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The Role of Cathepsin K in Hematopoiesis

Marianne Hackl

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Table of Contents

Zusammenfassung

In dieser Arbeit wurde die Auswirkung von Kathepsin K auf die Hämatopoese, insbesondere auf die frühen hämatopoetischen Stammzellen, untersucht. Hämatopoese geschieht in einer spezialisierten Mikroumgebung im Knochenmark, der sogenannten hämatopoetischen Nische. Verschiedene Zelltypen werden als Bestandteile dieser Nische diskutiert. Einer davon, die Osteoklasten, ist die Hauptquelle von Kathepsin K, einer Protease mit der Aufgabe, Knochenmatrix zu degradieren. Erste Hinweise auf eine mögliche weitere Rolle von Kathepsin K hatten sich in Untersuchungen von Stromazellen bezüglich ihrer Fähigkeit, hämatopoetische Stammzellen in kontaktlosen Co-Kulturen zu erhalten, ergeben. Hier zeigte sich, dass in Zellen mit einer solchen Fähigkeit unter anderem Kathepsin K vermehrt exprimiert wurde.

In der vorliegenden Arbeit wurde zunächst eine Charakterisierung der hämatopoetischen Organe von CTSK-defizienten Mäusen unternommen. Hier zeigten sich Veränderungen bei der Anzahl der CD8+ Lymphozyten und eine Verringerung der GMP (granulozytär-monozytäre Vorläufer) im Knochenmark der KO-Mäuse. Im CFU-Assay, der zur Detektion von koloniebildenden Einheit dient, bildeten sich hingegen mehr CFU-GM Kolonien (mit Stammzellen von Myeloblasten und Monoblasten) aus den Knochenmarks-Proben von KO-Mäusen.

In einem zweiten Schritt wurden serielle Transplantationen von Wildtyp-Knochenmark in CTSK-KO-Mäuse und die Weitertransplantation in einen zweiten Wildtyp-Empfänger durchgeführt. In den ersten, Kathepsin K-defizienten Empfängern fiel vor allem ein schlechteres Engraftment der Spenderzellen auf, ein Hinweis auf eine mögliche Bedeutung von Kathepsin K für den Erhalt und die Expansion des Stammzell-Pools. Signifikante Unterschiede bei der Anzahl der hämatopoetischen Vorläuferzellen zeigten sich jedoch nicht. Wieder fielen allerdings Unterschiede bei den Lymphozytenzahlen auf, die hier die B- und T-Lymphozyten wie auch die NK1.1 positiven Zellen betrafen. Auch bei den sekundären Empfängern zeigte sich ein schlechteres Engraftment mit signifikanten Ergebnissen für die Milz bei den Zellen, die zuvor Kathepsin K Knockout-Knochenmark populiert hatten. Hier zeigten sich außerdem signifikant geringere Zellzahlen bei frühen CD34-negativen Stammzellen im Knochenmark. Dies kann als Hinweis auf eine Bedeutung von Kathepsin K für die Regulierung des Pools an hämatopoetischen Stammzellen unter Stressbedingungen gewertet werden. Wieder wurden

darüber hinaus Veränderungen bei der Anzahl von B-und T-Lymphozyten sowie von NK1.1-Zellen in den hämatopoetischen Organen der Empfänger festgestellt.

Ergänzend wurden Experimente zur Mobilisierung und zum Homing von hämatopoetischen Stammzellen in CTSK-defizienten Mäusen durchgeführt. In den Versuchen zum Stammzell-Homing ergaben sich keine wegweisenden Hinweise auf einen diesbezüglichen Defekt der KO-Mäuse. In den Mobilisierungsversuchen mit Filgrastim betrafen die Veränderungen bei den adulten Zellen vor allem Thrombozyten und Lymphozyten. Bei der Stammzellmobilisierung ergaben sich Unterschiede bezüglich der Anzahl der CFU-GM mit über 1000 Zellen sowie der Anzahl der MPP (multipotente Vorläufer) im peripheren Blut.

Zusammenfassend lässt sich sagen, dass Kathepsin K die murine Hämatopoese und deren Stammzell-Pool beeinflusst. Weitere Experimente zu den beteiligten Mechanismen stehen zum aktuellen Zeitpunkt noch aus.

Summary

In this thesis, the effect of cathepsin K on hematopoiesis, especially on the early hematopoietic stem cells, was examined. Hematopoiesis occurs in a specialized microenvironment in the bone marrow called hematopoietic niche. Different cell types are being discussed as components of that niche. One of the presumed components, the osteoclasts, is the main source of cathepsin K, a protease which degrades bone matrix. The first indications of a possible further role of cathepsin K originated from studies which examined stromal cells and their ability to maintain hematopoietic stem cells in non-contact co-cultures. It was shown that cells with the capability to do so expressed more cathepsin K than others.

In the present study, at first a characterization of the hematopoietic organs of CTSK deficient mice was pursued. There, changes in the number of CD8+ lymphocytes and of GMP (granulocyte-monocyte progenitors) showed. In CFU-assays, which detect colony-forming units, a higher number of CFU-GM colonies (representing monoblast and myeloblast precursors) from bone marrow samples that derived from KO mice formed.

In a further step, serial transplantations of wild-type bone marrow to CTSK-KO mice and its re-transplantation to secondary wild-type recipients were performed. In the first, cathepsin K deficient recipients poorer engraftment of donor cells was observed. This suggests a role of cathepsin K for the preservation and expansion of the hematopoietic stem cell pool. However, significant alterations concerning the number of hematopoietic progenitor cells did not show. Here again, alterations of the cell counts of B and T lymphocytes as well as of NK1.1 positive cells were noted. The secondary recipients of cells which had previously populated cathepsin K knockout bone marrow, too, exhibited worse engraftment with significant results for spleens. Moreover, significantly lower numbers of early stem cells (CD34- LSK) in the bone marrow were counted. This indicates that cathepsin K plays a role in the maintenance and self-renewal of the pool of hematopoietic stem cells under stress conditions. Again, changes in the numbers of B and T lymphocytes as well as in the numbers of NK1.1 cells in the hematopoietic organs of the recipients were observed.

Additional experiments examining mobilization and homing of hematopoietic stem cells were performed in CTSK deficient mice. In the experiments on stem cell homing, there were no indications of a respective defect in the knockout mice to be found. In the mobilization experiments with filgrastim, the changes in adult cells especially concerned platelets and

lymphocytes. In stem cell mobilization, differences in the numbers of CFU-GM with more than 1000 cells and the numbers of MPP (multipotent progenitors) in the peripheral blood were noted.

In summary it can be said that cathepsin K affects murine hematopoiesis and stem cell maintenance and self-renewal. Further experiments on the mechanisms involved are pending at the time of delivery of this thesis.

1 Introduction

1.1 The hematopoietic stem cell in its niche

Hematopoietic stem cells (HSCs) are the source of all blood cells an individual possesses. One single HSC has the capability to repopulate the bone marrow with stem cells and progenitors and to reconstitute blood cells from all linages after ablation (Ema H, 2006). For that purpose, it has to differentiate into diverging progenitors. But then, naturally, the process of proliferation and differentiation leads to a decrease in the number of HSCs itself. If not counteracted, this would eventually deplete the bone marrow. The ability of a HSC to self-renew and replenish the HSC compartment and regenerate the quiescent state of a large fraction of HSCs are therefore traits that are vital for maintaining live-long hematopoiesis.

HSCs differentiate into hematopoietic progenitors and based on the steps of differentiation, a hematopoietic hierarchy can be established. At the top of this hierarchy, a discrimination is made between long-term repopulating HSCs (LT-HSCs) and short-term repopulating HSCs (ST-HSCs). LT-HSCs are responsible for maintaining the HSC pool for the duration of the lifetime of the host, ST-HSCs differentiate into hematopoietic progenitors and contribute to the hierarchy for a limited time. While both are CD48 and lineage negative and positive for CD150, Sca-1 and c-Kit (Ikuta K, 1992; Kiel MJ, 2005), ST-HSCs additionally express CD34 (Osawa M, 1996). Among the LT-HSC not expressing CD34 (CD34-) HSCs, those with a high expression of CD150 hold the largest self-renewal potential (Morita Y, 2010). Multipotent progenitors (MPPs) lose their self-renewal potential and divide into lineage progenitors. Common lymphoid progenitors (CLPs) develop into precursors for NK, B and T cells and express interleukin 7 receptor (Il7-R) (Kondo M, 1997). Common myeloid progenitors (CMPs) are lin-, Sca-1-, c-kit+, CD34+ and CD16/32=FcγR^{mid} (Akashi K, 2000). They give rise to megakaryocyte-erythrocyte progenitors (MEPs; lin- Sca-1- c-kit+ CD34- CD16/32^{low}) from which derive platelets and erythrocytes, and granulocyte-monocyte progenitors (GMPs; lin- Sca-1- c-kit+ CD34+ CD16/32^{hi}) which develop into precursors of granulocytes and monocytes. Lately the strict validness of this classification has been doubted. On the one hand, there is prove that even early hematopoietic stem cells yet have a lineage bias. The bias makes them likely to differentiate into either lymphoid or myeloid progenitors. Epigenetic modifications have been identified as a correlate for a myeloid or a lymphoid commitment (Muller-Sieburg CE, 2004; Ji H, 2010). On the other hand, there are indications of a so called

lineage infidelity. It has been demonstrated that early thymic progenitors possess lymphoid as well as myeloid potential (Wada H, 2008; Bell JJ, 2008).

For a long time, there has been a consent that HSC behavior is controlled in the presence of a niche (Schofield, 1978). This niche includes all cells in the surroundings of stem cells and the microenvironment provided by them. This environment is decisive for the fate of HSCs - that is whether and how they must differentiate and proliferate, self-renew or remain quiescent. Disagreement remains about the exact location of the niche, or whether different niches exist for different types of HSC (Kiel MJ, 2008). First, an endosteal location of the niche was proposed (Nilsson SK, 2001). It was found that HSC often localize in trabecular bone where there is a large endosteal surface. (Nombela-Arrieta C, 2013; Guezguez B, 2013). After their transplantation to irradiated mice, HSCs were found in close proximity to the endosteum (Lo Celso C, 2009; Xie Y, 2009). Osteoblasts were the first cells that were closely observed in matters of their influence on HSCs; their ablation alters hematopoiesis (Calvi LM, 2003; Visnjic D, 2004). Lately, however, the central role of osteoblasts has been challenged. For example, recent data suggests that there is no crucial role for N-cadherin, an adhesion molecule expressed by osteoblasts and HSCs, in maintaining hematopoiesis (Kiel MJ, 2007; Li P, 2010; Bromberg O, 2012). There are findings which indicate that mature osteoblasts are not crucial for hematopoiesis but that immature osteoblasts play an important role (Calvi LM, 2012). It is also being speculated that the osteoblastic influence might rather be an indirect one (Morrison SJ, 2014). Plausible also is the existence of a vascular niche which is composed of perivascular and endothelial cells. The majority of HSCs reside in close proximity to sinusoidal vessels (Kiel MJ, 2005). The adhesion molecule E-selectin, expressed by bone marrow endothelial cells, has been identified as a mediator of HSC proliferation (Winkler IG, 2012). In addition, factors like SCF (see below) (Ding L, 2012; Sasaki T, 2010) and pleiotrophin (Himburg HA, 2012), both of which have been found to be influential factors for hematopoiesis, are also expressed by endothelial and perivascular cells. It is possible that endosteum and blood vessels constitute two functionally different niches, the former supporting quiescence and the latter attracting HSCs that are about to differentiate. Apart from that, further influential factors like innervation (Katayama Y, 2006; Kollet O, 2012) and the interaction of HSCs with mesenchymal stem cells (Méndez-Ferrer S, 2010) have been identified.

The questions whether there exist distinct niches for distinct subpopulations of HSCs or there is redundancy and if and how the cells that comprise the respective niche influence each other remain largely unresolved.

A number of factors have been identified as influential on hematopoiesis. Hematopoietic cytokines like G-CSF (Taichman RS, 1994), GM-CSF and IL-6 (Taichman RS, 1997; Rieger MA, 2009) promote the proliferation of hematopoietic progenitors. A significance of Notch/Jagged1 interaction for maintaining the HSC pool was demonstrated in vitro (Li L, 1998). In vivo, however, Notch signaling appears to be dispensable (Benveniste P, 2014). The interaction of angiopoietin 1 (Ang-1) with its receptor Tie-2 promotes the maintenance of quiescent HSCs (Arai F, 2004). Thrombopoietin is a cytokine that not only stimulates megakaryocyte differentiation but also supports HSC maintenance (Hitchcock IS, 2014; Chou FS, 2011). Osteopontin (OPN) suppresses cell cycling of hematopoietic stem and progenitor cells and promotes HSC migration towards the endosteum. Especially OPN fragments cleaved by thrombin interact with HSCs (Grassinger J, 2009; Nilsson SK, 2005). Bone morphogenetic protein 4 (BMP4) is important for hematopoietic commitment of mesodermal stem cells. Moreover, it plays a role in adult hematopoiesis, as in BMP4 deficient mice the number of LSKs is reduced and WT cells exposed to a BMP4 deficient milieu have an impaired repopulation capacity (Goldman DC, 2009). There are indications of a link between BMP4 and hedgehog (Hh) signaling in erythropoiesis (Perry JM, 2009). Hh signaling is another pathway that has been controversially discussed in hematopoiesis (Mar BG, 2011). However, there is evidence that it is important for B lymphopoiesis (Cooper CL, 2012). It is widely recognized that canonical as well as noncanonical Wnt signaling is important in the hematopoietic niche (Reya T, 2003; Schreck C, 2014). Overexpression of Catenin β (CTNNB1), a protein upregulated by canonical Wnt signaling that activates transcription factors, increases HSC numbers transiently and leads to a differentiation block (Kirstetter P, 2006). Wnt3A, in vivo expressed by HSCs, in the presence of stromal cells can inhibit hematopoietic differentiation in vitro (Yamane H, 2009). There also seems to be a role for noncanonical Wnt signaling in maintaining quiescent LT-HSCs (Sugimura R, 2012). Interestingly, the noncanonical ligand Wnt5A also enhances osteoclastogenesis (Maeda K, 2012) and is required for T-cell migration mediated by CXCL12, suggesting a link between the two pathways (Ghosh MC, 2009). Stem cell factor (SCF; steel factor) and its receptor, tyrosine kinase c-Kit, was one of the first signaling pathways described in hematopoiesis (Bernstein A, 1991). The expression level of c-Kit is very stable during all stages of hematopoiesis and the pathway contributes to the self-renewal and maintenance of

HSCs in vivo (Keller JR, 1995; Kent D, 2008). Moreover, it plays a role in homing of hematopoietic stem cells (Nervi B, 2006). Another cytokine with an evident role in hematopoiesis is stromal cell-derived factor 1 (SDF-1; CXCL12), ligand of the CXCR4 receptor (Nagasawa T, 1996; Bleul CC, 1996). In the BM it is mainly produced by immature osteoblasts and in endothelial cells (Ponomaryov T, 2000). SDF-1 signaling plays a pivotal role in the migration of HSCs and their localization in the BM through direct adhesion and chemotactic activity (Imai K, 1999; Lapidot T, 2002) It has been shown that G-CSF administration decreases SDF-1 levels in the bone marrow; this might constitute a mechanism by which G-CSF induces mobilization of HSCs (Petit I, 2002). SDF-1 and CXCR4 deficiency, both of which are lethal around birth, result in an impaired myeloid and lymphoid hematopoiesis in the fetal liver and the absence of myelopoiesis in the perinatal bone marrow (Ma Q, 1998). Transplanted CXCR4-/- cells proved to be unable to durably repopulate bone marrow in serial transplantations (Kawabata K, 1999). The interaction between SDF-1 and CXCR4 is essential for the quiescence especially of primitive LT-HSCs (Sugiyama T, 2006; Nie Y, 2008). In addition, SDF-1 is a strong growth factor for pre-B cells (Ma Q, 1998; Nagasawa, 2007). SDF-1 enhances platelet formation by promoting the transendothelial migration of megakaryocytes, to the extent that it is capable of counteracting thrombopenia caused by myelosuppression. This effect is supposedly mediated by an augmentation of the interaction between vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) (Hamada T, 1998; Avecilla ST, 2004).

The contribution of osteoclasts to hematopoiesis is not fully understood yet and the results are somewhat contradictory. Osteoclasts are required to form bone marrow cavities, the place where adult hematopoiesis mainly takes place. M-CSF, c-Fos and RANKL (receptor activator of NF-κB ligand) simulate osteoclast differentiation (Nijweide PJ, 1986; Teitelbaum, 2000; Miyamoto T, 2001). Mice that lack osteoclasts have no bone marrow cavities and show extramedullary hematopoiesis but also residual HSCs in the bone tissue (Miyamoto K, 2011). It was reported that inhibition of osteoclast activity by bisphosphonate administration reduces the number of HSCs in the bone marrow and leads to an impaired engraftment of treated BM cells (Lymperi S, 2011). In a different study, it was reported that G-CSF administration leads to increased bone resorption by osteoclasts. In this set-up, bisphosphonate, while inhibiting bone resorption, at the same time increased HSC mobilization. It was concluded that osteoclasts are not necessary for HSC mobilization (Takamatsu Y, 1998). Consistently, in osteopetrotic M-CSF, RANKL as well as c-Fos deficient murine models, G-CSF mediated mobilization was

found to be even increased instead of impaired. In analogy, in mice deficient in osteoprotegerin, a cytokine that inhibits osteoclast activity, HSC mobilization was reduced (Miyamoto K, 2011). On the other hand, it was reported that osteoclast activity is necessary for the regulation of B lymphopoiesis. Administration of zoledronic acid decreased the occurrence of B cells in the bone marrow, they were retained outside of the bone marrow. This was not a direct effect of zoledronic acid but mediated by a decrease in the expression of CXCL12 and IL-7 by stromal cells and a reduced osteoblast activity. The same effect was observed in the osteopetrotic oc/oc (osteosclerotic spontaneous mutation) mouse model (Mansour A, 2011). Another study by the same group suggests that osteoclasts contribute to the hematopoietic niche beyond B lymphopoisis. Oc/oc mice exhibited an increased proportion of mesenchymal progenitors but reduced osteoblastic commitment. This was associated with a dramatic reduction of LSKs and impaired homing of hematopoietic progenitors (Mansour A, 2012).

When osteoclasts degrade bone matrix, the local calcium concentration increases. HSCs deficient in calcium-sensing receptor were found to be incapable of localizing to the endosteum, suggesting a role for calcium gradients in HSC migration within the bone marrow (Adams GB, 2006). Matrix metalloproteinase 9 (MMP-9) is a proteolytic enzyme produced by osteoclasts that degrades bone matrix. It was reported that MMP-9 mediated release of soluble kit-ligand promotes the recruitment of HSCs from the quiescent niche, thereby favoring their differentiation (Heissig B, 2002). MMP-9 KO mice, however, did not show mobilization defects (Robinson SN, 2003; Levesque JP, 2004).

Cathepsin K, subject of this study, is the major protease produced by osteoclasts and its involvement in hematopoiesis has so far not been studied thoroughly.

1.2 Cathepsin K, a component of the hematopoietic niche?

Cathepsin K (CTSK, CatK) is a secreted lysosomal cysteine proteinase and a member of the peptidase C1 protein family. It is highly expressed in osteoclasts and plays an important role in the degradation of bone matrix. The human *Ctsk* gene is located on chromosome 1 (1q21) (Polymeropoulos MH, 1995). Deficiency of cathepsin K causes pycnodysostosis, a rare disease that shows in osteosclerosis and a short statue (Gelb BD, 1996) but can also lead to hematological failure (Del Fattore A, 2008).

Ctsk knockout mice have first been established as a model for this condition. Mouse cathepsin K is a 46kDA protein and the gene is mapped to chromosome 3 (Saftig P, 1998; Rantakokko J, 1999). It cosegregates with cathepsin S which is responsible for degrading cathepsin K and thereby reduces its collagenolytic activity (Barry ZT, 2012). Bone collagen I, osteopontin and osteonectin as well as elastin have been identified as substrates for cathepsin K (Bossard MJ, 1996; Saftig P, 1998). *Ctsk* KO mice show osteopetrosis and despite having a high bone mass, they have fragile bones (Li CY, 2006). In addition, mice deficient of cathepsin K show a reduced resistance against bleomycin-induced lung fibrosis (Srivastava M, 2008); newborn *Ctsk*-/- mice develop lung fibrosis (Zhang D, 2011). Cathepsin K is also expressed by fibroblasts in the skin, its absence results in skin fibrosis (Quintanilla-Dieck MJ, 2009). This indicates that cathepsin K is not restricted to osteoclasts as originally believed and some research about the functions of cathepsin K remains to be done.

