiology, Charité, Berlin). During the first days of infection, *N.brasiliensis* larvae migrate through the lung causing the inflammatory reaction. In the later stages, mature parasites reside in the gut lumen, which leads to the infiltration of the small intestines with inflammatory cells, including high numbers of eosinophils (Finkelman et al., 1997). The infection stimulates the production of eosinophils in the bone marrow, which peaks at day 7 and leads to peripheral blood eosinophilia, evident already at day 4 and culminating between day 11 and 14 (Rennick et al., 1990).

In order to assess the effect of *N.brasiliensis* infection in Icsbp$^{-/-}$ mice, the animals were sacrificed on day 13 and peripheral blood and small intestines were sampled (as described in Materials and Methods). The blood smears were stained according to May-Grünwald-Giemsa and the number of eosinophils was determined by microscope examination. As shown in Figure 36A, the percentage of eosinophils in non-infected mice of both genotypes (Icsbp$^{+/+}$ and Icsbp$^{-/-}$) showed no difference and comprised approximately 1% of all peripheral blood leukocytes. In response to the parasite infection, the percentage of eosinophils in wild type mice increased 10 fold, while in the Icsbp$^{-/-}$ mice it remained below 2% (without significant increase). In order to rule out the possibility of the inefficient parasite infection, the number of intestinal goblet cells was analyzed in naïve and infected mice (analysis performed by D.Struck, Institute for Microbiology, Charité, Berlin). The increased mucus secretion from intestinal goblet cells is another prominent feature of the inflammatory reaction caused by *N. brasiliensis* (apart from eosinophilia), important for the spontaneous expulsion of the parasite at the end of the second week of the infection (Levy et al., 1983). In our experiments, the number of goblet cells increased approximately 4 fold in infected mice of both genotypes, suggesting a successful infection procedure (Figure 36B).

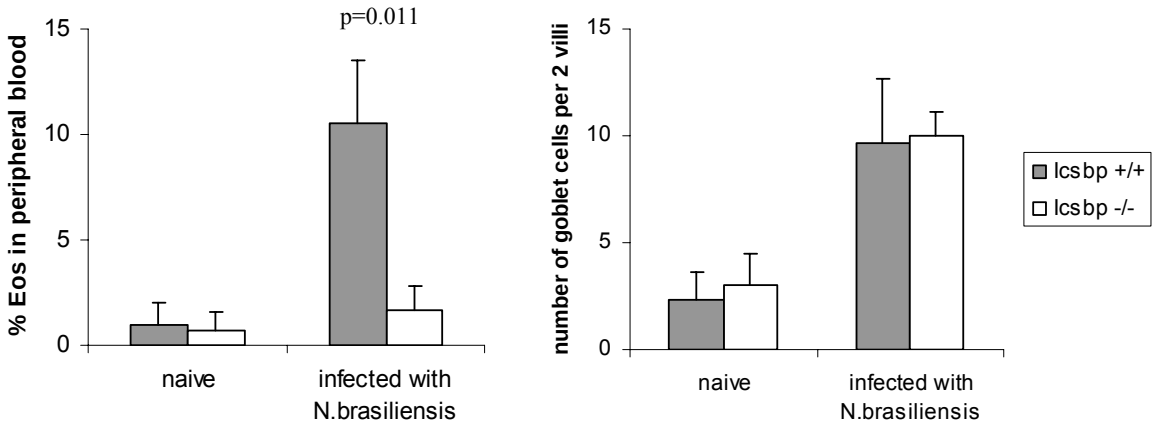


Figure 36: The response of Icsbp$^{-/-}$ mice to the parasite infection: reduced peripheral blood eosinophilia, but normal increase of intestinal goblet cells
The peripheral blood and small intestines were sampled from the mice 13 days after the *N.brasiliensis* infection. A) The blood smears were stained according to May-Grünwald Giemsa and the percent of eosinophils was determined by counting the cells with characteristic eosinophil morphology. B) The number of intestinal goblet cells was determined by analyzing 2 villi from the small intestine sections stained with hematoxylin and eosin

The functional immune response to the parasite infection depends on the cytokines produced by Th2 subset of helper T-lymphocytes, Il-4, Il-5, Il-9 and Il-13 (Finkelman et al., 1997). Out of those, Il-4 is necessary for Th2 cell polarisation and secretion of other Th2 type cytokines (Kopf et al., 1993) and Il-5 is most directly involved in stimulating eosinophil production, survival and activation (Yamaguchi et al., 1988). Coffman et al. demonstrated the *in vivo* significance of Il-5 in inducing eosinophilia in response to the *N.brasiliensis* infection (Coffman et al., 1989). Il-4 is required for the generation of other Th2-derived cytokines (Kopf et al., 1993). It also regulates the antigen switching to IgE isotype, facilitating the parasite recognition and enhances the expression of adhesion molecules on endothelial cells, stimulating the eosinophil recruitment to the site of infection (review Lampinen et al., 2004).