Recently, cathepsin K inhibitors have been developed against osteoporosis. They prove to be able to raise bone mineral density and bone volume in mice (Yamane H, 2009). At the time this thesis was written, clinical trials had not yet been finalized. In vivo studies in mice further have shown that cathepsin K deficient mice have a lower risk for developing arteriosclerosis. This might be another application area for cathepsin K inhibitors (Lutgens SP, 2007)

For a broad range of tumors, cathepsin K immunoreactivity has been shown. Many mesenchymal neoplasms like giant cell tumor of the bone, melanoma, Kaposi sarcoma or liposarcoma are reactive. Only a few carcinomas like the renal MiTF/TFE family translocation carcinomas express cathepsin K, but its expression is common in the stroma that surrounds epithelial neoplasms (Zheng G, 2013). Examples for such carcinomas are breast tumors (Kleer CG, 2008) and lung adenocarcinomas (Rapa I, 2006). Those findings suggest that cathepsin K is a factor for invasiveness of carcinomas and their progression. Under investigation is the use of cathepsin K inhibitors for breast cancer bone metastasis (Clézardin, 2011; Jensen AB, 2010).

All of this shows that cathepsin K does not simply play a role in bone resorption but is important in many other tissues in the body. But why did we decide to investigate the role of cathepsin K in hematopoiesis?

In order to determine the components of the hematopoietic niche, a number of cell lines which are able to maintain HSCs in culture have been established. Two lines of embryonic stromal

cells, urogenital-ridge derived UG26-1B6 and embryonic-liver derived EL08-1D2, are clones from the aorta gonads mesonephros (AGM) region where HSCs first appear in the embryo. Those two clones were the first found to be able to maintain HSCs in contact as well as in noncontact cultures, thus making it possible to study soluble factors which might be able to support long-term maintenance of HSCs. In gene expression arrays, where the profiles of UG26-1B6 and EL08-1D2 were compared to nonsupportive stromal cell lines, several genes were expressed higher in the supportive lines, among them secreted frizzled-related protein 1 (SFRP1), pleiotrophin (Ptn) and cathepsin K. (Oostendorp RA, 2005; Oostendorp RA, 2002). So far, it has been demonstrated that SFRP1 and Ptn play a role in hematopoiesis. SFRP1 extrinsically regulates the homeostasis of LSKs and MPPs through ß-catenin dependent cell cycle regulation (Renström J, 2009). Ptn deficient mice showed an increase in the generation of HSCs in serial transplantations (Istvanffy R, 2011; Himburg HA, 2010).

It has previously been shown that the stimulation of osteoclastic proliferation with RANKL also stimulates the mobilization of HSCs from the bone marrow. This was associated with a reduction of the expression of osteopontin and SCF by bone-lining osteoblasts as well as a loss of the expression of SDF-1 by osteoblasts along the endosteum in proximity to osteoclasts. In the presence of an inhibitor of cathepsin K (E-64), RANKL mediated migration was reduced. Further examination of cathepsin K then revealed its ability to cleave the signaling N terminus of SDF-1, suggesting this as a mechanism to facilitate mobilization. Also, SCF is cleaved by cathepsin K (Kollet O, 2006; Staudt ND, 2012).

This could be one possible mechanism by which CSTK influences the mammalian HSC pool. However, not much is known about the role of cathepsin K in hematopoiesis and it is the aim of this study to further clarify if there is evidence for an involvement.

2 Materials and Methods

2.1 Materials

2.1.1 Mice

C57BL/6 Cathepsin K knockout mice

C57BL/6.Pep3b.Ptpcr (CD45.1), Ly5.1+

C57BL/6.J (B6, B6 CD45.2); Ly5.2+

All mice were bred at the Klinikum rechts der Isar in Munich except for the Ly5.2+ C57BL/6.J (B6, B6 CD45.2) needed for secondary transplantations which were purchased from Harlan laboratories (Indianapolis, USA). The animals were kept according to FELASA guidelines. All experiments were approved by the government of Upper Bavaria (Az.: 55.2-1-54-2531-132/06 and 85/10).

2.1.2 Antibodies for flow cytometry

Antigen	**Host**	**Ig subtype**	**Clone**	**Conjugate**	**Conc. (mg/ml)**	**Manufacturer**	**Cat. No.**
CD3e	Armenian Hamster	IgG	145-2C11	Biotin	0,5	eBioscience	13-0031
				PE-Cy5.5	0,2		35-0031
CD4	Rat	IgG2b, κ	GK1.5	PE	0,2	eBioscience	12-0041
				PE-Cy5	0,2		15-0041
				PE-Cy7	0,2		25-0041
CD8a	Rat	IgG2a, κ	53-6.7	PE-Cy5	0,2	eBioscience	15-0081
				PE-Cy5.5	0,2		35-0081
CD11b	Rat	IgG2b, κ	M1/70	APCeFlour® 780	0,2	eBioscience	47-0112
				PE-Cy7	0,2		25-0112
CD16/CD32 (FcγR)	Rat	IgG2a, λ	93	PE	0,2	eBioscience	12-0161
				PECy-5.5	0,2		35-0161

CD34 (Mucosialin)	Rat	IgG2a, κ	RAM34	eFluor® 450	0,2	eBioscience	48-0341
				FITC	0,5		11-0341
CD41 (gpIIb)	Rat	IgG1, κ	MWReg30	eFluor® 450	0,2	eBioscience	48-0411
				PE-Cy7	0,2		25-0411
CD42d (GP5)	Armenian Hamster	IgG	1C2	APC	0,2	eBioscience	17-0421
CD43 (Leukosialin)	Rat	IgG2a, κ	S7	FITC	0,5	BD Biosciences	553268
CD45 (Ly5)	Rat	IgG2b, κ	30-F11	APC	0,2	eBioscience	17-0451
				APCeFlour® 780	0.2		47-0451
				eFluor® 450	0,2		48-0451
				eFluor® 605NC	0,5		93-0451
				FITC	0,5		11-0451
				PE	0,2		12-0451
				PE-Cy5	0,2		15-0451
				PE-Cy5.5	0,2		35-0451
				PE-Cy7	0,2		25-0451
CD45.1 (Ly5.1)	Mouse	IgG2a, κ	A20	APCeFlour® 780	0,2	eBioscience	47-0453
				eFluor® 605NC	0,5		93-0453
				FITC	0,5		11-0453
				PE	0,2		12-0453
CD45.2 (Ly5.2)	Mouse	IgG2a, κ	104	FITC	0,5	eBioscience	11-0454
				PE	0,2		12-0454
CD45R (B220)	Rat	IgG2a, κ	RA3-6B2	eFluor® 450	0,2	eBioscience	48-0452
				PE	0,2		12-0452
				PE-Cy5.5	0,2		35-0452
				PE-Cy7	0,2		25-0452
CD117 (c-Kit)	Rat	IgG2b, κ	2B8	APC	0,2	eBioscience	17-1171
				APCeFlour® 780	0,2		47-1171
				PE	0,2		12-1171
CD127 (IL7Rα)	Rat	IgG2a, κ	A7R34	APC	0,2	eBioscience	17-1271
CD150	Rat	IgG1	9D1	APC	0,2	eBioscience	17-1501
CD161 (NK1.1)	Mouse	IgG2a, κ	PK136	FITC	0,5	eBioscience	11-5941
Gr-1 (Ly-6G)	Rat	IgG2b, κ	RB6-8C5	eFluor® 450	0,2	eBioscience	48-5931
IgM	Mouse	IgG1, κ	DS-1	PE	0,2	BD Biosciences	553517

Sca-1 (Ly-6A/E)	Rat	IgG2a, κ	D7	PE-Cy7	0,2	eBioscience	25-5981
Ter-119	Rat	IgG2b, κ	TER-119	PE	0,2	BD Biosciences	553673
Lineage cocktail (CD3,B220, Ter119 Gr-1 CD11b)			145-2C11 RA3-6B2, M1/70, TER-119, RB6-8C6	Biotin		eBioscience	88-7774-75

Streptavidin PB (Life Technologies; Cat. No. S11222) or PECy5.5 (Life Technologies; Cat. No. SA1018) conjugates were used for secondary staining of biotinylated antibodies.

APC= Allophycocyanin, Cy= Cyanine, FITC= Fluorescein isothiocyanate, PB= Pacific Blue PE= R-phycoerythrin

2.1.3 Buffers and media

HF2+ buffer: 1 x HBSS
2% FCS
1% 1M Hepes
1% Pen/Strep

FACS buffer: 1 x DPBS
0,5% Albumin Fraction V

Freezing medium: 1 x FCS
10% Dimethyl sulfoxide (DMSO)

Stroma medium 400 ml MEM α
75 ml FCS

25 ml HS

5 ml Pen/Strep (dil. 1:100)

100 µl 2-Mercaptoethanol

2.1.4 Chemicals

ACK Lysing Buffer, 280 – 320 mol/l (Life Technologies, USA; Catalog No. A10492-01)

Albumin Fraction V, ≥ 98 %, powdered (BSA) (Carl Roth GmbH & Co. KG, Germany; Catalog No. 8076.5)

Ciprofloxacin (2mg/ml solution; Fresenius Kabi, Germany)

Dimethyl sulfoxide (DMSO) (SERVA electrophoresis GmbH, Germany; Catalog No. 39757.01)

DMEM (Life Technologies, USA; Catalog No. 21063-029)

DPBS (Sigma Aldrich, USA; Catalog No. 59331C)

Fetal Calf Serum (FCS) (PAA, Austria; Catalog No. A15-108)

Filgrastim (Neupogen®; Amgen, USA)

Glutamax (Life Technologies, USA, Catalog No. A12860-01)

HBSS (Life Technologies, USA; Catalog No. 14175-095)

Hepes (Life Technologies, USA; Catalog No. 15630049)

Horse Serum (Stemcell Technologies, Canada; Catalog No. 06850)

Isofluran (Forene® 100%; Abbott Laboratories, USA; Catalog No. 4831867)

Lineage Cell Depletion Kit mouse (MACS, Miltenyi Biotec, Germany, Cat. No. 130-090-858)

- Lineage Biotin Antibody Cocktail
- Anti–Biotin MicroBeads

MEM α (Life Technologies, USA; Catalog No. A10490-01)

MethoCult™ GF M3434 (Stemcell Technologies, Canada; Catalog No. 03434)

2-Mercaptoethanol 50mM (Life Technologies, USA; Catalog No. 31350-010)

Penicillin-Streptomycin 10,000 U/mL (Pen/Strep) (Life Technologies, USA; Catalog No. 15140-148)

Propidium iodide (PI) (Life Technologies, USA; Catalog No. P1304MP)

2.1.5 Consumable materials

Cell Strainer 100µm (BD Falcon™, BD Biosciences, USA)

Filcon 30µm for Syringe (BD Biosciences, USA)

MACS® Cell Separation Columns (Miltenyi Biotec, Germany)

Needles 30 gauge BD Microlance™ 3 (BD Biosciences, USA)

Needles 27 gauge BD Microlance™ 3 (BD Biosciences, USA)

S – Monovette® (Sarstedt AG & Co., Germany)

Supra Blood Lancet (megro GmbH & Co KG, Germany)

Syringes with Needle 1 ml BD Plastipak™ (BD Biosciences, USA)

2.1.6 Equipment

Blood counter	Scil Vet Abc™ Animal Blood Counter (Scil Animal Care Company GmbH, Germany)
Clean bench	ANTAES 48/72, (Biohit, Germany)
Counting chamber	Neubauer improved, depth 0.1 mm (Marienfeld, Germany)
Cell Separator	QuadroMACS (Miltenyi Biotec, Germany)
Centrifuges	Megafuge 3.0 RS, Multifuge 3S (Heraeus, United Kingdom)
Flow Cytometer	CyAn ADP Lx P8 (Beckman Coulter, Miami, USA)
Incubator	Heracell 240 (Thermo Fisher Scientific, USA)
Linear accelerator	KD2 Mevatron, (Siemens, Germany)
Microscope	Axiovert 25 (Carl Zeiss, Jena, Germany)
Water bath	SUB Water Bath (Grant, UK)
Shaker	IKA®, MS1 Minishaker (IKA® Werke GmbH & Co. KG, Germany)

2.2 Methods

2.2.1 Transplantation of bone marrow stem cells

For this study, primary and secondary transplantations of hematopoietic cells were performed. Like in humans, after the elimination of bone marrow through irradiation or cytostatic treatment, the hematopoietic system in mice can be restored completely after transplantation of hematopoietic stem cells. That is why by means of transplantation the integrity of hematopoietic stem cells can be tested, regarding their ability to differentiate as well as to maintain themselves. In primary transplantations this ability is tested in a milieu deficient of the target substance. Long-term changes like alterations of the HSC pool due to exposure to this environment are examined in secondary transplantations.

For the primary transplantation, *Ctsk* KO mice were used with C57BL/6 (CD45.2+) wild type mice as age- and sex-matched controls. So, in this experimental set-up, extrinsic effects of the microenvironment in *Ctsk* KO mice on transplanted wild type bone marrow was tested. Cathepsin K deficient HSCs were also transplanted to wild type mice in order to detect intrinsic effects of the lack of cathepsin K. However, the analysis of these mice is not part of this study, but of the one of Miriam Daldrup (III. Medizinische Klinik und Poliklinik, Klinikum rechts der Isar, Munich).

First, 8 - 10-week-old mice of both groups were lethally irradiated with 8.5Gy (KD2 Mevatron). The next day, 0.25ml PBS together with the donor cells were injected into their tail veins using 1ml syringes. All recipients of the primary transplants were given $2.5x10^5$ cells obtained from the femora and tibiae of C57BL/6 (CD45.1+) mice. After 16 weeks, the recipients were sacrificed. Bone marrow, spleen, thymus, peripheral blood and lymph nodes were analyzed as described below (2.2.4.) and a blood count was performed on the Scil Vet Animal Blood Counter. The remaining bone marrow of the respective groups was pooled for transplantation to the secondary recipients.

For the secondary transplantation both groups consisted of C57BL/6.J (CD45.2+) wild type mice. After irradiation, one group was given $1x10^6$ BM cells from the primary *Ctsk* KO recipients to the tail veins; the other group was given the same amount of cells from the bone marrow of wild type primary recipients. Then, bone marrow, spleen and peripheral blood were analyzed and a blood count was conducted as well.

All mice received Ciprofloxacin (5mg in 250ml of drinking water) as an antibiotic for the 4 weeks following transplantation.

2.2.2 Homing of lineage negative bone marrow cells

The term homing means the migration of hematopoietic stem cells and precursors into the bone marrow. This happens within the first hours after transplantation. Constant migration of these cells from and to the bone marrow occurs physiologically in the body (Méndez-Ferrer S, 2008). So, alterations in the mechanisms involved can affect hematopoiesis and the maintenance of stem cells. To detect such effects, homing and mobilization experiments (see below) were performed.

The 8 – 10-week-old recipient mice (*Ctsk* KO and wild type C57BL/6; all CD45.2+) were irradiated with 9Gy. Seven hours later the same day, they were transplanted $1x10^6$ lineage negative (lin-) bone marrow cells obtained from 8 – 10-week-old CD45.1+ mice.

For gaining the lin- cells, the MACS Lineage Cell Depletion Kit (Miltenyi Biotec) was used according to the protocol provided by the company. First, bone marrow was prepared as described below (2.2.4). All BM cells were then labeled with biotin-conjugated antibodies against CD3 (expressed on B and T cells), CD45R (=B220, expressed on B cells), CD11b (expressed on granulocytes and monocytes), Gr-1 (expressed on granulocytes), 7-4 (expressed on neutrophil granulocytes) and Ter-119 (expressed on erythrocytes). Those antibodies were then labeled with anti-biotin-antibodies conjugated to magnetic MicroBeads. Then, the cells were flushed through MACS columns attached to a QuadroMACS separator. Thus, the magnetically labeled cells were retained in the columns and the lin- cells, that had passed the columns, could be collected.

The following day, 24h after irradiation, the mice were sacrificed. Bone marrow, spleen and peripheral blood were prepared (2.2.4.). Blood counts and flow cytometry with antibodies against Ly5.1 (donor cells) and stem cell markers such as Sca-1, CD34 and c-Kit were conducted. In this experiment, $1x10^7$ bone marrow and spleen cells and all PB cells were stained in order to detect the donor cells.

2.2.3 Mobilization of hematopoietic stem cells

Like homing, mobilization of hematopoietic stem cells from the bone marrow is a physiological process. The subject of this study, cathepsin K, is a protease. Proteases are substantial for migration processes throughout the body. That is one possible reason why defects of mobilization in cathepsin K KO mice could be a factor of influence for the alterations in hematopoiesis observed in these models.

For the mobilization assay, four experimental groups were formed. Two groups consisted of wild type or *Ctsk* KO mice that did not receive treatment and two groups of wild type or *Ctsk* KO mice that were treated with 125µg/kg/injection of filgrastim (recombinant G-CSF) (Winkler IG, 2010). G-CSF (granulocyte-colony stimulating factor) is a peptide hormone which causes the mobilization of HSCs from the bone marrow and in recombinant form is used to treat neutropenia in chemotherapy patients. The drug was administered via subcutaneous injections to the neck region of the animals every 12 hours for 4 consecutive days. On day 5, the mice were sacrificed and blood counts, CFA (2.2.6) and flow cytometry of bone marrow, spleen and peripheral blood were performed. For flow cytometry, HSC markers were stained.

2.2.4 Preparation and analysis of murine tissue

For the investigation of the effects of cathepsin K, various tissues which are involved in hematopoiesis were analyzed: Bone marrow, spleen, lymph nodes, thymus and peripheral blood in primary transplantation experiments and bone marrow, spleen and peripheral blood in secondary transplantation, homing and mobilization experiments. All above mentioned organs were also analyzed in 8 – 10-week-old *Ctsk* knockout mice and wild type controls in order to examine steady-state hematopoiesis.

Bone marrow cells were won from femora and tibiae. The ends of the bones were cut open and flushed with 3ml of HF2+ buffer using a 27 Gauge cannula. After that, they were filtered through a 30µm Filcon (BD) and centrifuged. The pellet was then resuspended in 1ml of HF2+. Like the cells obtained from the other organs, BM cells were counted with Turk's solution and Trypan blue in a Neubauer chamber. Turk's solution stains the nuclei of white blood cells and contains acetic acid that lyses erythrocytes and Trypan blue stains only non-viable cells, both

of which would distort counting results. The bone marrow cells were subsequently used for transplantation or flow cytometry analysis.

Blood was obtained from the facial veins of live mice or directly from the heart after euthanasia and collected in S – Monovettes® (Sarstedt) containing EDTA to prevent the samples from clotting. After drawing the blood, a differential blood count was performed on the Scil Vet Abc™ Animal Blood Counter (Scil Animal Care Company). Then erythrocytes in the probes were lysed by adding 5ml of ACK lysing buffer (Life Technologies) and incubating the samples on ice for 15 minutes. This procedure was performed twice. Afterwards, the samples were centrifuged at 2100rpm and resuspended in 1ml of HF2+ buffer. The cells were then also counted and stained for flow cytometry.

Spleen, lymph nodes and thymus were mashed and then flushed through a 100µm cell Strainer (BD). Then, they were also filtered (30µm Filcon, BD), centrifuged and resuspended in 1ml of HF2+. Like blood and bone marrow samples, cells were counted and stained for flow cytometry.

2.2.5 Flow cytometry

Flow cytometry is an established method of distinguishing cells in a sample by detecting characteristic structures on their surface. Those antigens can be marked with antibodies that are conjugated with fluorescent agents (fluorochromes) and then be quantified with a flow cytometer. For that purpose, single cells pass a laser-based measuring system in a stream of fluid that detects light emitted by the above mentioned florescent agents as well as other parameters such as forward scatter (commensurate with the size of a cell) and side scatter (commensurate with the granularity of a cell). In this study, surface markers characteristic of hematopoietic stem cells and progenitors as well as of adult blood cells were aimed at.

$1x10^6$ cells were stained when looking for mature cell populations, $6x10^6$ when looking for HSCs and progenitors. The cells were suspended in 100µl of FACS buffer (see 2.1.4.) and 1µl per $1x10^6$ cells of each antibody was added (exception: Sca-1 and c-Kit antibodies: 2µl per $1x10^6$ cells). The samples were then mixed on a shaker and incubated at 4°C for 15 minutes. Then, 2ml of FACS buffer were added in order to wash away free antibodies. The samples were

then centrifuged. If necessary, the process was repeated with secondary antibodies. Finally, 500µl of FACS buffer and 1µg propidium iodide per ml (accumulates in non-viable cells and can be detected by flow cytometry) were added. Color controls were made from wild type bone marrow cells that were stained with all fluorochromes used in the experiment bound to CD45 antibodies.

Flow cytometry was performed on a CyAn ADP Lx P8 (Beckman Coulter) and data was analyzed with the program FlowJo (TreeStar Inc., USA).

2.2.6 Colony forming unit assay

In order to determine not only the numbers of hematopoietic cells in the respective tissues, but also their proper functioning, colony forming unit assays (CFA) were performed. A CFA tests the capability of a stem cell or progenitor to form colonies of cells in various stages of differentiation by providing it with a mixture of necessary cytokines. In this study the M3434 methylcellulose medium (Stemcell Technologies) was used on cells obtained from peripheral blood or bone marrow. M3434 contains recombinant cytokines and erythropoietin for the stimulation of erythroid progenitors (bust-forming unit – erythroid; BFU-E), granulocyte-macrophage progenitors (colony-forming unit – granulocyte macrophage; CFU-GM) and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitors (colony-forming unit - granulocyte, erythroid, macrophage, megakaryocyte; CFU-GEMM). CFA were set up following the sacrifice of wild type mice and *Ctsk* KO mice with bone marrow for characterization as well as after the mobilization of stem cells from the bone marrow with peripheral blood cells.

The CFAs were prepared as described in the M3434 user's manual. After gaining blood cells (see 2.2.4) and counting them, $2.5x10^4$ bone marrow cells in characterization and $2.5x10^5$ peripheral blood cells in mobilization experiments were resuspended in 250µl of stroma medium and then thoroughly mixed with 2.5ml of the M3434 medium using a pipette and a shaker. After letting the tubes rest for several minutes until all visible bubbles had disappeared, duplicates of the assay were set up by applying 1ml of the mixture to each of two 35mm dishes. Together with a third dish filled with sterile water to humidify the medium, they were put in a 100mm dish and then incubated at 37°C for 10 days. Then the BFU-E, CFU-GEMM, small

CFU-GM (50-1000 cells per colony) and large GFU-GM colonies (>1000 cells per colony) were counted under a microscope.

2.2.7 Statistics

Data was statistically analyzed with Excel (Microsoft). The arithmetical mean, the standard deviation and the standard error of the mean (SEM) were calculated. Unpaired Student's t-tests were applied and a value was considered significant when the probability of error was below 5%. Data obtained from repetitive experiments was normalized.

3 Results

3.1 Hematopoietic characterization of cathepsin K knockout mice

3.1.1 Blood count and cell numbers

To learn something about the phenotype of cathepsin K knockout mice with regard to hematopoiesis, 8 – 10-week-old KO mice and controls were characterized repeatedly. For that purpose, immediately after their sacrifice, blood samples were taken and analyzed on a blood counter. The complete blood counts showed a significantly lower mean corpuscular hemoglobin and a significantly higher red cell distribution width (i.e. the range of different sizes in the erythrocyte fraction) in the KO group (Figure 3.1). Those changes in both parameters are indicators for anemia. However, both parameters were within the reference range and the number of red blood cells and the hemoglobin were not reduced in the KO mice (Table 3.1).

After taking the blood samples, even though they were quickly mixed with EDTA, a high rate of clotting in the peripheral blood from *Ctsk* KOs was observed. Also, the blood counts of many of the KO mice showed very high numbers. But probably as a result of the great variation within the KO group, its mean platelet number was not significantly higher. Nevertheless, because of these observations, it was decided to add antibodies for staining platelet markers in flow cytometry (see figures 3.18 – 3.20).

Table 3.1 Complete blood count of 8 – 10-week-old *Ctsk* KO mice and wild type controls.
Results of three independent experiments; arithmetic means ±SEM, $n_{(+/+)}=12$, $n_{(-/-)}=12$, *p<0.05, **p<0.01.

	CTSK +/+	CTSK -/-
WBC [10³/µl]	5.21 ± 0.51	4.75 ± 0.53
Lymph. [10³/µl]	3.93 ± 0.36	3.29 ± 0.37
Mono. [10³/µl]	0.18 ± 0.02	0.15 ± 0.02
Gran. [10³/µl]	1.10 ± 0.14	1.26 ± 0.19
Eos. [10³/µl]	0.07 ± 0.01	0.10 ± 0.02
RBC [106/µl]	8.56 ± 0.43	9.20 ± 0.47
HGB [g/dl]	14.23 ± 0.66	14.72 ± 0.73
HCT [%]	51.25 ± 2.65	54.65 ± 3.00
MCV [µm³]	59.75 ± 0.44	59.35 ± 0.49
MCH [pg]	16.65 ± 0.13	16.03 ± 0.23 *
MCHC [g/dl]	27.92 ± 0.33	27.13 ± 0.47
RDW [%]	13.04 ± 0.18	13.99 ± 0.24 **
PLT [10³/µl]	985 ± 213	1208 ± 280
MPV [µm³]	5.72 ± 0.08	5.49 ± 0.14

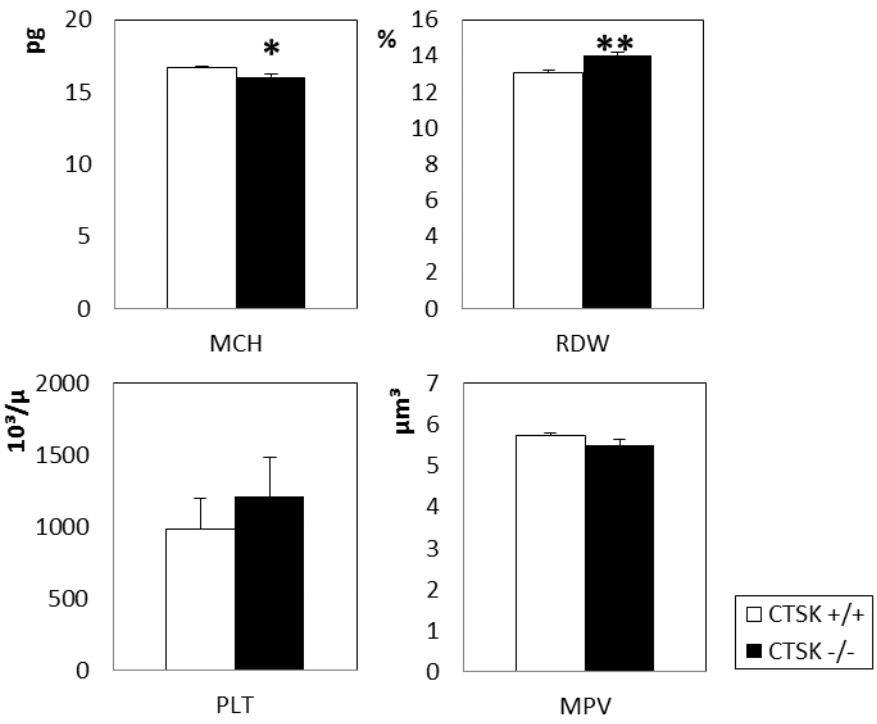

Figure 3.1. Mean corpuscular hemoglobin, red cell distribution width, number of platelets and mean platelet volume in blood counts of 8 – 10-week-old *Ctsk* KO mice and wild type controls. Means +SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=12, *p<0.05, **p<0.01.

Next, all spleens were weighed and absolute cell numbers of all organs were determined as described in 2.2.4. Cell numbers of bone marrow, spleen, thymus or lymph nodes (Figure 3.2) as well the spleen weights (Figure 3.3) did not show significant differences.

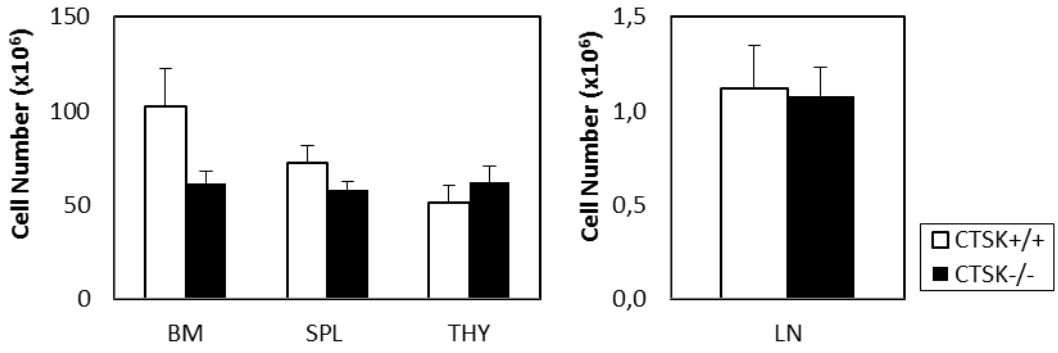

Figure 3.2. Means of cell numbers in bone marrow, spleen and thymus and in lymph nodes from 8 – 10-week-old mice +SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

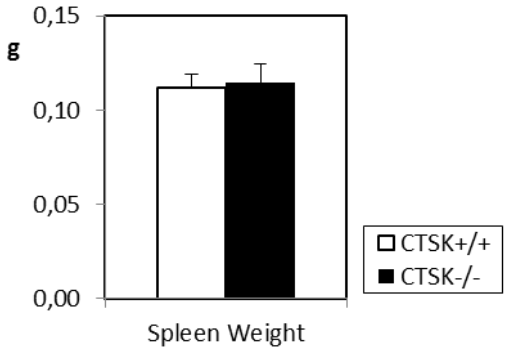

Figure 3.3. Spleen weight, means of wild type and *Ctsk* KO samples in characterization of 8 – 10-week-old mice. +SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=12.

3.1.2 Flow cytometry

To further investigate whether cathepsin K deficiency in mice has an effect on hematopoiesis, cells from bone marrow, spleen, peripheral blood, thymus and lymph nodes were marked with antibodies for flow cytometry as described in 2.2.5.

To examine the early stages of hematopoiesis, lineage negative cells, CLPs (common lymphoid progenitors), MPPs (multipotent progenitors), LSKs (lineage-negative, Sca-1+, c-Kit+ cells), LT-HSCs (long-term repopulating hematopoietic stem cells), CMPs (common myeloid progenitors), GMPs (granulocyte-monocyte progenitors) and MEPs (megakaryocyte-erythrocyte progenitors) in bone marrow from tibiae and femora of both hind legs and in spleens were identified. The gating strategy and the antigens targeted in bone marrow and spleen samples respectively is depicted in figures 3.4 and 3.6.

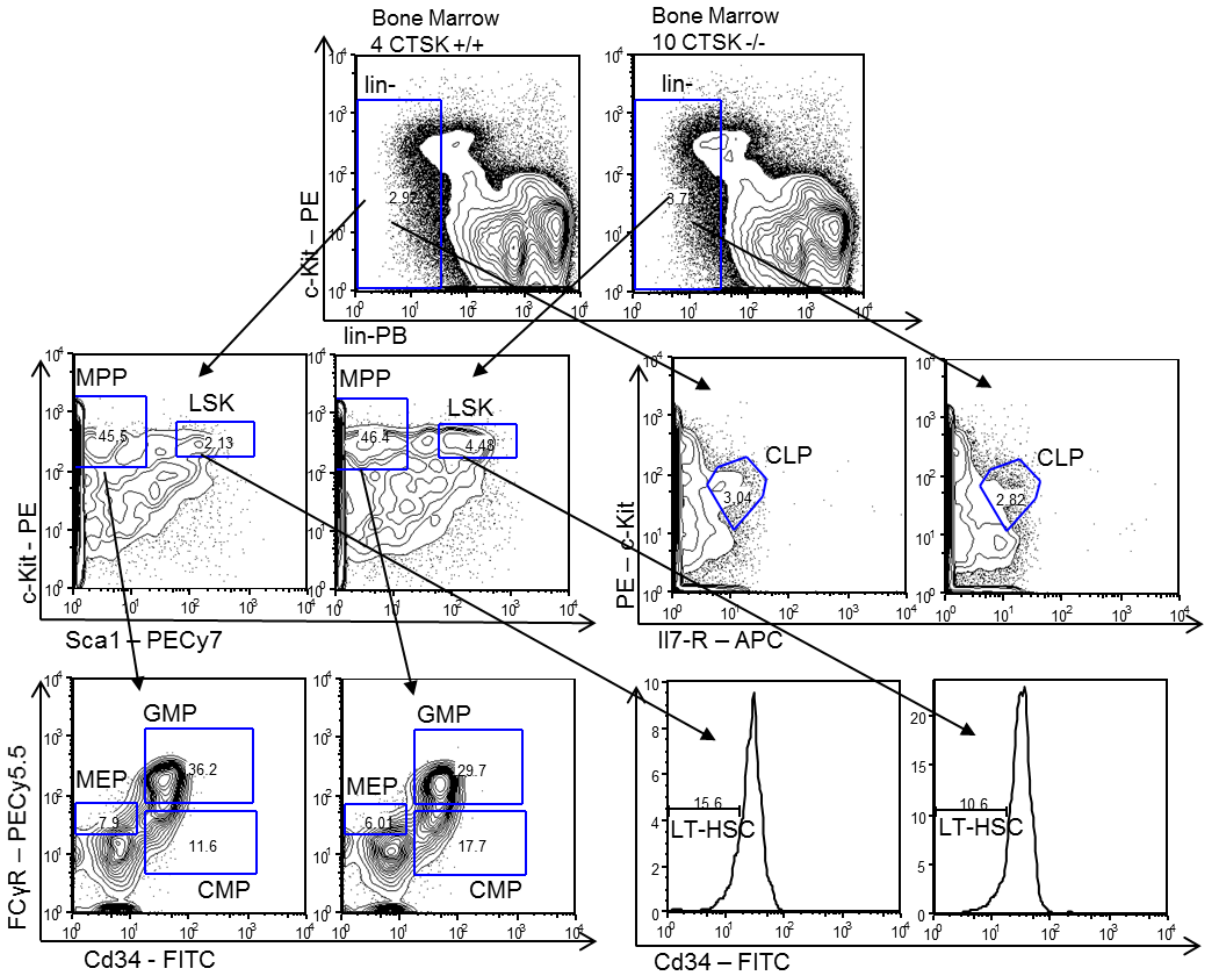

Figure 3.4. Gating strategy for hematopoietic stem cells, bone marrow. Lineage negative cells (CD3- B220- Ter119- Gr-1- CD11b-), MPP (c-Kit+ Sca-1- lin-), LSK (c-Kit+ Sca-1+ lin-), CLP (Il7-R+ c-Kit^{mid} lin-), MEP (CD34- FcγR^{low} MPP), GMP (CD34+ FcγR+ MPP), CMP (CD34+ FcγR- MPP) and LT-HSC (CD34- LSK).

The only alteration found here was a lower number of GMPs in the bone marrow of the cathepsin K deficient mice (Figure 3.5). In particular, there was no change in the common lymphocyte progenitor populations or the megakaryocyte-erythrocyte progenitors.

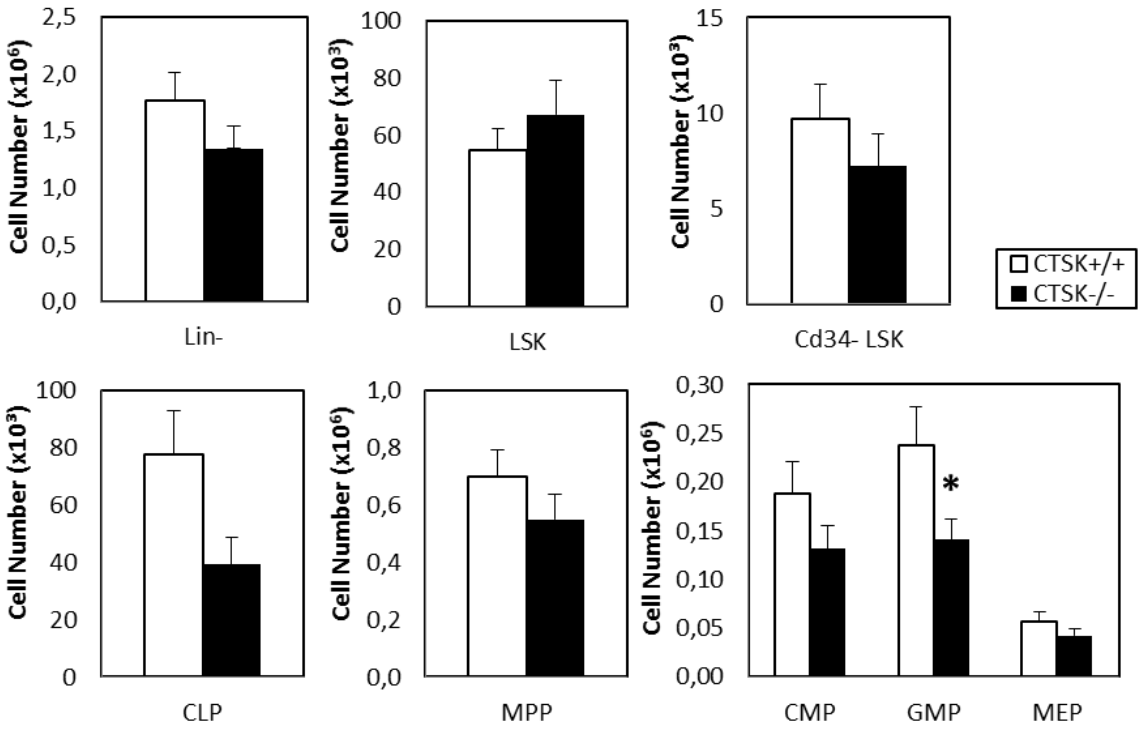

Figure 3.5. Number of stem cells and progenitors in bone marrow of *Ctsk* KO and wild type controls. Long bones of both hind legs. CD34- LSK = LT-HSC. Means +SEM, $n_{(+/+)}$=15, $n_{(-/-)}$=15, *p<0.05.

GMPs differentiate into monocyte and macrophage progenitors on the one hand and granulocyte progenitors on the other hand. To find out if the adult cells that originate from this lineage are affected, monocytes and granulocytes were examined by flow cytometry as well (see below). Also, the functional capacity of the progenitors was tested in a colony forming unit assay (see 3.1.3).

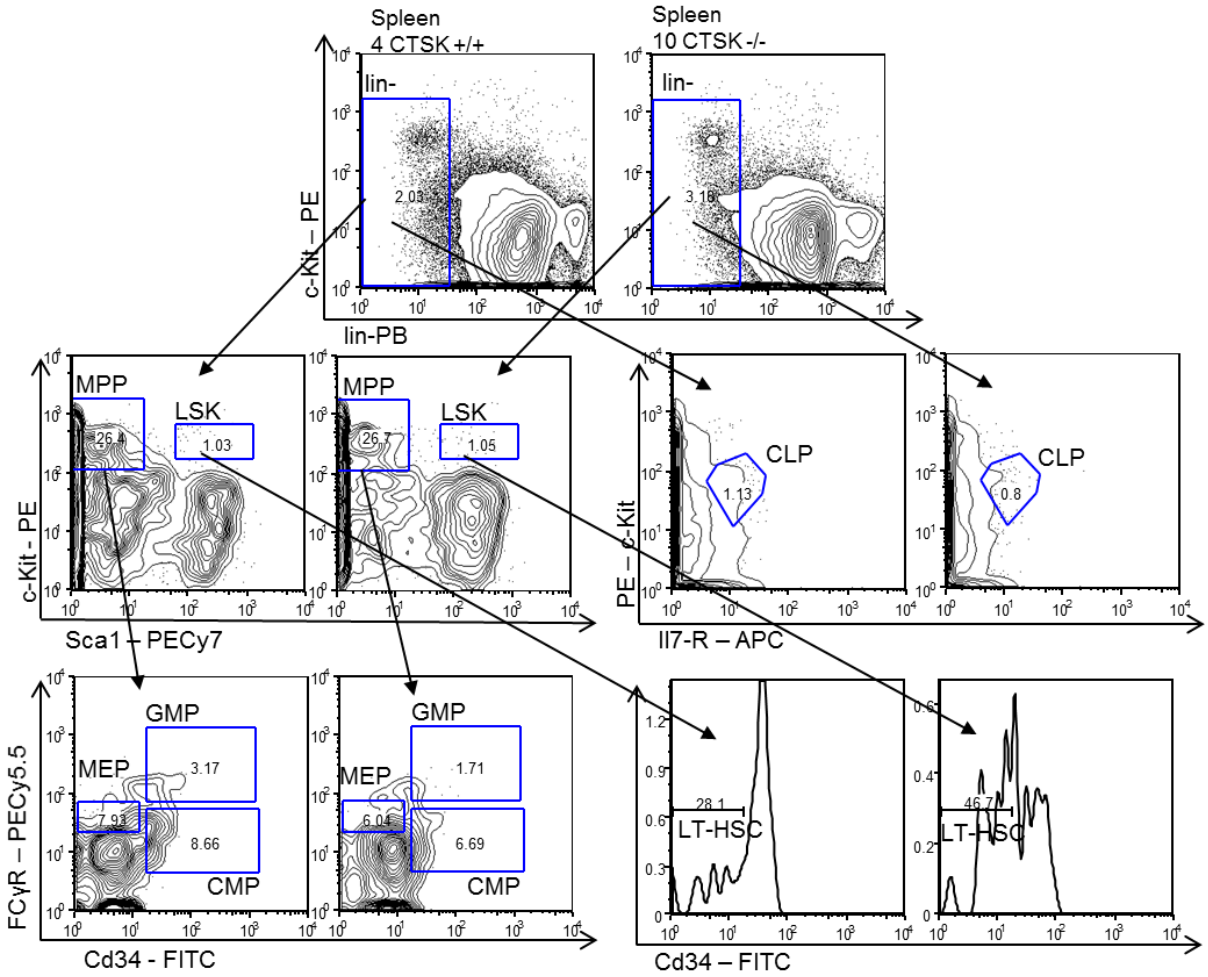

Figure 3.6. Gating strategy for hematopoietic stem cells, spleen. Lineage negative cells (CD3- B220- Ter119- Gr-1- CD11b-), MPP (c-Kit+ Sca-1- lin-), LSK (c-Kit+ Sca-1+ lin-), CLP (Il7-R+ c-Kit^{mid} lin-), MEP (CD34- FcγR^{low} MPP), GMP (CD34+ FcγR+ MPP), CMP (CD34+ FcγR- MPP) and LT-HSC (CD34- LSK)

No significant alterations regarding HSCs and progenitor populations were detected in the spleen samples (Figure 3.7). The variation in cell numbers in *Ctsk*-/- mice was much larger than in the WT mice.