It was previously reported that Icsbp$^{-/-}$ mice have a defect in generating the Th1 type cytokines, based on the intrinsic failure to produce Il-12, a key inducer of Th1 cell polarization (Giese et al., 1997). The production of Th2 type cytokines is, however, not perturbed by Icsbp deletion (Giese et al., 1997). In order to test the production of two key Th2 type cytokines, Il-4 and Il-5, in *N. brasiliensis* infected Icsbp$^{-/-}$ mice, lymphocyte cultures were prepared from spleens (as described in Materials and Methods) and the cytokine concentrations in the cell-free supernatants were determined by ELISA (analysis performed by D.Struck, Institute for Microbiology, Charité, Berlin).

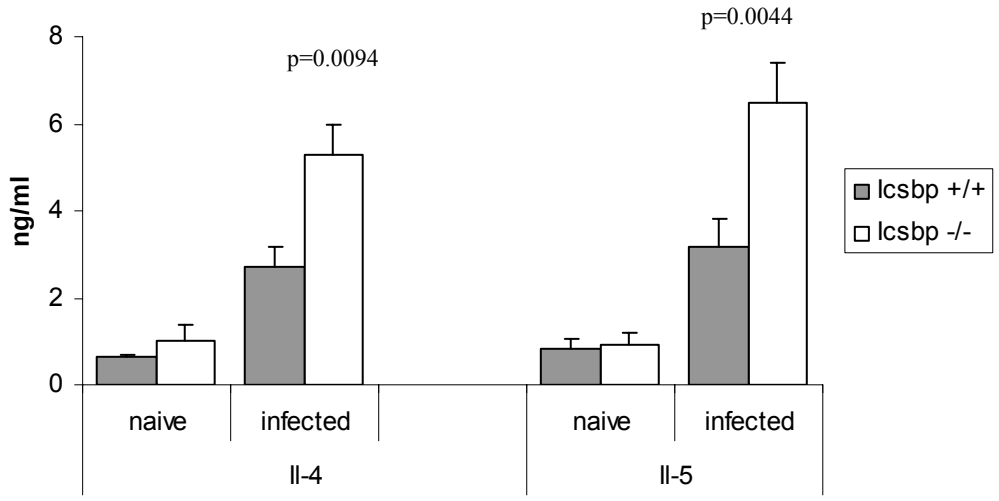


Figure 37: Icsbp$^{-/-}$ mice show strong humoral response to the parasite infection
The cytokine concentrations were determined by ELISA in spleen cell cultures prepared from *N.brasiliensis* infected Icsbp$^{+/+}$ or Icsbp$^{-/-}$ mice (as described in Materials and Methods).

As shown in Figure 37, Icsbp$^{-/-}$ mice developed strong humoral response to the *N.brasiliensis* infection, reflected in high Il-5 and Il-4 production. Moreover, the concentrations of measured cytokines were significantly higher in the infected Icsbp$^{-/-}$ compared to the infected wild type mice, most likely explained by the lack of cross-inhibition of Th1 cytokines, which have a suppressive effect on Th2 polarization and cytokine secretion. Therefore, the lack of eosinophilia observed in parasite infected Icsbp$^{-/-}$ mice is not caused by the defective cytokine secretion, but by inefficient eosinophil production in response to growth factors.

5.6.2. The generation of eosinophils from the bone marrow progenitors in response to Il-5 is impaired in Icsbp$^{-/-}$ mice

The data shown in the previous two sections indicate that the production of eosinophils in response to their main growth factor (Il-5) is defective in Icsbp$^{-/-}$ mice. In order to analyze this observation further, the ability of the bone marrow progenitors to differentiate into eosinophils in response to Il-5 was tested *in vitro*. The bone marrow cells from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were isolated and mononuclear cells were selected by Ficoll gradient centrifugation (as described in Materials and Methods). This separation method eliminates the mature granulocytes which constitute up to 80% of the bone marrow of Icsbp$^{-/-}$ mice (in comparison to approximate 30% in wild type mice) and enables the normalization of the cell plating numbers between the two genotypes. The immature lineage negative cells obtained by MACS lineage depletion showed poor growth in response to Il-5 alone or combined with other cytokines, like SCF and IL-3 (data not shown), therefore the density gradient centrifugation was used as the preferred separation method.

The isolated mononuclear cells from Icsbp$^{+/+}$ and $^{-/-}$ mice were plated in the liquid medium supplemented with 50ng/ml Il-5. The percentage of eosinophils developing in the culture was determined by the microscope examination of May-Grünwald-Giemsa stained samples after 0, 4, 7 and

11 days of culturing. The results are shown in Figure 38. The cultures obtained from Icsbp$^{+/+}$ bone marrow cells contained significantly higher number of mature eosinophils than those obtained from Icsbp$^{-/-}$ cells.