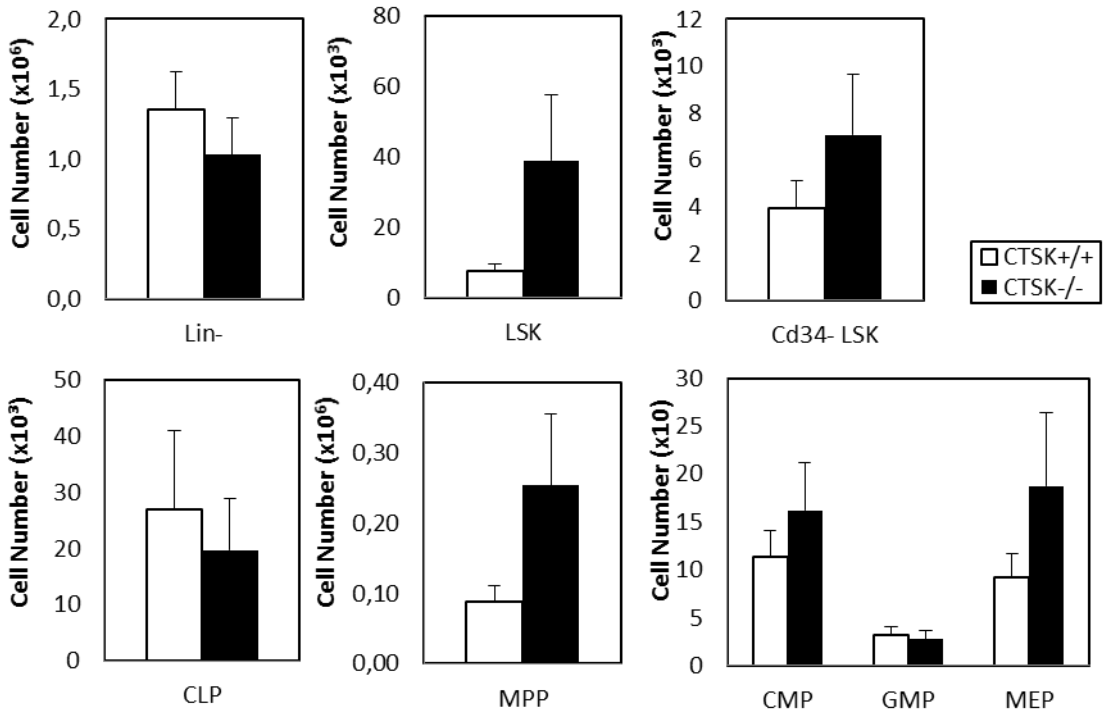

Figure 3.7. Number of stem cells and progenitors in spleens of *Ctsk* KO and wild type controls. Means + SEM, $n_{(+/+)}$=15, $n_{(-/-)}$=15.

B220, an isoform of CD45 that is expressed on B lymphocytes, was used as a marker antigen for adult B cells. In *Ctsk* KO mice no alterations concerning B220+ cells were found compared to C57BL/6 wild type mice in any of the organs analyzed (Figures 3.8, 3.9, 3.10, 3.11 and 3.12)

Monocytes and granulocytes are defined by their expression of Gr-1 and CD11b. Surprisingly, no difference in cell numbers of the respective populations in bone marrow, spleen, peripheral blood, thymus or lymph nodes was discovered. So, the diminution of their common progenitor, GMP, did not seem to have an effect on the number of adult cells. This suggests a higher differentiation rate of each GMP in cathepsin K deficient mice so as to be able to compensate a lower number of progenitors. The functional capacity necessary to do so was examined by Colony-Forming Assay (CFA, see 3.1.3).

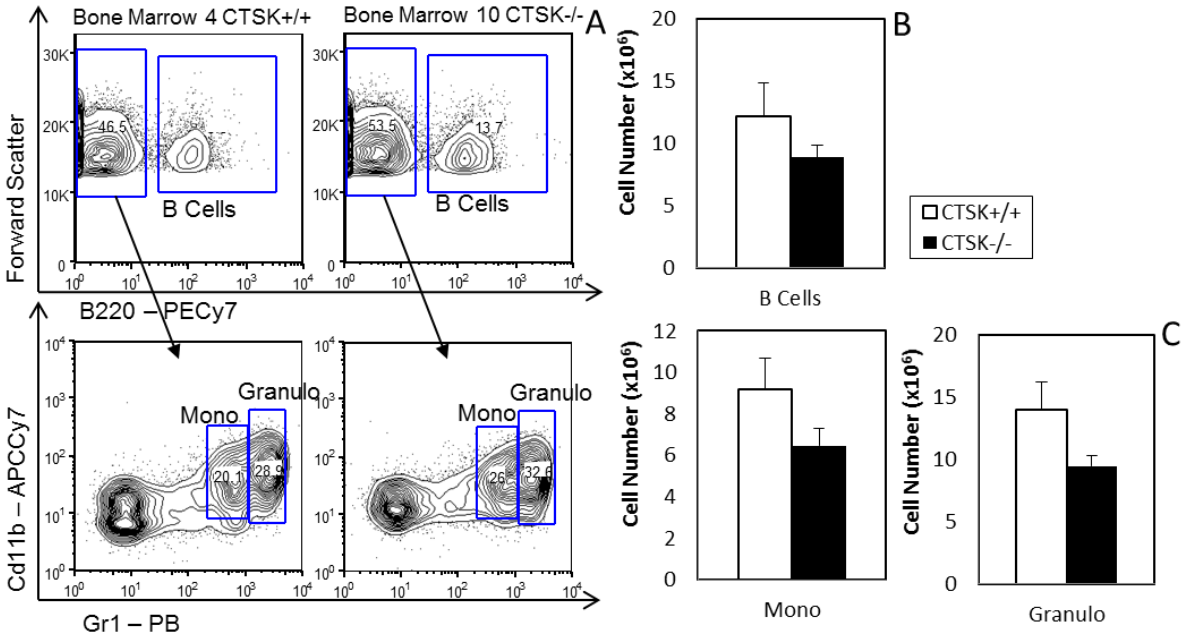

Figure 3.8. B cells, granulocytes and monocytes in bone marrow obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of B cells (B220+ cells) C. Number of monocytes ($Gr1^{mid}$ and CD11b+, B220-) and granulocytes (Gr1+ and CD11b+, B220-). Means + SEM, $n_{(+/+)}=5$, $n_{(-/-)}=5$.

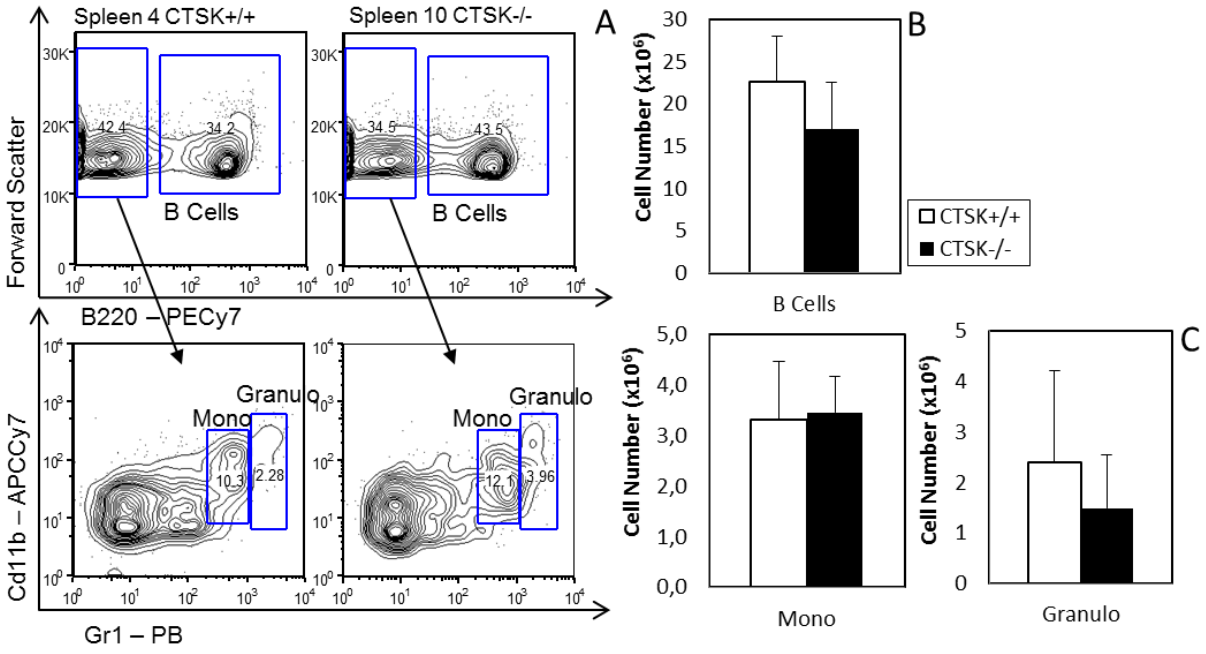

Figure 3.9. B cells, granulocytes and monocytes in spleens obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of B cells (B220+ cells) C. Number of monocytes (Gr1^{mid} and CD11b+, B220-) and granulocytes (Gr1+ and CD11b+, B220-). Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

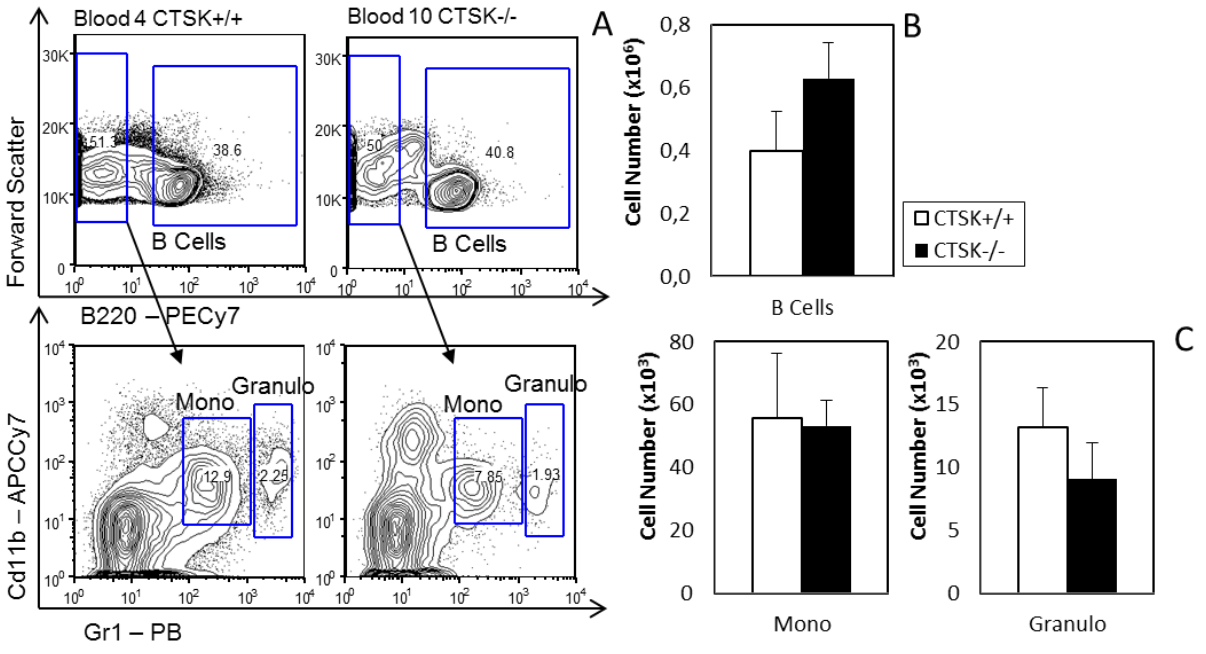

Figure 3.10. B cells, granulocytes and monocytes in peripheral blood samples (300µl) obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of B cells (B220+ cells) C. Number of monocytes (Gr1^{mid} and CD11b+, B220-) and granulocytes (Gr1+ and CD11b+, B220-). Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

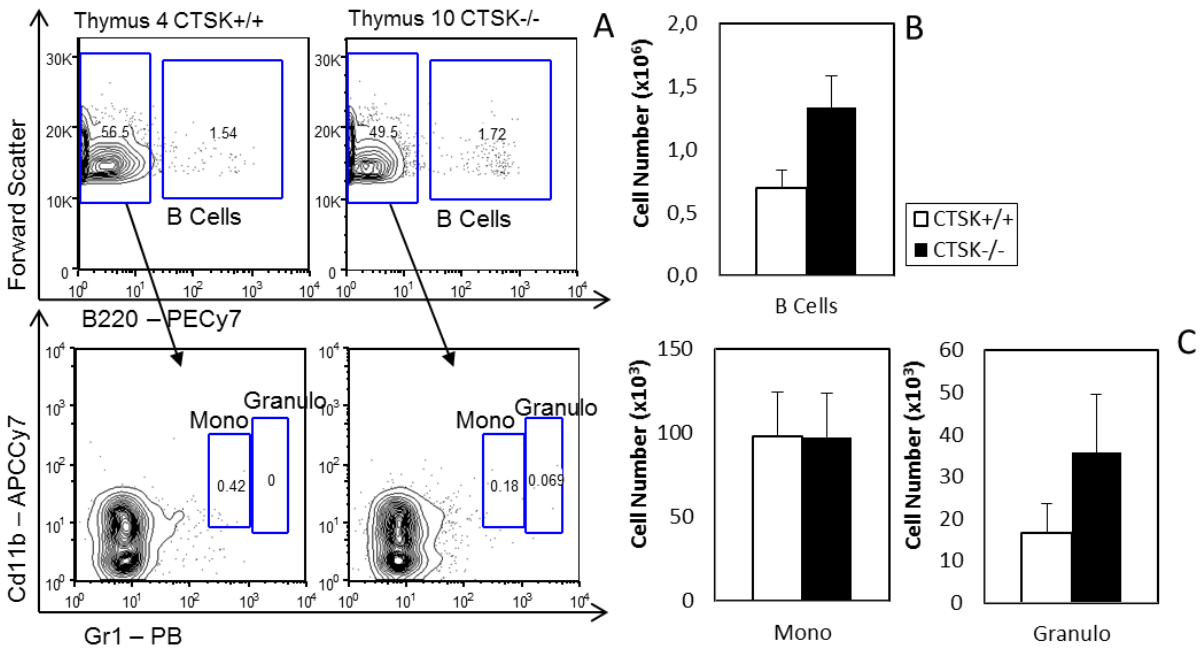

Figure 3.11. B cells, granulocytes and monocytes in thymi obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of B cells (B220+ cells) C. Number of monocytes (Gr1^{mid} and CD11b+, B220-) and granulocytes (Gr1+ and CD11b+, B220-). Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

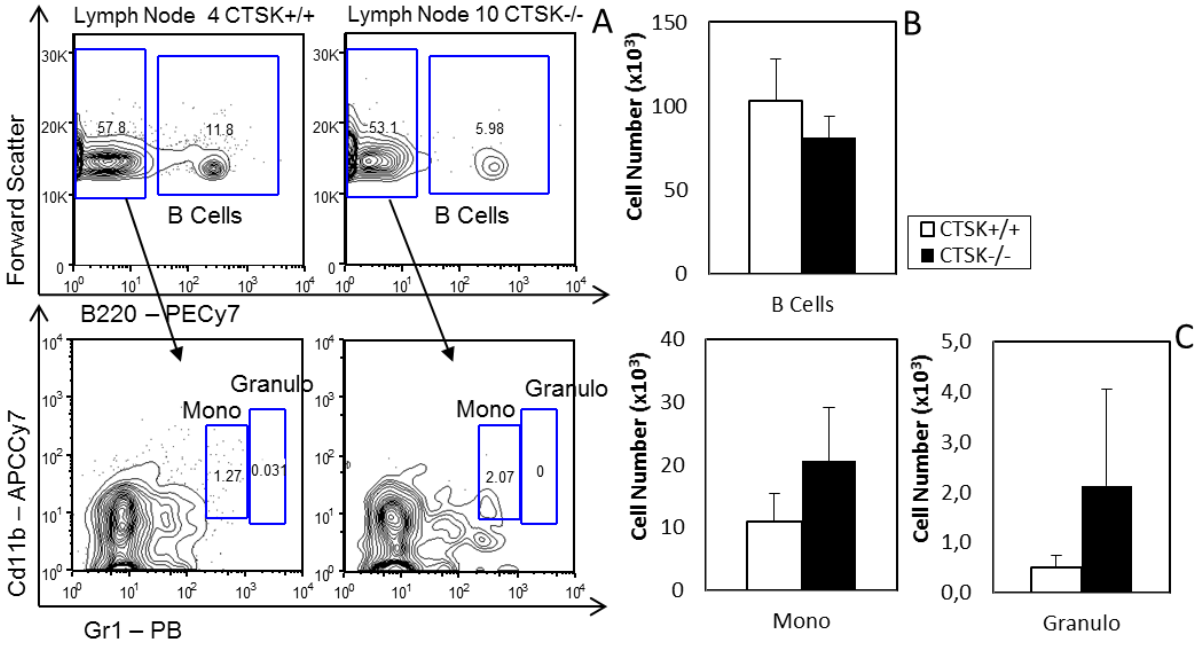

Figure 3.12. B cells, granulocytes and monocytes in lymph nodes obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of B cells (B220+ cells) C. Number of monocytes (Gr1^{mid} and CD11b+, B220-) and granulocytes (Gr1+ and CD11b+, B220-). Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

T lymphocyte populations were in this experiment classified by their expression of CD4 or CD8a. CD4 is expressed mainly by T helper cells, but also by monocytes, macrophages and dendritic cells. CD8 is found on the surface of cytotoxic T lymphocytes. There is also a subset of CD4/CD8 double positive cells that physiologically appears in the thymus during the maturation of thymocytes (Figure 3.16) before becoming either CD4 or CD8 positive. But there are small numbers of double positive T cells that can be found in other lymphatic tissue and in the blood as well. These lymphocytes might play a role in autoimmune processes (Parel Y, 2004).

In steady-state hematopoiesis of *Ctsk* KO mice, no changes were discovered in CD4 or double positive populations (Figures 3.13 to 3.17). Yet, there were significant differences concerning CD8a+ cells. Less CD8a+ cells were detected in the bone marrow samples of KO mice (Figure 3.13) while more of them appeared in their peripheral blood (Figure 3.15). As there was no alteration in the number of cytotoxic T cells in the thymus where they first appear, or more generally in CLPs (see above), the alteration of CD8 positive cells does likely not originate from their development but rather from either an altered life span or differences in the migration between compartments. It is conceivable that CD8+ cells cannot move from the peripheral blood to the bone marrow easily in the absence of the protease cathepsin K.

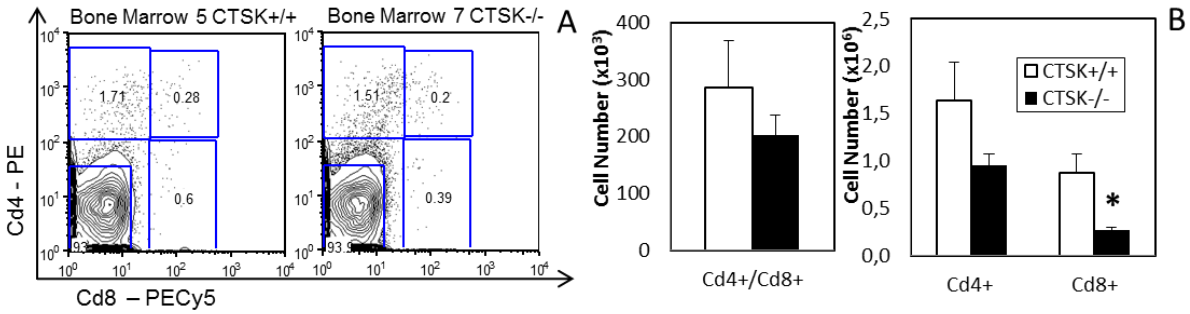

Figure 3.13. T cells in bone marrow obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of CD4/CD8a+, CD4+/CD8a- and CD8a+/CD4- T cells. Means + SEM, $n_{(+/+)}=5$, $n_{(-/-)}=5$, *$p<0.05$.

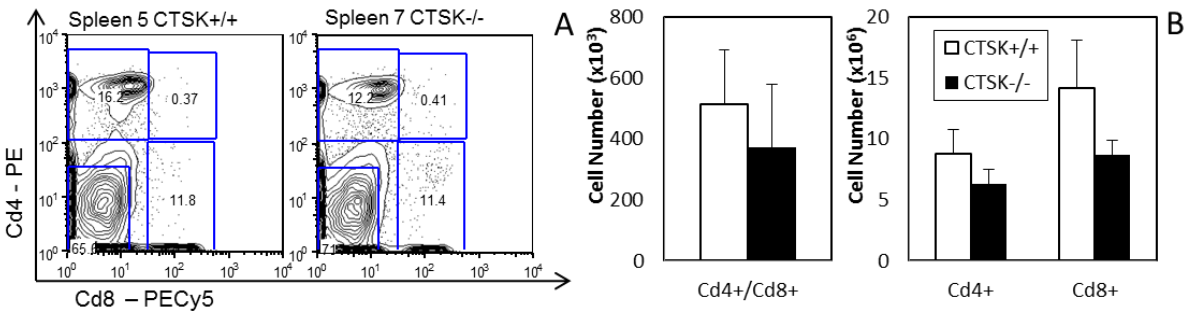

Figure 3.14. T cells in spleens obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of CD4/CD8a+, CD4+/CD8a- and CD8a+/CD4- T cells. Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

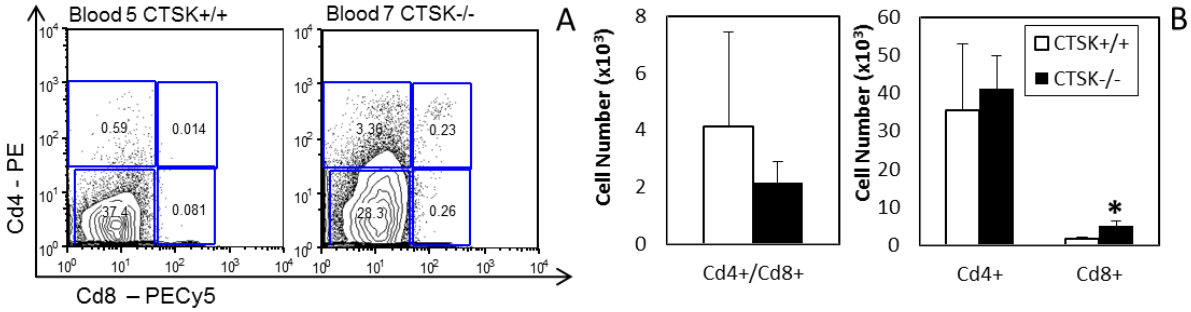

Figure 3.15. T cells in peripheral blood (samples of 300µl) obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of CD4/CD8a+, CD4+/CD8a- and CD8a+/CD4- T cells. Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5, *p<0.05.

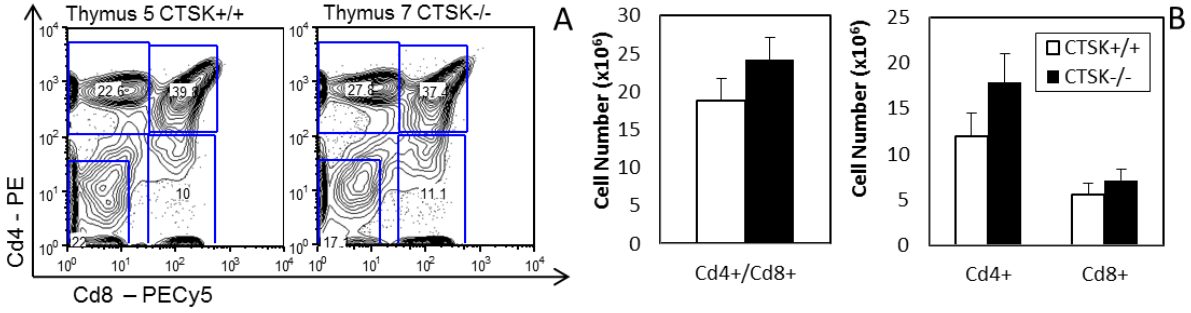

Figure 3.16. T cells in thymi obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of CD4/CD8a+, CD4+/CD8a- and CD8a+/CD4- T cells. Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

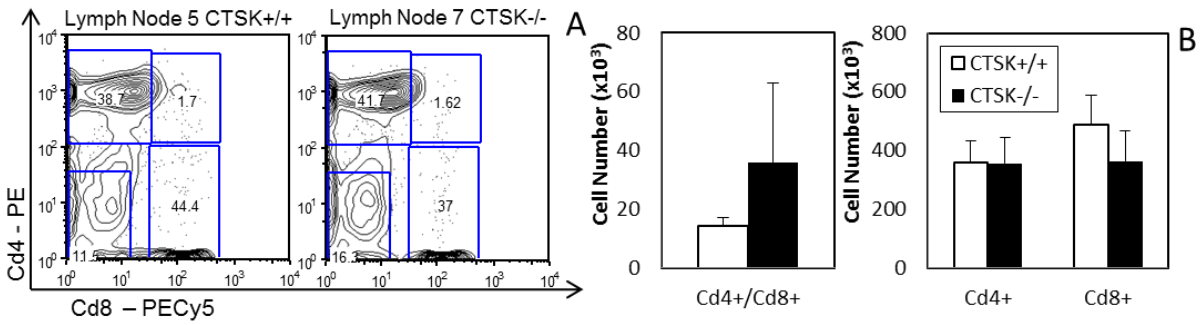

Figure 3.17. T cells in lymph nodes obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. Number of CD4/CD8a+, CD4+/CD8a- and CD8a+/CD4- T cells. Means + SEM, $n_{(+/+)}$=5, $n_{(-/-)}$=5.

Because there seemed to be an enhanced likeliness for clotting in *Ctsk* -/- mice, it was decided to also look into platelet numbers in bone marrow, spleens and peripheral blood. CD41 (glycoprotein IIb/IIIa), the fibrinogen receptor, and CD42d (glycoprotein V) which is part of the receptor for von Willebrand factor are both present on platelet surfaces. As it is known that CD41 is expressed on hematopoietic progenitors as well (Mitjavila-Garcia MT, 2002), CD42d antibodies were added. GP5 is restricted to megakaryopoiesis, where it appears after CD41, and adult platelets (Sato N, 2000). Results are shown in percent of live cells here as the cell counts which were performed with Turk's solution do not give any information about platelet numbers.

In bone marrow, a higher percentage of CD41+/CD42d+ cells was detected (Figure 3.18). This means that the observed high number of platelets in the blood counts of some KO mice and the overshooting coagulation in the samples of others seems to be the result of an overproduction of thrombocytes in the bone marrow.

CD41+/CD42d+ cells in spleens (Figure 3.19) and, as to be expected because of the similar results in blood counts, in peripheral blood (Figure 3.20) were not altered.

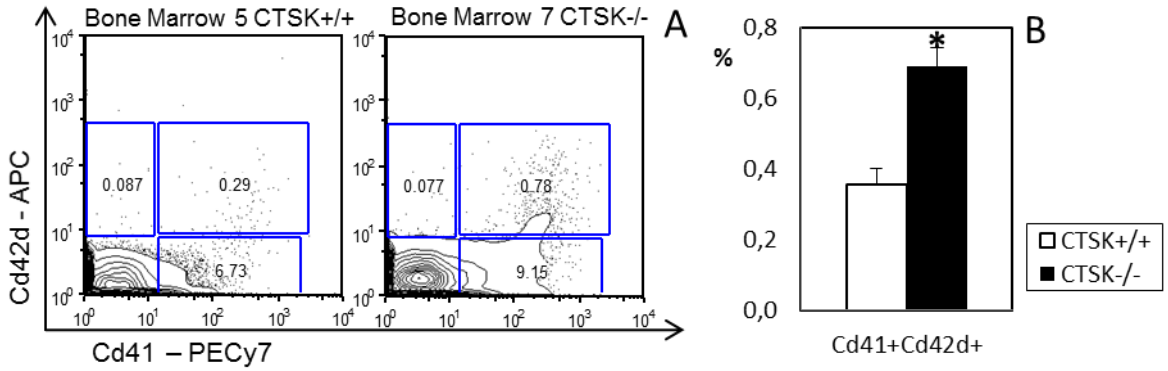

Figure 3.18. Platelets in bone marrow obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. CD41+/CD42d+ platelets, percentage of live cells. Means + SEM, $n_{(+/+)}=5$, $n_{(-/-)}=5$, *p<0.05.

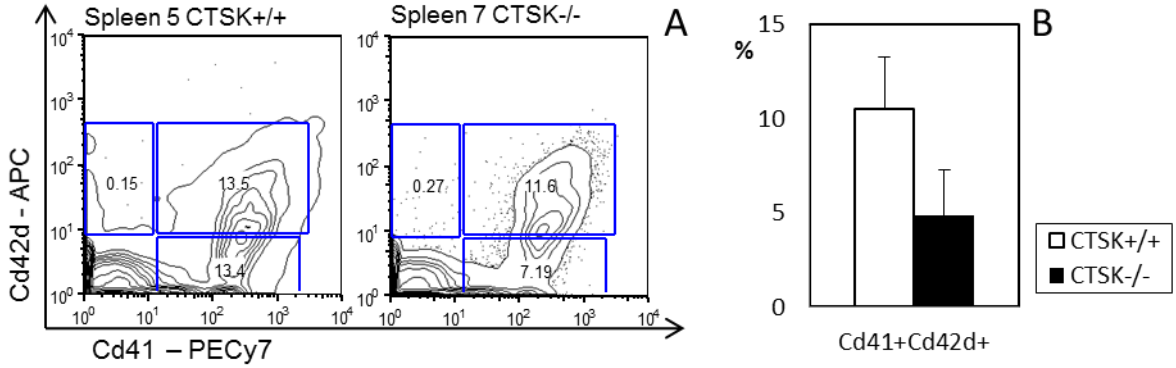

Figure 3.19. Platelets in spleens obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. CD41+/CD42d+ platelets, percentage of live cells. Means + SEM, $n_{(+/+)}=5$, $n_{(-/-)}=5$.

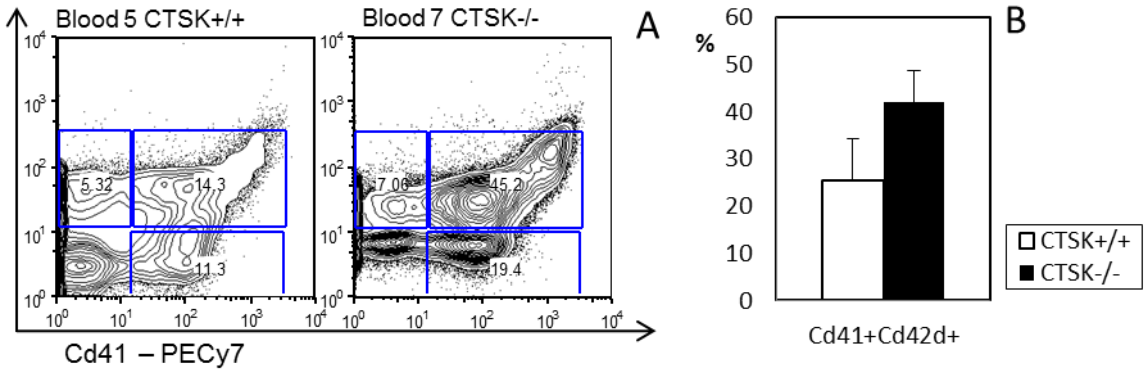

Figure 3.20. Platelets in peripheral blood (samples of 300µl) obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls. A. Flow cytometry plots. B. CD41+/CD42d+ platelets, percentage of live cells. Means + SEM, $n_{(+/+)}=5$, $n_{(-/-)}=5$.

3.1.3 Colony forming unit assay

For the more functional assessment of hematopoietic progenitors, CFU assays were conducted. They did not show a difference concerning the erythroid lineage (BFU-E) or CFU-GEMM, which are formed by multipotent myeloid progenitors.

However, more CFU-GM formed in the dishes with *Ctsk* KO bone marrow. There were more small colonies consisting of 50 – 1000 cells as well as large colonies with more than 1000 cells. This did also result in a higher total colony number in cathepsin K deficient mice. It seems that GMPs, whilst existing in a smaller number in the bone marrow, have a higher functional capacity here than in wild type mice. This apparently results in unmodified numbers of granulocytes and monocytes in the KOs.

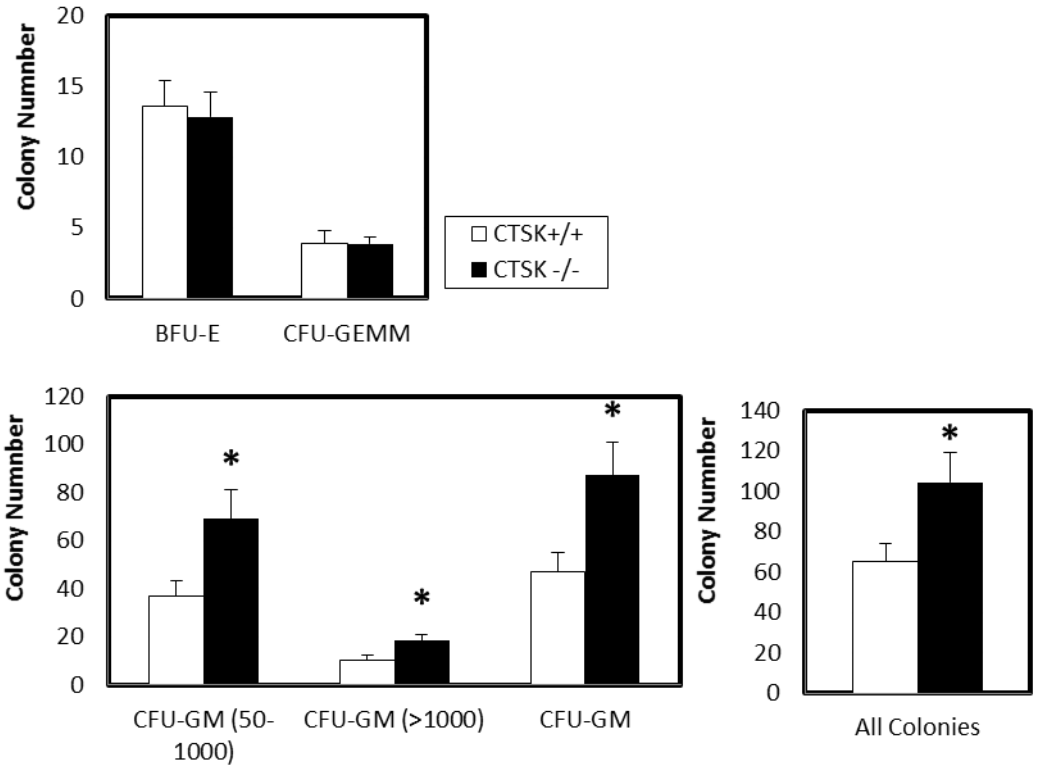

Figure 3.21. Colony forming unit assays prepared with bone marrow obtained from 8 – 10-week-old *Ctsk* KO mice and wild type controls showing colonies of erythroid progenitors, granulocyte-macrophage progenitors and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitors. BFU-E (bust-forming unit - erythroid), CFU-GEMM (colony-forming unit - granulocyte, erythroid, macrophage, megakaryocyte), CFU-GM (colony-forming unit - granulocyte macrophage) and total number of colonies. Means + SEM, $n_{(+/+)}=11$, $n_{(-/-)}=11$ *$p<0.05$.

3.2 Primary transplantation of hematopoietic stem cells

3.2.1 Blood count and cell numbers

In the blood counts conducted after the sacrifice of the KO mice and controls transplanted with bone marrow cells 16 weeks in advance, no significant differences were detected in the white cell lineage. One alteration concerned the red cells. The mean cell volume of the erythrocytes (Figure 3.22) was higher in *Ctsk* KO mice than in wild type mice. Red cell distribution width and mean corpuscular hemoglobin, which had both been altered in the characterization of *Ctsk* KO mice (Figure 3.1), showed no significant differences in the primary transplantations.

A notable change was also found in the platelet fraction. Again, like in the characterization of the *Ctsk*-/- mice under steady-state conditions (see 3.1.1) the platelet number was found to be higher in average in the *Ctsk* KO recipients. And also a raise in clotting in the KO mouse samples could be observed. The differences between the values, however, were again not significant. The mean platelet volume (Figure 3.22) was lower in *Ctsk* KOs (highly significant).

Table 3.2. Complete blood count of primary transplantations.
Results of two independent experiments; arithmetic means ±SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$, *p<0.05, **p<0.01.

	CTSK +/+	CTSK -/-
WBC [10³/µl]	8.01 ± 0.97	6.61 ± 0.89
Lymph. [10³/µl]	5.86 ± 0.71	4.31 ± 0.58
Mono. [10³/µl]	0.31 ± 0.04	0.31 ± 0.07
Gran. [10³/µl]	1.82 ± 0.25	1.99 ± 0.43
Eos. [10³/µl]	0.11 ± 0.02	0.13 ± 0.04
RBC [10⁶/µl]	9.37 ± 0.32	8.97 ± 0.39
HGB [g/dl]	13.91 ± 0.31	13.86 ± 0.55
HCT [%]	45.68 ± 1.17	45.70 ± 1.86
MCV [µm³]	48.89 ± 0.82	51.06 ± 0.36 *
MCH [pg]	14.92 ± 0.35	15.54 ± 0.39
MCHC [g/dl]	30.47 ± 0.24	30.43 ± 0.72
RDW [%]	13.77 ± 0.15	13.49 ± 0.33
PLT [10³/µl]	863 ± 109	2507 ± 841
MPV [µm³]	5.88 ± 0.12	5.29 ± 0.15 **

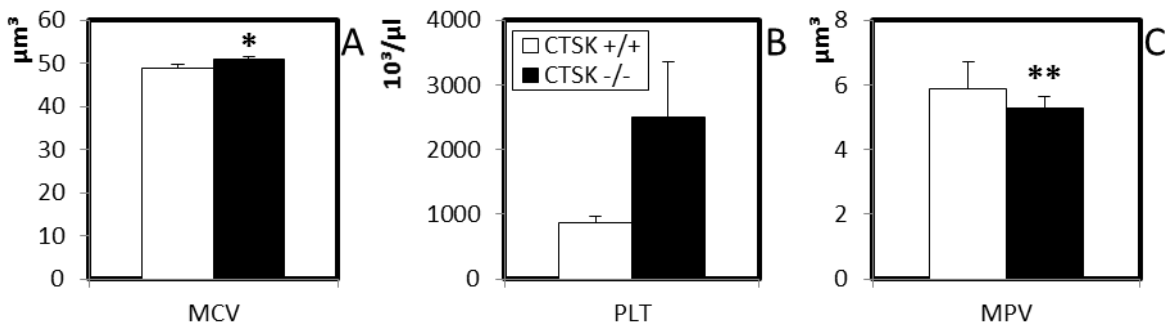

Figure 3.22. Mean cell volume (A), platelets (B) and mean platelet volume (C) in blood counts of primary transplantations. Significant difference between wild type and *Ctsk* KO MCV and MPV; means +SEM, $n_{(+/+)}$=9, $n_{(-/-)}$=9 *p<0.05, **p<0.01.

Absolute numbers of bone marrow, spleen, thymus and lymph node cells were not significantly altered in *Ctsk* KOs (Figure 3.23). The same thing applies to spleen weights (Figure 3.24).

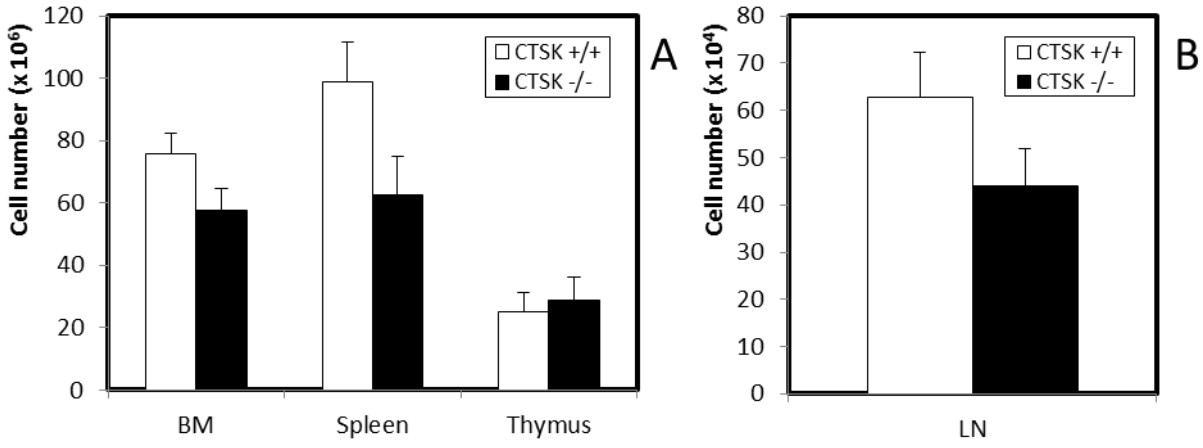

Figure 3.23. Means of cell numbers in bone marrow, spleen and thymus (A) and in lymph nodes (B) from primary transplantations +SEM, $n_{(+/+)}$=9, $n_{(-/-)}$=9.