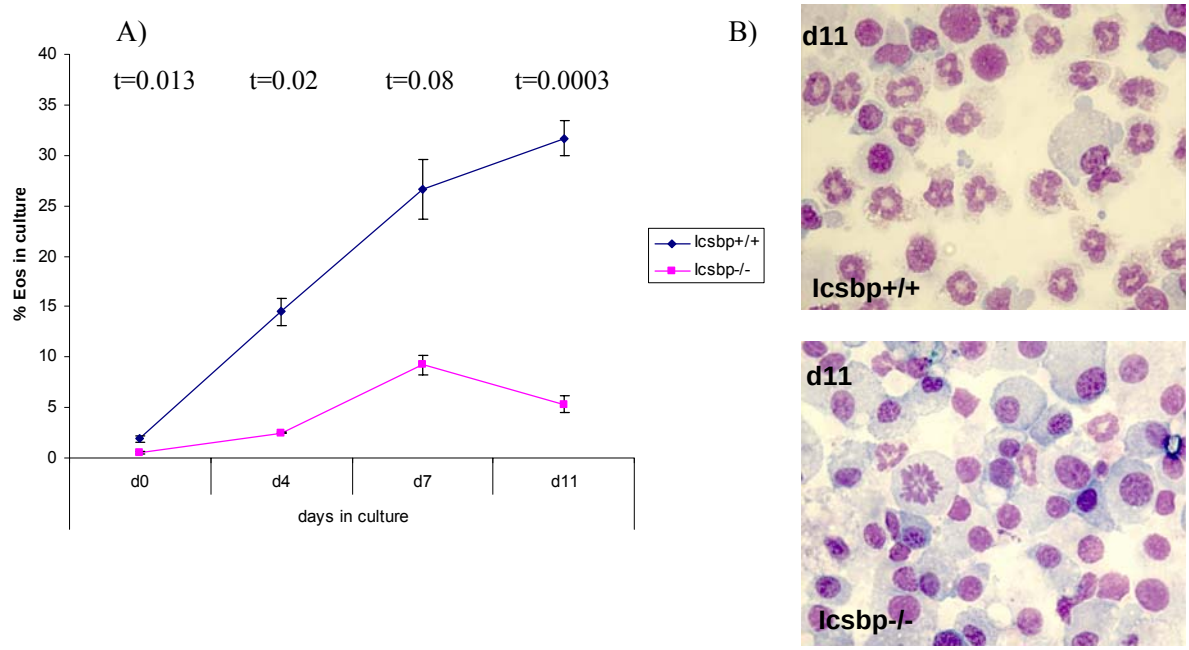


Figure 38: The differentation of Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mononuclear bone marrow cells in Il-5 supplemented culture
The mononuclear cells were isolated from the bone marrow of Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice by density gradient centrifugation and plated in liquid medium supplemented with 50ng/ml Il-5. Three mice were used per group and analysed individually. The samples from cultured cells were taken on day 0, 4, 7 and 11. Microscope slides stained according to May-Grünwald –Giemsa were made and the percentage of eosinophils was determined by counting the cells with typical eosinophil morphology. A) The mean values of eosinophil counts (with standard deviations) from two independent experiments are shown. B) Representative microscope slides after 11 days of cluturing.

5.6.2.1. The number of eosinophil committed progenitors is reduced in Icsbp$^{-/-}$ mice

The percentage of eosinophils in liquid cultures described above depends on several factors: 1) the number of progenitor cells with eosinophil differentiation potential in the starting bone marrow cell population, 2) the ability of eosinophil committed cells to receive and transduce the signals coming from Il-5 and 3) the survival of eosinophils.

In order to address the first possibility, the representation of eosinophil progenitors in Icsbp$^{-/-}$ bone marrow was tested by the CFU-assay. The selected mononuclear cells from the bone marrow of Icsbp$^{-/-}$ and $^{+/+}$ mice were plated (10^5 cells/ml) in the methylcellulose medium supplemented with 50ng/ml Il-5 and incubated 11 days. As shown in Figure 39A, plating 10^5 mononuclear bone marrow cells gave rise to average 17 eosinophil colonies in wild type samples and only 2 eosinophil colonies in Icsbp$^{-/-}$ samples (8.5 fold difference). Considering that each eosinophil colony descends from the eosinophil committed progenitor, the number of these progenitors could be estimated in the total bone marrow recovered per one mouse (from 2 femurs and 2 tibias). As shown in Figure 39C, the number of eosinophil progenitors is approximately $5x10^3$ in the bone marrow of wild type mouse and less than

$1x10^3$ in the $Icsbp^{-/-}$ mouse, which indicates that $Icsbp^{-/-}$ mice bear a reduced pool of cells with the eosinophil differentiation potential. Additionally, analyzing the morphology of the cells isolated from the methylcellulose and stained according to May Grünwald Giemsa showed that the colonies derived from $Icsbp^{+/+}$ progenitors consisted mostly of eosinophils (although macrophages and mast cells were also observed), while the majority of cells derived from $Icsbp^{-/-}$ progenitors showed monocyte/macrophage morphology (Figure 39B). The eosinophils which developed in $Icsbp^{-/-}$ samples had normal morphology (Figure 39B).