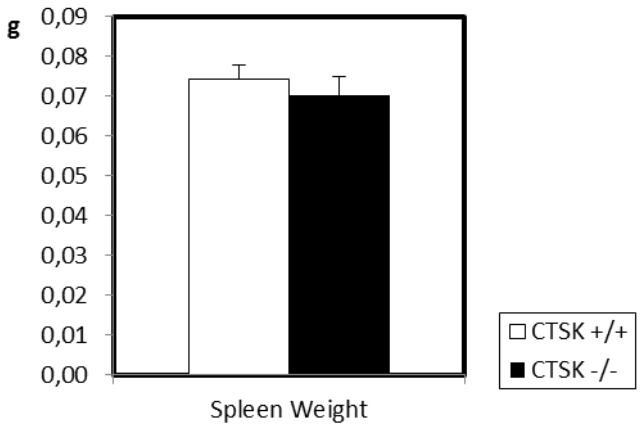

Figure 3.24. Spleen weight, means of wild type and *Ctsk* KO samples in primary transplantations +SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$.

3.2.2 Flow cytometry

Bone marrow, spleen, peripheral blood, lymph nodes and thymus of all mice which had received a transplant were analyzed on the flow cytometer. First, the engraftment of hematopoietic cells to the bone marrow was examined. It would have been desirable to also know about the development of the engraftment during the weeks following transplantation. Unfortunately, this was not possible as some of the knockout mice did not recover from the anesthesia that is necessary to take blood samples, so it was decided to investigate engraftment only after sacrifice. A reason for the high sensitivity could be the known affinity of cathepsin K knockout mice to lung fibrosis.

It turned out that there were differences compared to wild type mice in the engraftment of HSCs. While no significant changes could be found in bone marrow or spleens, the KO mice exhibited a smaller percentage of Ly5.1 positive donor cells in peripheral blood as well as in thymi and lymph nodes (Figure 3.25).

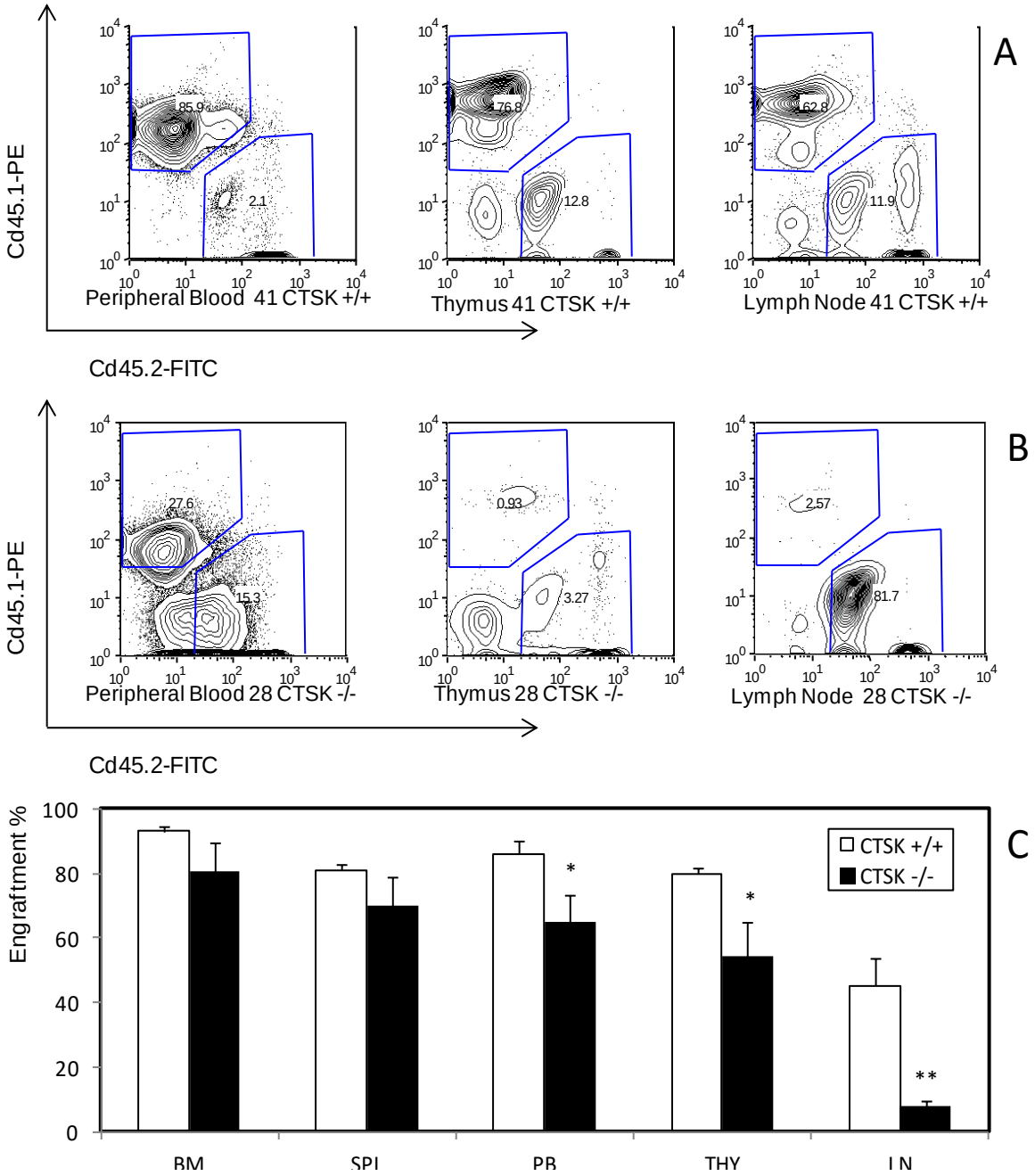

Figure 3.25. Engraftment of CD45.1+ cells in *Ctsk* KOs and controls. A. Flow cytometry plots of peripheral blood, thymus and lymph nodes of a representative wild type mouse. B. Flow cytometry plots of peripheral blood, thymus and lymph nodes of a representative *Ctsk* KO mouse; y-axis: CD45.1+ cells (donor cells), x-axis: CD45.2+ (recipient cells). C. Relation of donor and recipient cells (engraftment; shown in percent); +SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$, *p<0.05, **p<0,01.

Figure 3.26 shows the gating strategy for HSCs in the bone marrow of representative wild type and *Ctsk* KO mice. The same gates were used for spleen samples.

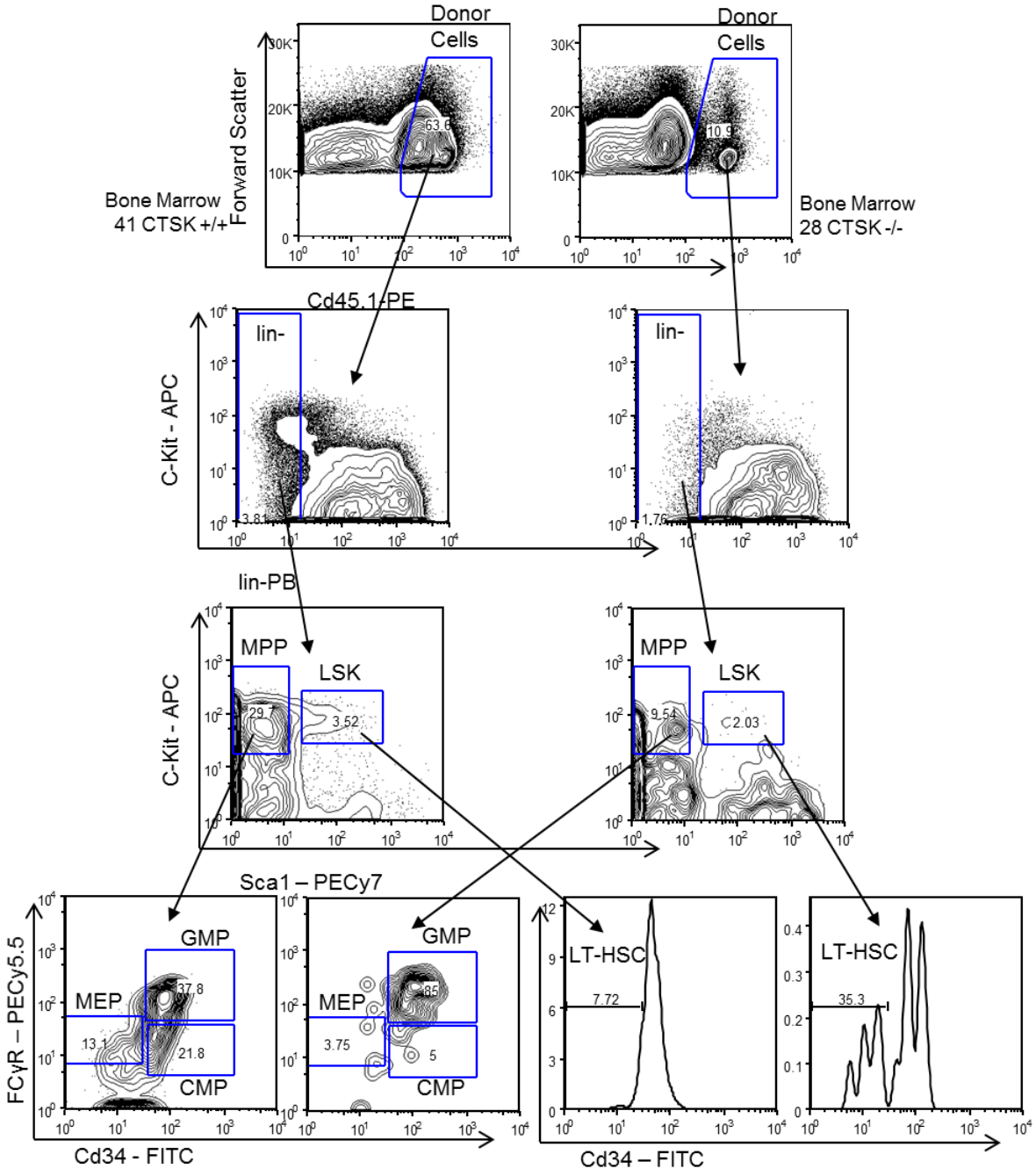

Figure 3.26. Gating strategy for hematopoietic stem cells and progenitors of donor origin, bone marrow. Donor cells (CD45.1+), lineage negative donor cells (CD3- B220- Ter119- Gr-1- CD11b- CD45.1+), donor MPP (c-Kit+ Sca-1- lin- CD45.1+), donor LSK (c-Kit+ Sca-1+ lin- CD45.1+), donor MEP (CD34- FcγR^{low} donor MPP), GMP (CD34+ FcγR+ donor MPP), CMP (CD34+ FcγR- donor MPP) and LT-HSC (CD34- donor LSK).

There were no significant differences between the experimental groups regarding donor HSCs or progenitors (Figure 3.27). Like in our characterization of steady-state *Ctsk*-/- mice, the inter-individual variation was larger in the *Ctsk*-/- mice. In particular, GMPs were not lower in the KOs like they had been in steady-state hematopoiesis. This suggests that the reduction of GMPs there may not be caused by extrinsic factors.

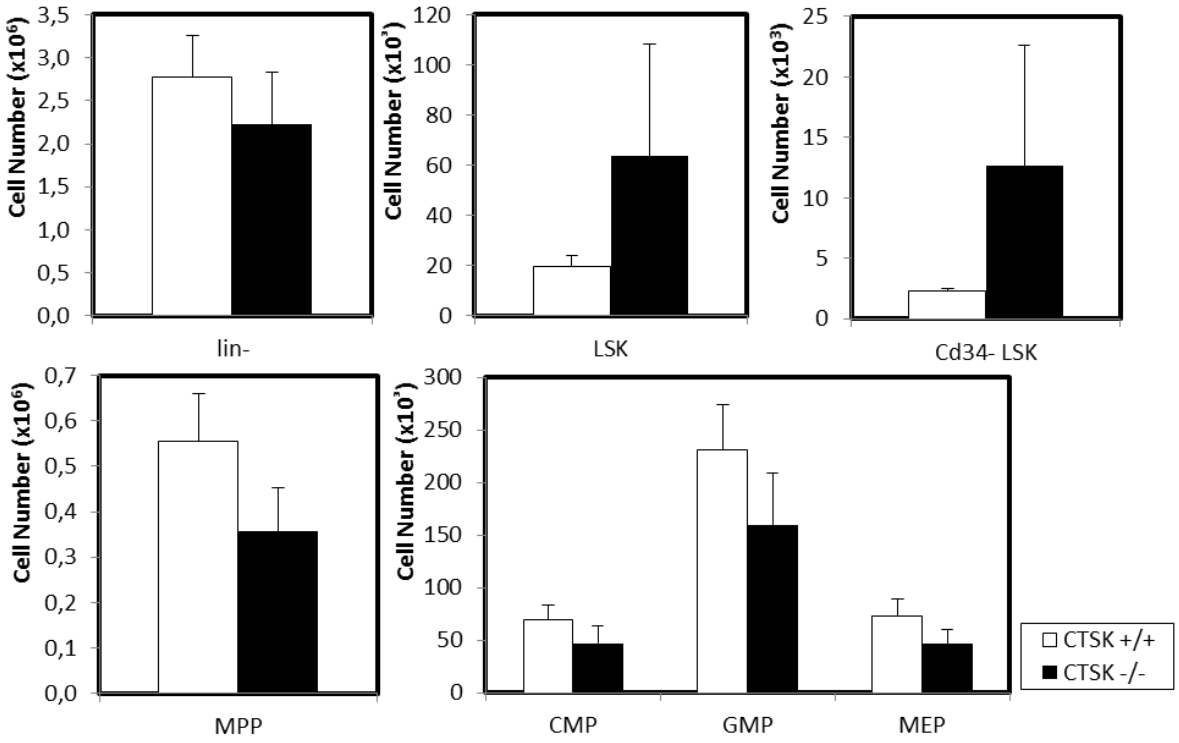

Figure 3.27. Number of donor stem cells and progenitors in bone marrow of *Ctsk* KO and wild type controls. Long bones of both hind legs. Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=8$.

Just like in the 8 – 10-week-old cathepsin K deficient mice, there were no alterations found concerning the number of donor HSCs or progenitors in the spleens of the KO mice that had received a transplant (Figure 3.28).

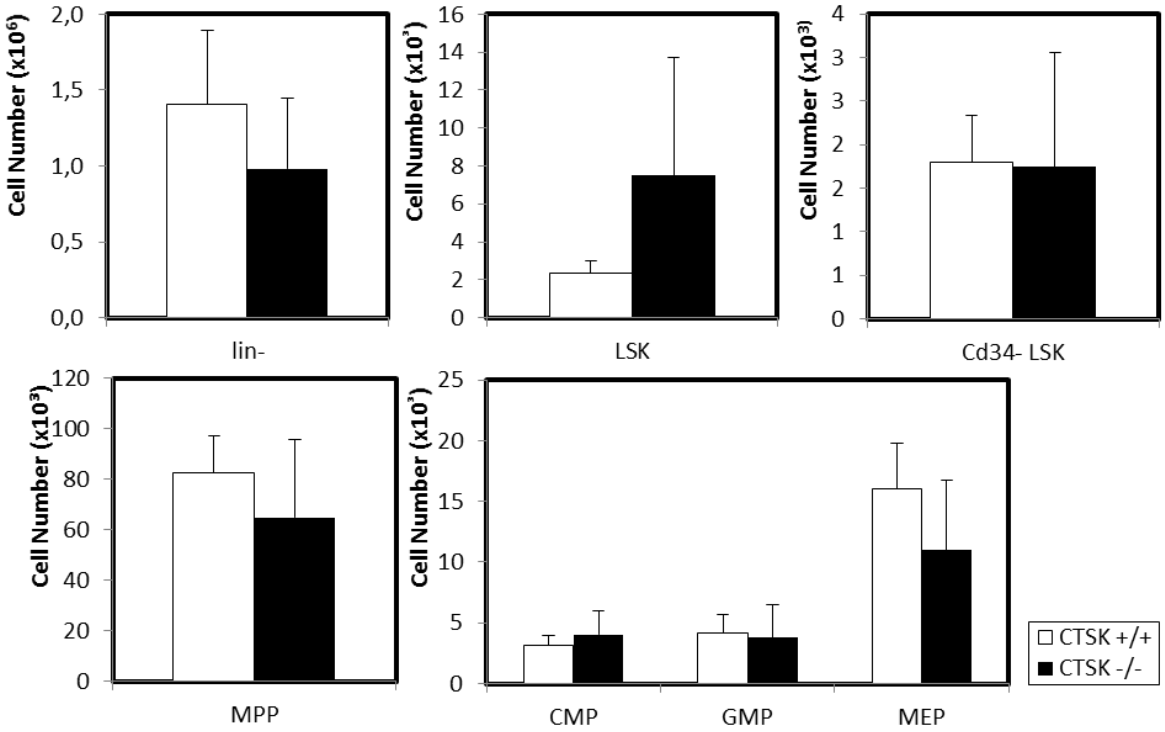

Figure 3.28. Number of donor stem cells and progenitors in spleens of *Ctsk* KO and wild type controls. Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$.

In the donor lymphocyte populations there were no changes found in the bone marrow of *Ctsk* KO mice compared to WT mice (Figure 3.29). Because there is a restriction to the number of fluorochromes that can be used in one flow cytometry stain and the need to detect donor and recipient cells in this experiment, CD4 and CD8a were both labeled with the same color. Because of that it is not possible to make a statement about the number of CD8+ cytotoxic T cells that had been lower the in young *Ctsk* KOs.

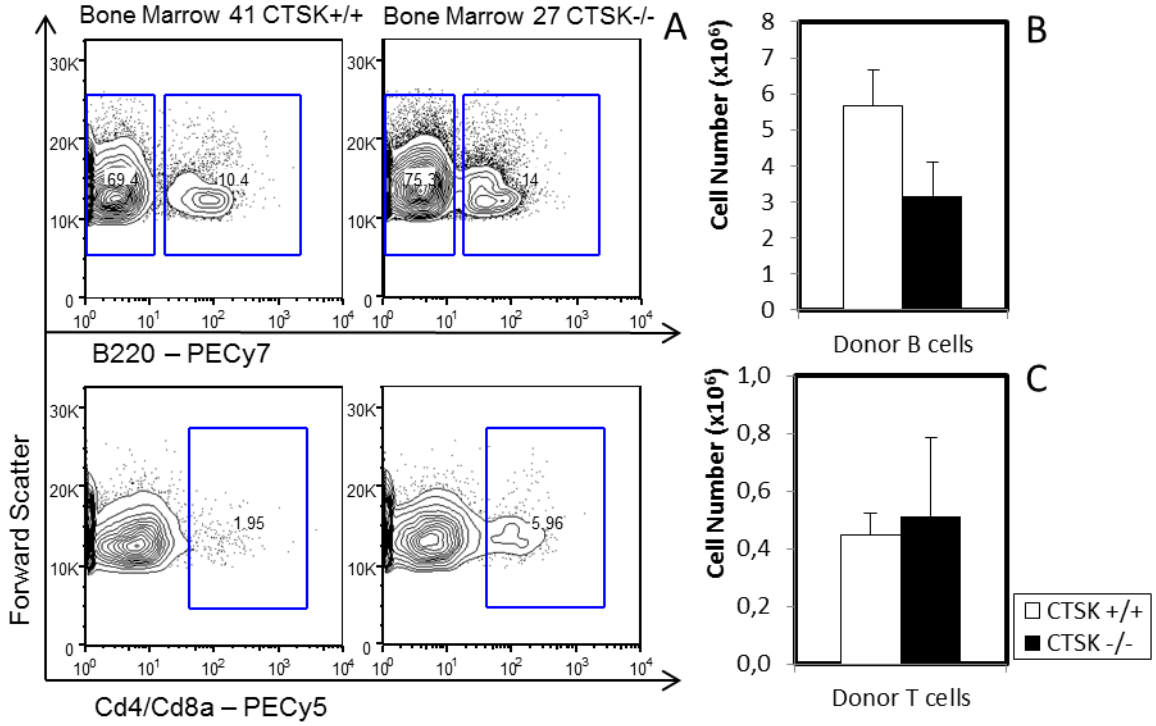

Figure 3.29. Donor B and T cells in bone marrow from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells) C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$.

In the spleen samples however, there were significantly lower numbers of B as well as of T cells of donor origin (Figure 3.30). This extrinsic effect of the loss of cathepsin K was not found in the above characterization of *Ctsk*-/- mice under steady-state conditions.

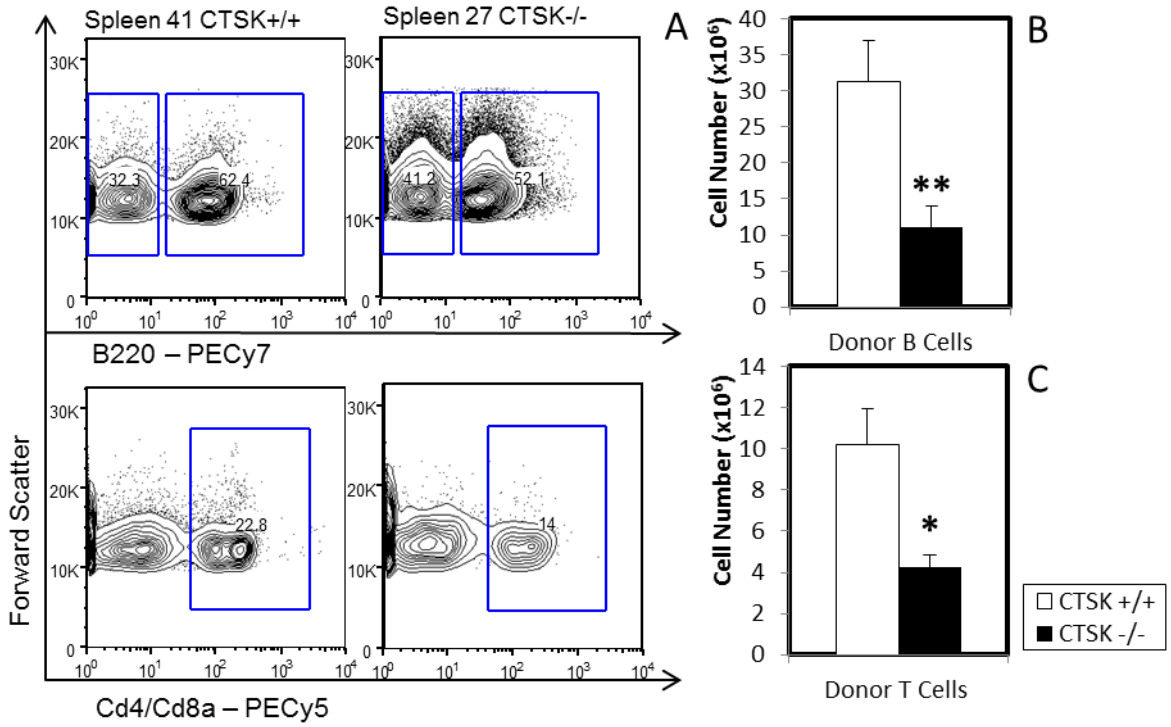

Figure 3.30. Donor B and T cells in spleens from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells). C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$, *$p<0.05$, **$p<0.01$.

In the peripheral blood samples there was no significant difference found concerning the number of donor B cells. Donor T cells were elevated compared to the transplanted cells in a wild type environment (Figure 3.31). This suggests that the previously observed increase is based on an extrinsic effect of cathepsin K. As the protease is likely to play a role in the migration of many cell types including tumor metastases in humans (Novinec M, 2013), it can be argued that cathepsin K is necessary for the migration of T cells. If only CD8+ or also CD4+ cells are affected cannot be told in this set-up.

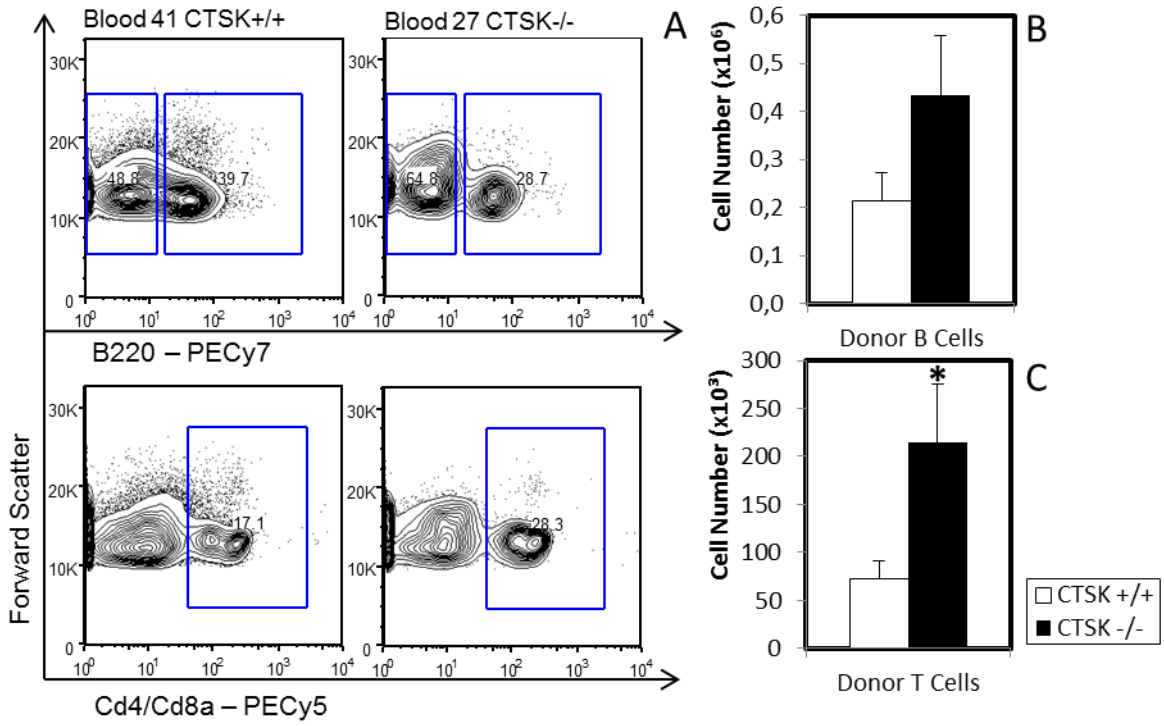

Figure 3.31. Donor B and T cells in blood samples (300µl) from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells). C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}=8$, $n_{(-/-)}=9$, *$p<0.05$.

No alterations that apply to the numbers of donor B and T cells in thymus and lymph node samples were found in this experiment (Figures 3.32 and 3.33).

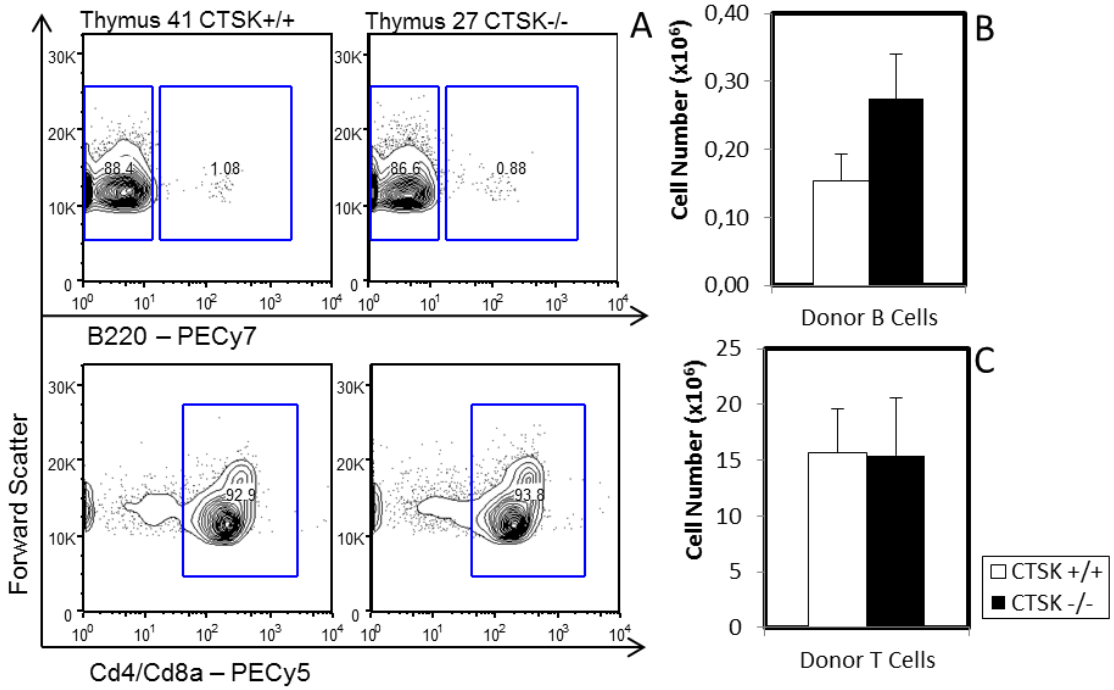

Figure 3.32. Donor B and T cells in thymi from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells) C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}$=9, $n_{(-/-)}$=8.

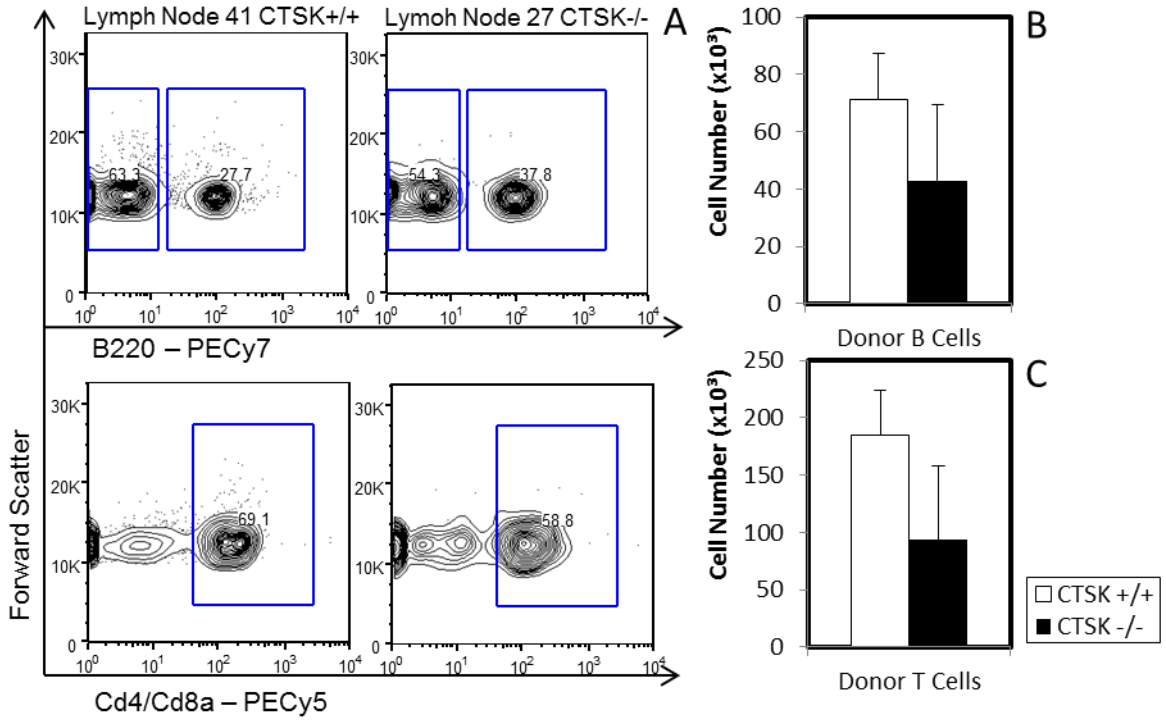

Figure 3.33. Donor B and T cells in lymph nodes from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells). C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}$=8, $n_{(-/-)}$=9.

NK1.1 is a marker for natural killer cells but is also expressed on a small subset of T cells, namely natural killer T cells (Godfrey DI, 2004). That is why it cannot be told for sure if the NK1.1+ cells, which were lowered significantly in the KO mice in the transplantation experiments, are natural killer cells or NKT cells. However, there were notable alterations found in the donor cells retrieved from *Ctsk* -/- recipient mice. In their bone marrow, NK1.1+ were lower than those from WT mice (Figure 3.34).

Granulocyte and monocyte numbers were not altered.

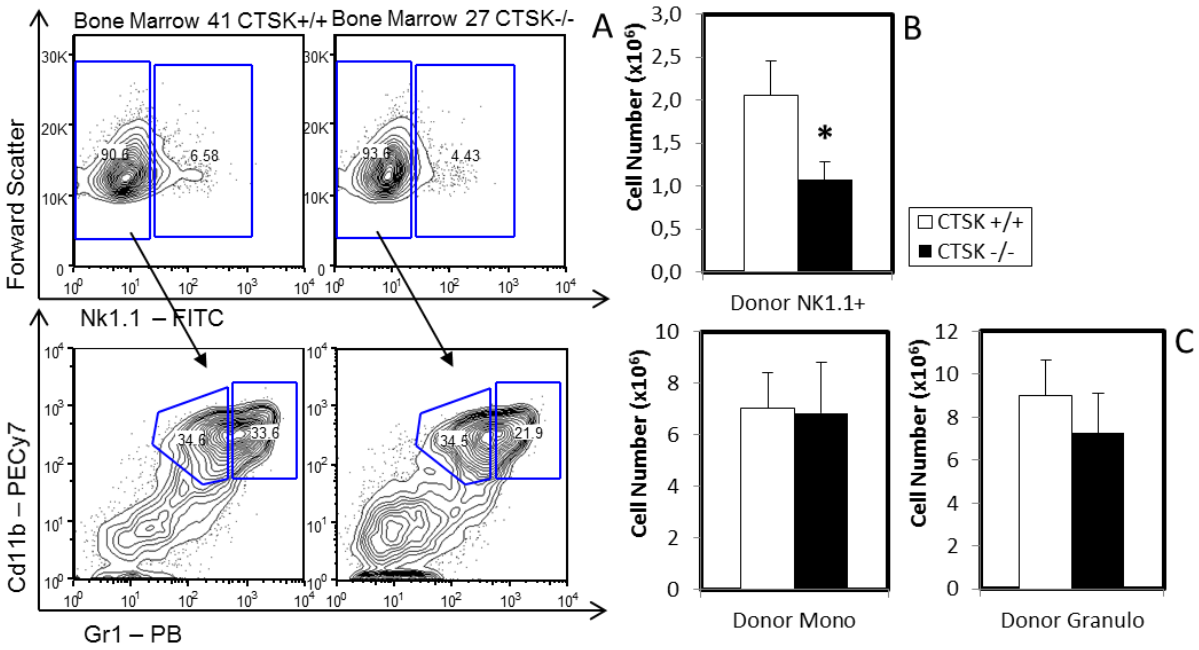

Figure 3.34. Donor NK1.1+ cells, monocytes and granulocytes in bone marrow from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ cells) and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes ($Gr1^{mid}$ and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$, *$p<0.05$.

In the spleens, the same thing occurred: Donor NK1.1+ cells were found to be lower in the KO mice than in wild type, monocytes and granulocytes did not differ (Figure 3.35).

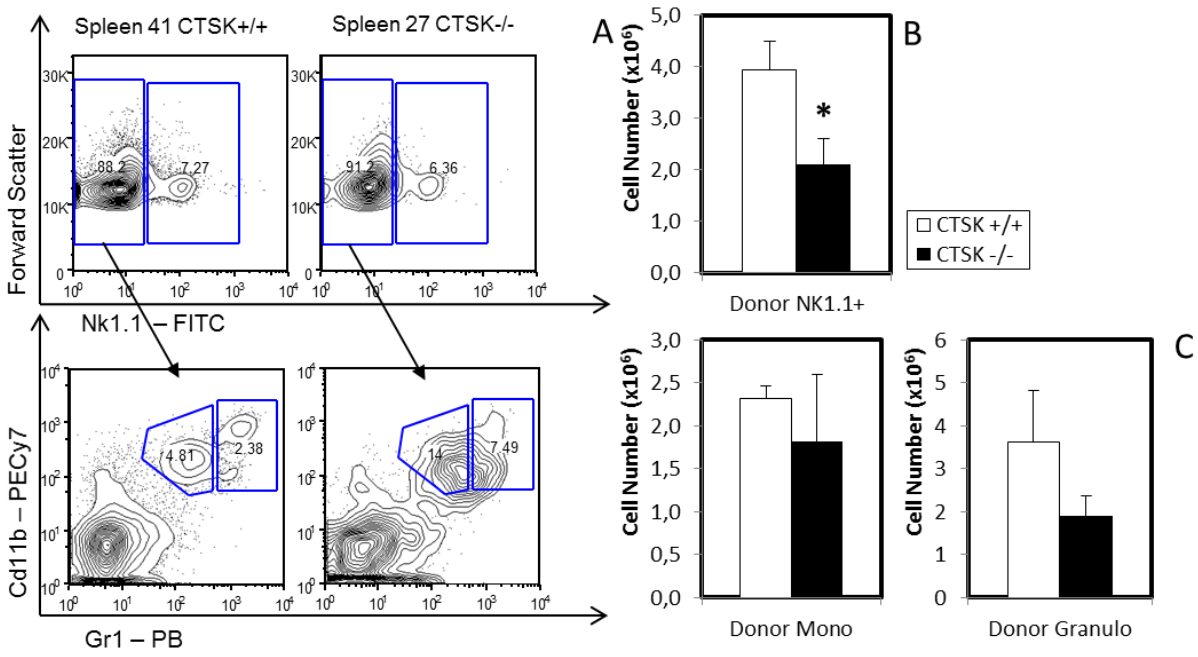

Figure 3.35. Donor NK1.1+ cells, monocytes and granulocytes in spleens from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ cells) and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes ($Gr1^{mid}$ and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}=9$, $n_{(-/-)}=9$, *$p<0.05$.

In peripheral blood, no changes relating to donor NK1.1+ cells, granulocytes or monocytes were detected (Figure 3.36).

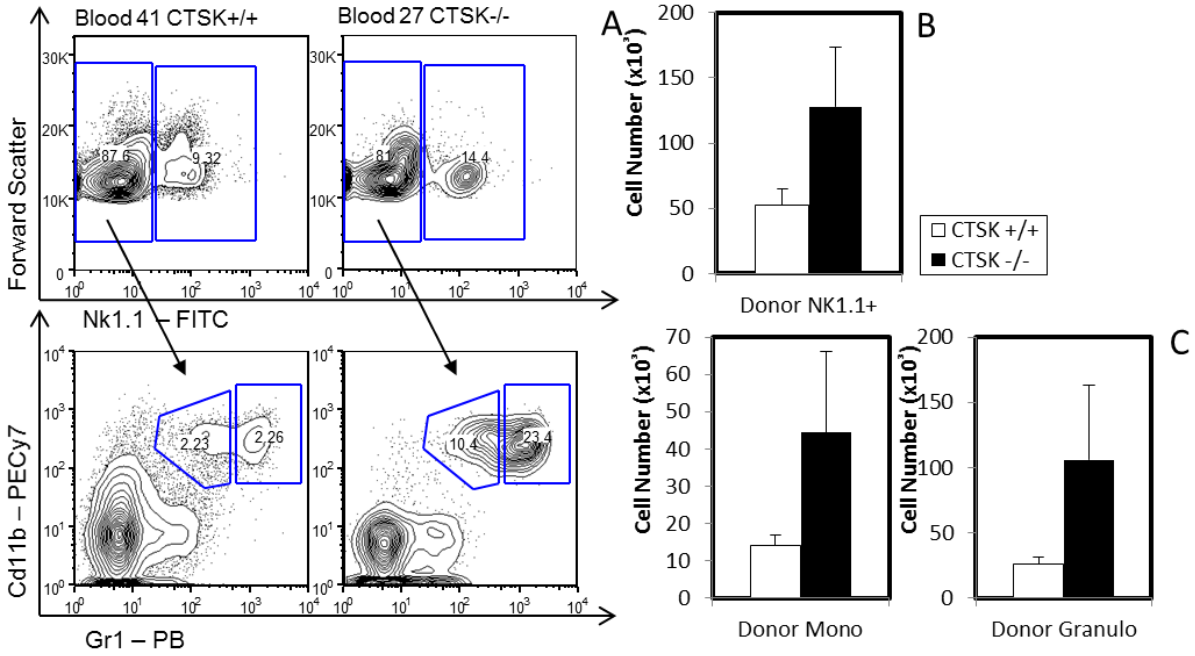

Figure 3.36. Donor NK1.1+ cells, monocytes and granulocytes in peripheral blood from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ cells) and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes ($Gr1^{mid}$ and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}=8$, $n_{(-/-)}=9$.