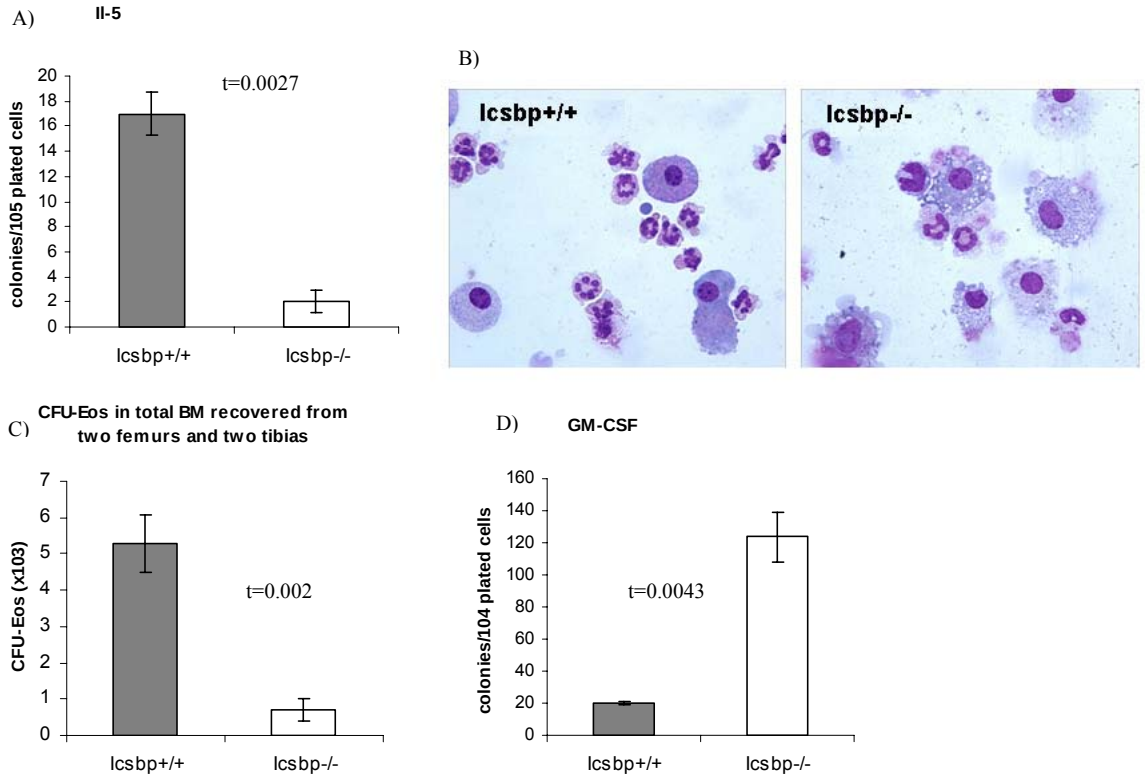


Figure 39: The bone marrow progenitors from $Icsbp^{-/-}$ mice show decreased responsiveness to Il-5 and increased responsiveness to GM-CSF

A) The bone marrow cells from $Icsbp^{+/+}$ and $Icsbp^{-/-}$ mice were plated in the Il-5 supplemented methylcellulose medium. The colonies were scored after 11 days. The mean values of colony counts from two independent experiments are shown, each performed with 3 mice per group (individually analysed). B) The colonies from methylcellulose plates were rinsed, pooled and analysed after the May-Grünwald-Giemsa staining. C) The total number of CFU-Eos was calculated by multiplying the colony number (A) with the total number of the bone marrow cells recovered per one mouse (from 2 femurs and 2 tibias). D) The same bone marrow samples used for culturing with Il-5 (A) were plated in methylcellulose medium with GM-CSF. The colonies were scored after 7 days. The mean values of colony counts from two independent experiments are shown, each performed with 3 mice per group.

It should be mentioned that at the time this study began, the eosinophil committed progenitor cell was not yet defined. Although eosinophils are historically categorized as granulocytes, their origin in the normal hematopoiesis is rather controversial, indicating GMP, MEP or even CMP as possible ancestors of eosinophil committed cells (reviewed in McNagny and Graf, 2002). In June 2005 Iwasaki et al. reported the isolation of the population which currently represents the earliest phenotypically defined eosinophil committed progenitors (Iwasaki et al., 2005). According to these authors, eosinophil committed progenitors descend from the GMP and posses characteristic Lin^-Cd34^+c-

kit^{low}Il-5Rα^{+} phenotype. The analysis of cells with this phenotype in our experimental model showed that Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} population constituted 0.073% of all nucleated cells in the bone marrow from wild type mice and only 0.008% in Icsbp$^{-/-}$ mice (9.7 fold difference; Figure 40). When the number of eosinophil progenitors was calculated as the percentage of Lin^{-}Cd34^{+} cells, it accounted to 5.8% of Icsbp$^{+/+}$ cells and 1.7% of Icsbp$^{-/-}$ cells (3.3 fold difference; experiment performed by G.Terszowski, Department for Immunology, University of Ulm, Germany). This is in accordance with the data obtained by the functional CFU-assay (Figure 39A) and further confirms that the pool of eosinophil progenitors is reduced in Icsbp$^{-/-}$ mice.

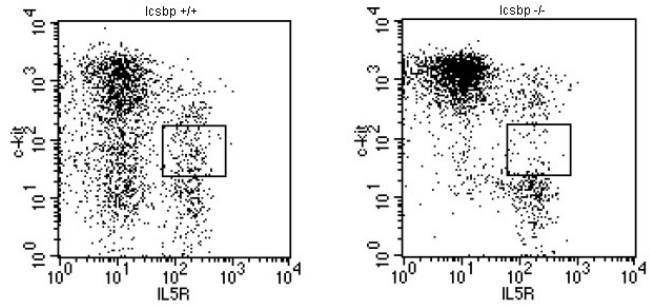


Figure 40: Icsbp$^{-/-}$ mice have reduced number of eosinophil progenitors
The bone marrow cells from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice were stained with antibodies against lineage markers (Lin), Cd34, c-kit and Il-5Rα. Lin^{-}Cd34^{+} population was gated and further analysed for c-kit and Il-5Rα expression (dot plots shown).

5.6.2.2. The signal transduction through β_c-unit of the Il-5 receptor is not disabled, but the expression of the cytokine-binding α-unit is reduced in Icsbp$^{-/-}$ eosinophils

Another conclusion coming from the two experiments described in the previous section is that the expression of the Il-5 receptor is not abolished in Icsbp$^{-/-}$ eosinophil progenitors, since they are detected by FACS as Il-5Rα^{+} (Figure 40) and that the signal transduction pathway utilized by Il-5 is functional, since the progenitor cells proliferate and form colonies when stimulated with Il-5 only (CFU assay, Figure 39A). However, the mean fluorescence intensity (MFI) of bound anti-Il-5Rα antibody in the FACS analysis amounted to 224 units for wild type and 182 units for Icsbp$^{-/-}$ cells (software analysis of the data shown in Figure 40), indicating a slight reduction of the Il-5Rα expression in the absence of Icsbp. Therefore, additional analysis was necessary in order to estimate the Il-5 receptor expression and functionality in the Icsbp$^{-/-}$ eosinophils.

The receptor for Il-5, along with Il-3 and GM-CSF belongs to the distinct family of cytokine receptors. They consist of two subunits: the cytokine specific α-subunit, responsible for ligand binding and the common β-subunit (β_c), shared by all three receptors, which is responsible for the signal transduction from the formed α-chain:ligand complex (Woodcock et al., 1999). Both subunits are necessary for the efficient cytokine stimulation.

Robb et al. (Robb et al., 1995) described a major reduction in the eosinophil numbers in mice with the targeted deletion of the β_c-chain. The functionality of the β_c-chain of the Il-5 receptor in our experimental model was tested by analyzing the response of Icsbp$^{-/-}$ cells to the GM-CSF stimulation. Since both cytokines use the same β_c-chain for activating further intracellular events, it was assumed

that the defect in the signal transduction would affect the response to either cytokine. Therefore, the same mononuclear cells used for the Il-5 based CFU-assay (Figure 39A) were plated in the methylcellulose medium supplemented with GM-CSF and the colony formation was observed. As shown in Figure 39D, the responsiveness to GM-CSF (measured by the number of colonies formed) was not reduced in Icsbp$^{-/-}$ mice (and was even increased), suggesting that the signal transduction through the common β_c-chain is not disabled in Icsbp$^{-/-}$ mice.

In order to analyze the Il-5-specific α-chain expression, mature eosinophils and their progenitors were isolated from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice. The eosinophil progenitors were sorted by FACS from the total bone marrow as Lin$^-$Cd34$^+$c-kit^{low}Il-5Rα$^+$ cells (performed by G.Terzsowski, Departemnt for Immunology, University of Ulm, Germany) and mature eosinophils were sorted as Cd11b$^+$F4/80^{low}Ccr3$^+$ fraction from the peritoneal exudates obtained 72 hours after thioglycolate stimulation (as described in Materials and Methods). The RNA was isolated from purified cell populations, converted to cDNA and the expression of the *Il-5Rα* was tested by RT-PCR. As shown in Figure 41, both mature eosinophils and their progenitors show reduction of *Il-5Rα* expression in Icsbp$^{-/-}$ mice.

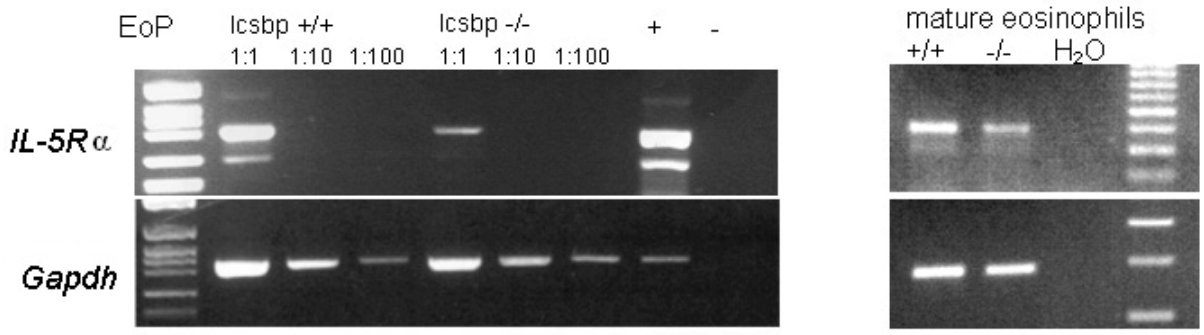


Figure 41: The *Il-5Rα* expression is reduced in Icsbp$^{-/-}$ eosinophils and their precursors
The eosinophil progenitors (EoPs) and mature eosinophils were isolated from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice by FACS (as described in Materials and Methods). Total RNA was isolated from the cells and *Il-5Rα* expression was analyzed by RT-PCR.

Therefore, the expression of the cytokine recognition Il-5 receptor subunit is decreased in Icsbp$^{-/-}$ eosinophils and their progenitors, but the signalling from the receptors which are present on the surface of the cell is functional.

5.6.2.3. Icsbp$^{-/-}$ eosinophils are more susceptible to starvation-induced apoptosis

Since Il-5 is not only the main growth, but also necessary survival factor of eosinophils, it could be assumed that lower Il-5Rα expression would effect the survival of Icsbp$^{-/-}$ eosinophils. In order to test this possibility, mature eosinophils were isolated from the peritoneal cavity of Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice in the same way as for the previous experiment and plated in the liquid medium supplemented with decreasing concentrations of Il-5 (as indicated in Figure 42). After 24 hours incubation, the percent of cells positive for apoptosis marker annexin V was determined by FACS. As shown in Figure 42, Icsbp$^{-/-}$ eosinophils show higher apoptosis rate at all Il-5 concentrations tested.

The percentage of apoptotic Icsbp$^{-/-}$ eosinophils decreases with the Il-5 concentration increase, indicating that these cells respond to Il-5 "rescue" effect, but they need higher doses than the wild type eosinophils. For example, the concentration of 0.1ng/ml Il-5 maintains 50% of Icsbp$^{-/-}$ cells viable, while the wild type cells show this survival rate already at 0.02ng/ml Il-5.

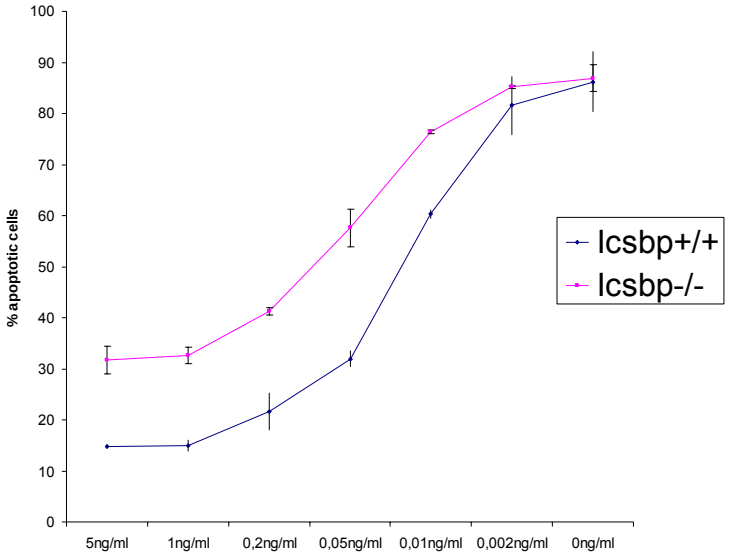


Figure 42: Icsbp$^{-/-}$ eosinophils are more susceptible to starvation induced apoptosis
The mature eosinophils were isolated by FACS from the peritoneal cavity of thioglycolate-stimulated Icsbp$^{+/+}$ or Iscbp$^{-/-}$ mice and plated in the liquid medium supplemented with decreasing Il-5 concentrations. After 24 hours, the cells were stained with apoptosis marker annexin V and analysed by FACS. The percentage of apoptotic cells (y-axis) was plotted as a function of Il-5 concentration used for culturing (x-axis). The summarized data from two independent experiments are shown.

Taken together, these data indicate a functional Il-5 signal transduction in Icsbp$^{-/-}$ eosinophils. On the other side, the reduced Il-5Rα expression in the absence of Icsbp suggests that Icsbp$^{-/-}$ eosinophils require higher Il-5 concentration in order to maintain the same physiological function as wild type eosinophils. This reduced responsiveness to Il-5 could explain the reduced proliferation and survival rate of Icsbp$^{-/-}$ eosinophils. However, the physiological significance of this *in vitro* finding is questionable. Icsbp$^{-/-}$ mice produce very high amounts of Il-5 in response to the parasite infection, which should compensate the lower cell responsiveness to Il-5, but they still lack the appropriate eosinophil production. The inability of high Il-5 concentration to rescue the deficient eosinophil production suggests an additional intrinsic defect of eosinophil-committed cells.

5.6.3. The differentiation of Icsbp$^{-/-}$ eosinophil progenitors is shifted towards other myeloid lineages

The Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} cell population defined by Iwasaki et al. as eosinophil progenitor population (EoP) consists of committed cells whose differentiation potential is limited to the eosinophil lineage (Iwasaki et al., 2005). In order to analyze the effect of Icsbp on the eosinophil differentiation, Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} cells were sorted by FACS from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ bone marrow and plated in the methylcellulose medium supplemented with SCF, Il-3, GM-CSF and Il-5. After 10 days, grown colonies were picked, stained according to May-Grünwald-Giemsa and the

cellular composition of each colony was analyzed. As shown in Figure 43**,** 80% of colonies grown from wild type EoP contained eosinophils (45% of those were pure eosinophil colonies and 35% contained eosinophils with other myeloid cells, like neutrophils, monocytes or mast cells). The remaining 20% of colonies consisted solely of monocytes (19%) or mast cells (1%). These data do not fully reproduce the finding of Iwasaki et al., who demonstrated the exclusive eosinophil differentiation of the EoP population. The reason for this discrepancy is not clear at the moment and could depend on the purity of the sorted population, difference in the culturing conditions or plasticity of selected progenitors. However, the EoP population isolated in our experiment is highly enriched for eosinophil-restricted progenitors (if not exclusively comprised of them, as shown by Iwasaki et al., 2005) and therefore represents useful starting point for studying the eosinophil differentiation of the primary progenitor cells.

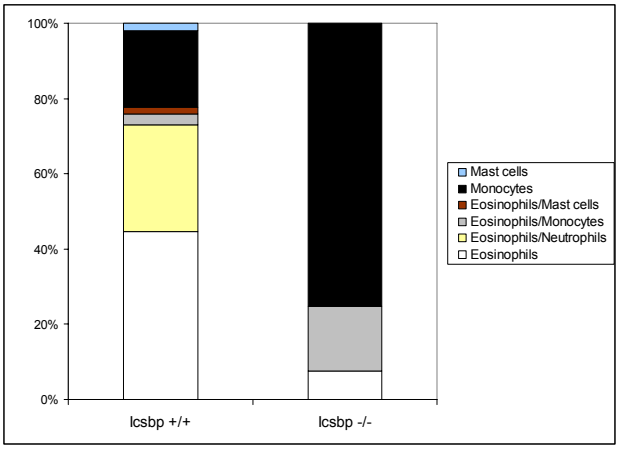


Figure 43: The Icsbp$^{-/-}$ eosinophil progenitors show aberrant differentiation potential
The eosinophil progenitors (EoPs) were isolated from the bone marrow of Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice by FACS (as described in Materials and Methods) and plated in the methylcellulose medium supplemented with SCF, Il-3, Il-5 and GM-CSF. After 10 days colonies were picked, stained according to May-Grünwald-Giemsa and analyzed.

In order to assess the eosinophil differentiation potential of Icsbp$^{-/-}$ EoPs, Lin^{-}Cd34^{+}c-kit^{low}Il-5Rα^{+} cells were isolated from Icsbp$^{-/-}$ bone marrow and cultivated under the same conditions as wild type EoPs. Analyzing the cellular composition of Icsbp$^{-/-}$ EoP derived colonies showed that less than 25% of all colonies contained eosinophils (only 7.5% of them with pure eosinophil composition), while the vast majority consisted of monocytes (75%). Given the propensity of Icsbp$^{-/-}$ phenotype to switch the differentiation of myeloid precursors to neutrophils on the expense of monocytes, this high monocyte developmental capacity of Icsbp$^{-/-}$ EoPs was quite unexpected and so far not explained. 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 cytokine signals from eosinophil-instructive toward monocyte/neutrophil-instructive. However, the similar monocyte/macrophage overgrow in Icsbp$^{-/-}$ samples was noticed when the cells were cultured in media supplemented with Il-5 only (Figure 38 and Figure 39), suggesting an aberrant differentiation potential of Icsbp$^{-/-}$ eosinophil progenitors. Additionally, numerous experiments show (as discussed in the Introduction) that cytokine signalling does not affect the commitment and that the cytokine receptor expression follows as a consequence of the lineage determination. Iwasaki et al. demonstrated

that 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 development. Therefore, the deviated differentiation potential of Icsbp$^{-/-}$ eosinophil progenitors most likely results from the deregulation of intrinsic factors involved in the lineage commitment.

5.6.4. The expression of Gata1 is reduced in Icsbp$^{-/-}$ eosinophils and their precursors

The transcriptional control of eosinophil development requires a combination of factors expressed in time and dose-controlled fashion. It was described previously that Pu.1, Gata1, Gata2 and transcription factors of C/ebp family stimulate eosinophil differentiation (McNagny et al., 1998; Hirasawa et al., 2002; Du et al., 2002). The combination of Pu.1 and C/ebp factors is characteristic for all myeloid cells. They are expressed at high level in the GMP population, which gives rise to all myeloid cells (Akashi, Nature, 2000). Gata1 expression, on the other side, is characteristic for the erythrocyte/megakaryocyte lineage and is not expressed (or only weakly) in the GMP population (Akashi et al., 2000; Terszowski et al., 2005). However, the development of eosinophils within the myeloid branch is strictly dependant on Gata1, as demonstrated by the Gata1 knock-out models (Yu et al., 2002). Therefore, the GMP population must switch on Gata1 expression at the certain point in the development order to enable eosinophil differentiation. Indeed, the experiments with transgenic mice harbouring the GFP marker under Gata1 promoter show that a small fraction of the GMP cells activates the Gata1 transcription and this is exactly the population which gives rise to eosinophils in functional assays (Iwasaki et al., 2005).

In order to assess the level of *Gata1* expression along the eosinophil developmental pathway in Icsbp$^{-/-}$ mice, several precursor cell populations were isolated from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ bone marrow: granulocyte-monocyte precursor (GMP), eosinophil precursors (EoP) and mature eosinophils. In addition, the non-myeloid precursor population (erythroid precursor, EP) which has high Gata1 expression was isolated. Total RNA was obtained from purified cells, converted to cDNA and analyzed by RT-PCR (cell sorting procedures, cDNA preparation and primers used for RT-PCR are described in Materials and Methods; the sorting of GMP, EoP and EP cells was performed by G.Terzsowski, Departement for Immunology, University of Ulm, Germany). As shown in Figure 44, the *Gata1* mRNA expression was not detected in the GMP population of either genotype. In the cells further developing along the eosinophil branch (EoP population and mature eosinophils), the *Gata1* signal was detected, but showed significantly reduced intensity in Icsbp$^{-/-}$ in comparison to wild type cells. The *Gata1* expression in the alternative differentiation pathway, erythroid lineage, was not affected by Icsbp deletion, suggesting that Icsbp has a role in regulating *Gata1* selectively in the eosinophil lineage.

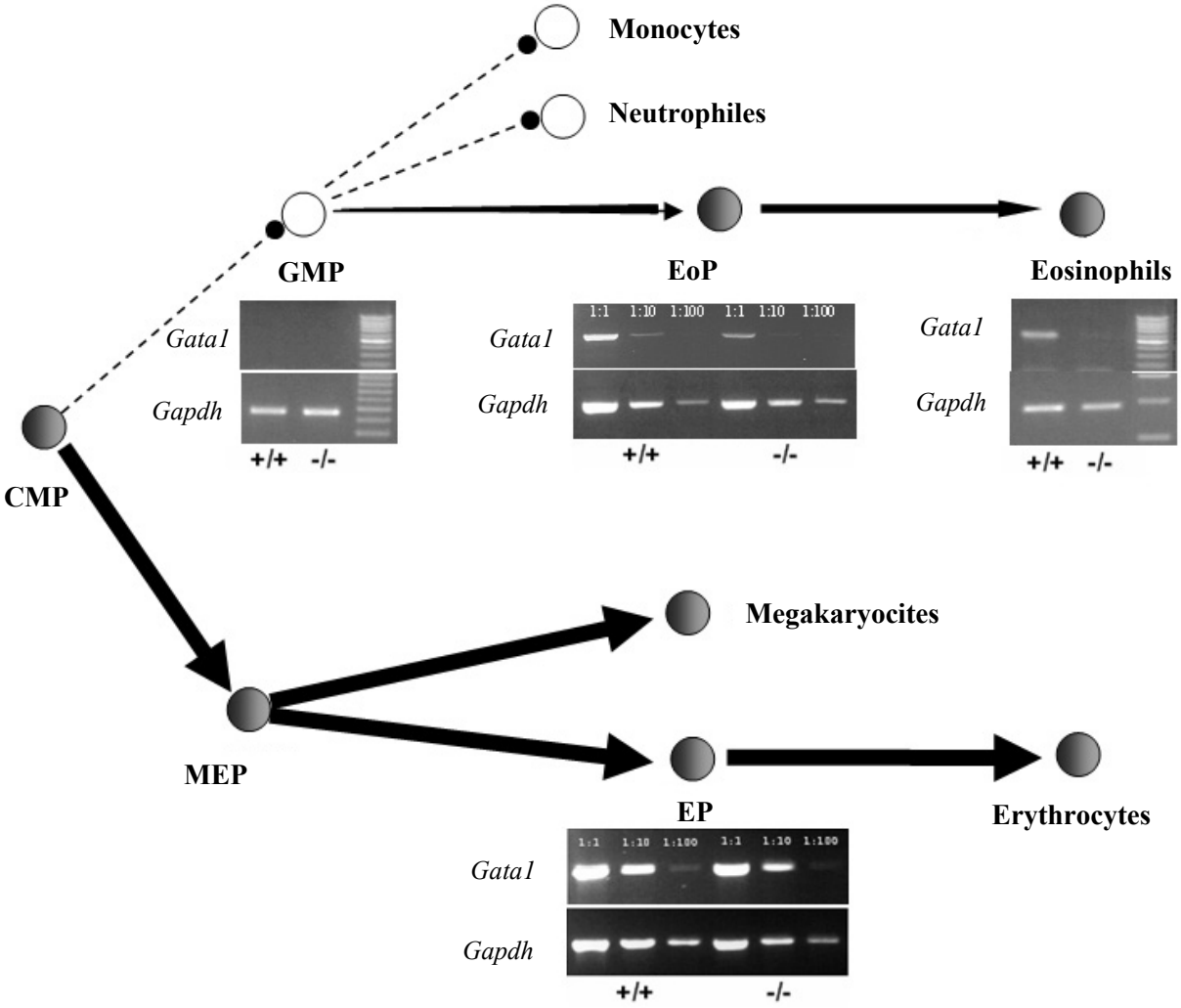


Figure 44: The expression of *Gata1* is reduced in Icsbp$^{-/-}$ cells of eosinophil lineage, but not in Icsbp$^{-/-}$ cells of the erythroid lineage

The GMP, EoP, EP populations and mature eosinophils were sorted by FACS from Icsbp$^{+/+}$ and Icsbp$^{-/-}$ mice (as described in Materials and methods). Total RNA was isolated, converted to cDNA and the expression of *Gata1* was analysed by RT-PCR.

Taken together, the Icsbp$^{-/-}$ mice have reduced capacity of generating eosinophils, based on the reduced number and aberrant differentiation pattern of eosinophil progenitors. Analysis of two main regulatory pathways influencing cell development, cytokine signalling and transcription factor expression showed that Icsbp$^{-/-}$ eosinophils have reduced responsiveness to Il-5 and reduced expression of a critical transcription factor regulating eosinophil development, Gata1.