Cell numbers of donor NK1.1+ cells, monocytes and granulocytes were unmodified in thymi of KO mice after primary transplantations (Figure 3.37).

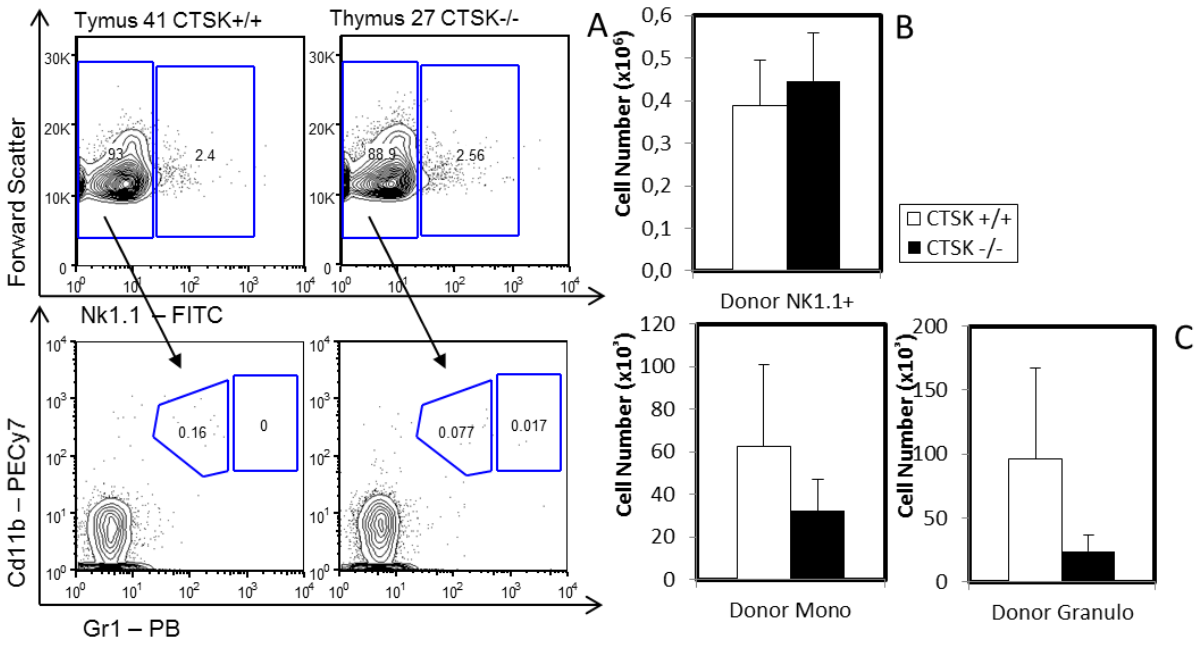

Figure 3.37. Donor NK1.1+ cells, monocytes and granulocytes in thymi from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ cells) and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes ($Gr1^{mid}$ and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}=8$, $n_{(-/-)}=8$.

Very low numbers (zero or close to zero) of donor granulocytes and monocytes were found in lymph nodes. That is why the relevance of the diminution of the latter is questionable.

Donor NK1.1+ cells, on the other hand, were significantly lower in KO mice than in those mice expressing cathepsin K (Figure 3.38).

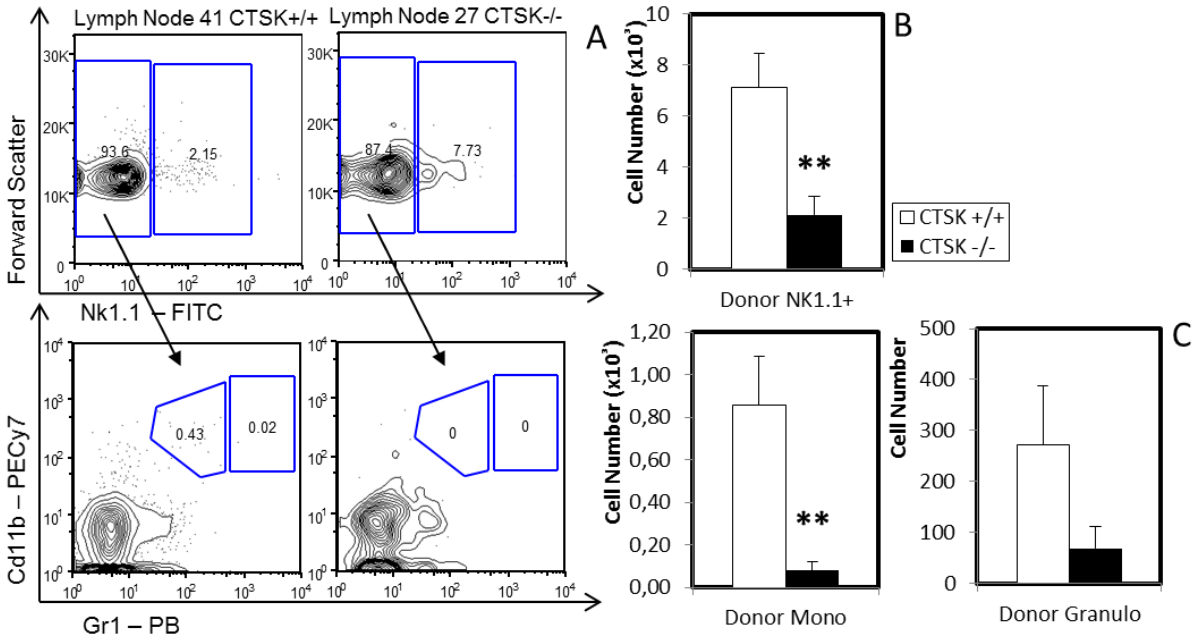

Figure 3.38. Donor NK1.1+ cells, monocytes and granulocytes in lymph nodes from primary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ cells) and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes (Gr1^{mid} and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}$=9, $n_{(-/-)}$=9, **p<0.01.

3.3 Secondary transplantation of hematopoietic stem cells

3.3.1 Blood count and cell numbers

16 weeks after their transplantation with bone marrow cells from the tibiae and femora of the mice from the primary transplantation experiments, all mice that had received their transplants from the KO group (from now on referred to as "*Ctsk*-/-" even though not *Ctsk* KO mice themselves) and the respective control groups were sacrificed. The only difference in their complete blood counts was a slightly higher mean corpuscular hemoglobin concentration in the *Ctsk* -/- group (Table 3.3, Figure 3.39).

Table 3.3. Complete blood count of secondary transplantations.
Results of two independent experiments; arithmetic means.
±SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=10, *p<0.05.

	CTSK +/+	CTSK -/-
WBC [10^3/µl]	6.82 ± 0.78	5.65 ± 0.64
Lymph. [10^3/µl]	4.89 ± 0.63	4.11 ± 0.57
Mono. [10^3/µl]	0.29 ± 0.04	0.21 ± 0.02
Gran. [10^3/µl]	1.65 ± 0.19	1.35 ± 0.15
Eos. [10^3/µl]	not done	
RBC [10^6/µl]	8.30 ± 0.23	8.14 ± 0.26
HGB [g/dl]	13.45 ± 0.28	13.63 ± 0.36
HCT [%]	43.09 ± 0.93	42.02 ± 1.17
MCV [µm³]	52.17 ± 0.53	51.83 ± 0.38
MCH [pg]	16.39 ± 0.18	16.93 ± 0.23
MCHC [g/dl]	31.45 ± 0.23	32.71 ± 0.41*
RDW [%]	13.82 ± 0.16	13.84 ± 0.14
PLT [10^3/µl]	1075 ± 100	1358 ± 191
MPV [µm³]	5.77 ± 0.09	5.92 ± 0.08

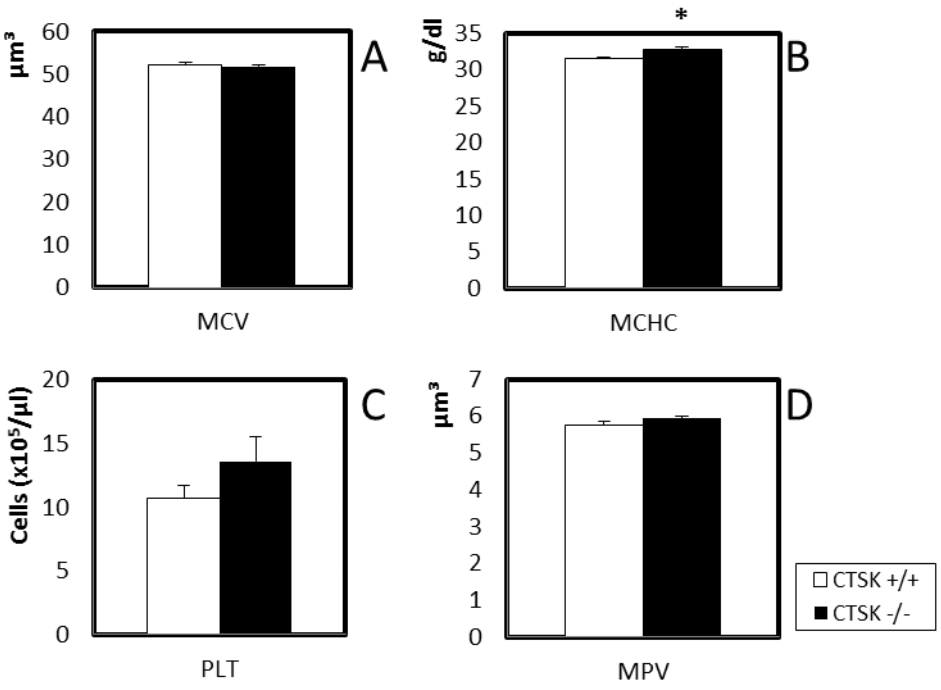

Figure 3.39. Mean cell volume (A), mean corpuscular hemoglobin concentration (B), number of platelets (C) and mean platelet volume (D) in blood counts of secondary transplantations. Significant difference between wild type and *Ctsk* KOs in MCHC. +SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=10, *p<0.05

Neither cell numbers in bone marrow or spleen (Figure 3.40) nor spleen weights (Figure 3.41) showed significant differences in secondary transplantations.

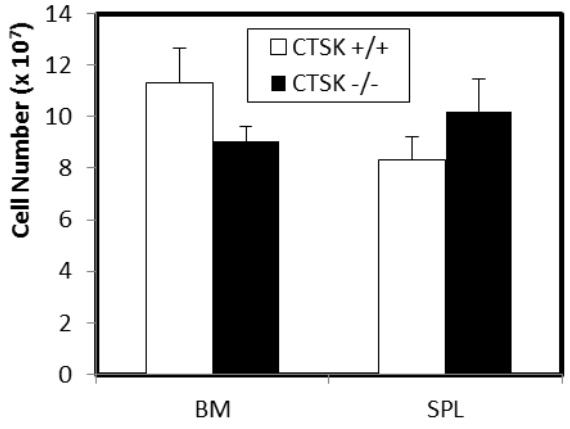

Figure 3.40. Means of cell numbers in bone marrow and spleen from secondary transplantations. +SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=11.

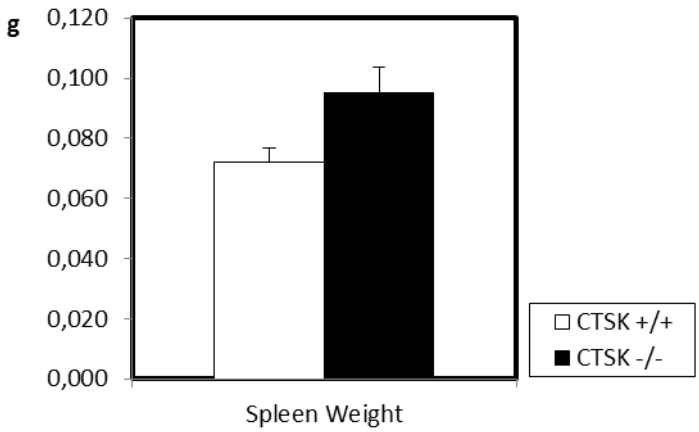

Figure 3.41. Spleen weight, arithmetic means in secondary transplantation experiments. +SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=11.

3.3.2 Flow cytometry

Bone marrow, spleens and peripheral blood cells were analyzed by flow cytometry as well. First, the engraftment of the CD45.1+ cells that had originally been transplanted to the *Ctsk*-/- mice and their wild type controls was studied. In primary transplantations, the engraftment in lymph nodes, thymi and peripheral blood had been less successful in the KO groups. Here it showed that there was less engraftment in spleens but that it was not significantly lowered in the peripheral blood (Figure 3.42).

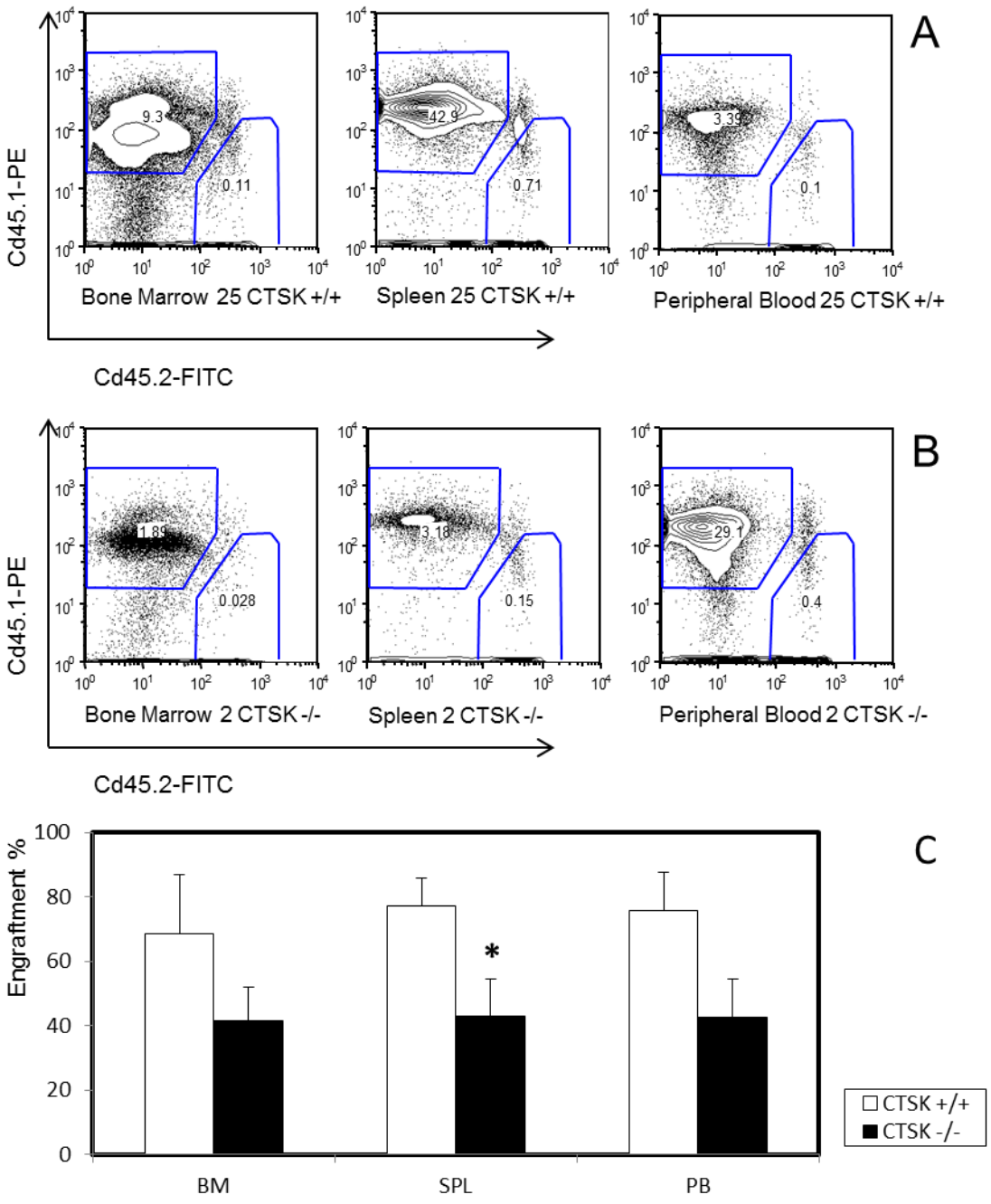

Figure 3.42. Engraftment of CD45.1+ cells in in secondary transplantation experiments. A. Flow cytometry plots of bone marrow, spleen and peripheral blood of a representative wild type mouse. B. Flow cytometry plots of bone marrow, spleen and peripheral blood of a representative *Ctsk* KO mouse; y-axis: CD45.1+ cells (donor cells), x-axis: CD45.2+ (recipient cells). C. Relation of donor and recipient cells (engraftment; shown in percent). +SEM, $n_{(+/+)}=12$, $n_{(-/-)}=11$, *$p<0.05$.

The goal of the secondary transplantation was to find out if the self-renewal capacity of the HSCs or their capacity to proliferate and differentiate into progenitors had changed after having been subjected to hematopoietic stress while repopulating in a *Ctsk* deficient niche. There were less lineage negative cells among the donor cells in the bone marrow. Also, the CD34 negative LSKs were reduced significantly in the bone marrow of the KO mice (Figure 3.43). Apparently, the long term repopulating hematopoietic cells had partially lost their ability to self-renew after engrafting to a cathepsin K deficient mouse. This possibly means that cathepsin K is necessary for enabling LT-HSCs to sustain their pool when being forced to proliferate after a transplantation as a loss of cathepsin K leads to an early exhaustion of HSCs in serial transplantations.

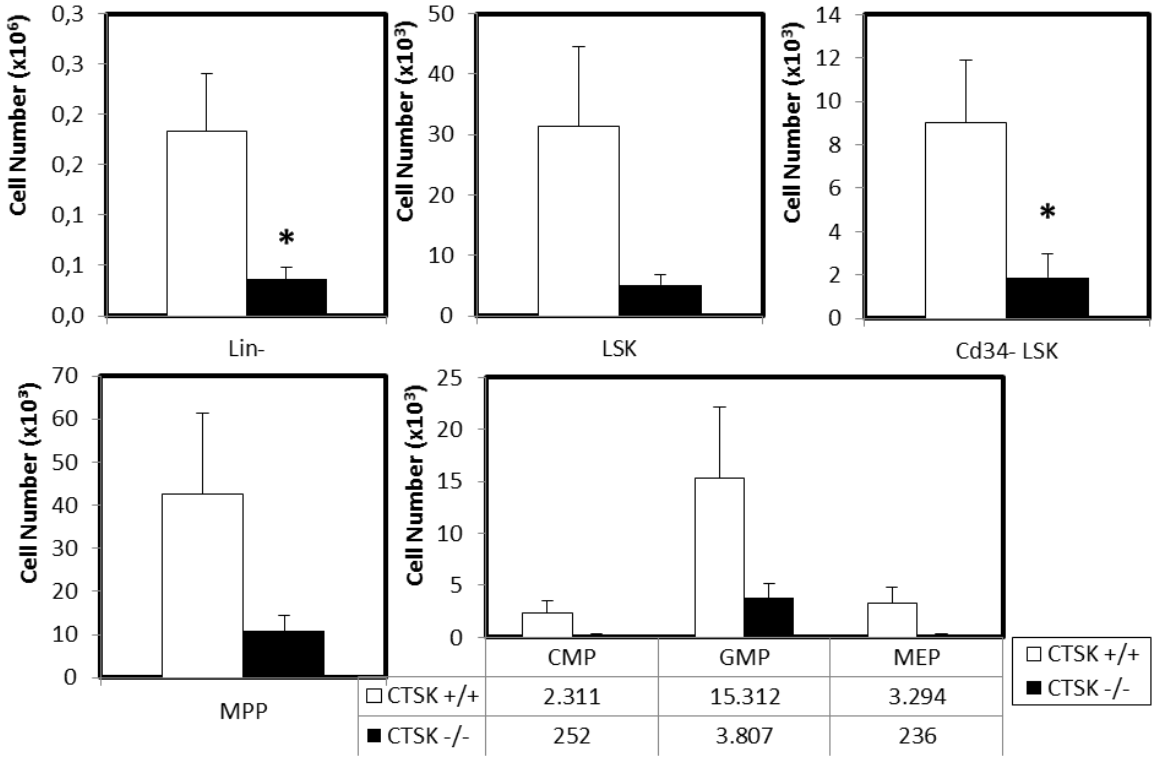

Figure 3.43. Stem cells and progenitors in bone marrow from secondary transplantation experiments. Long bones of both hind legs. For gating strategy see Figure 3.26. Means + SEM, $n_{(+/+)}=12$, $n_{(-/-)}=11$, *$p<0.05$.

The spleen samples, though exhibiting a lower overall engraftment when coming from *Ctsk* KO mice, did not show alterations in the numbers of HSCs and hematopoietic progenitors comparing cells from primary KO and WT grafts (Figure 3.44).

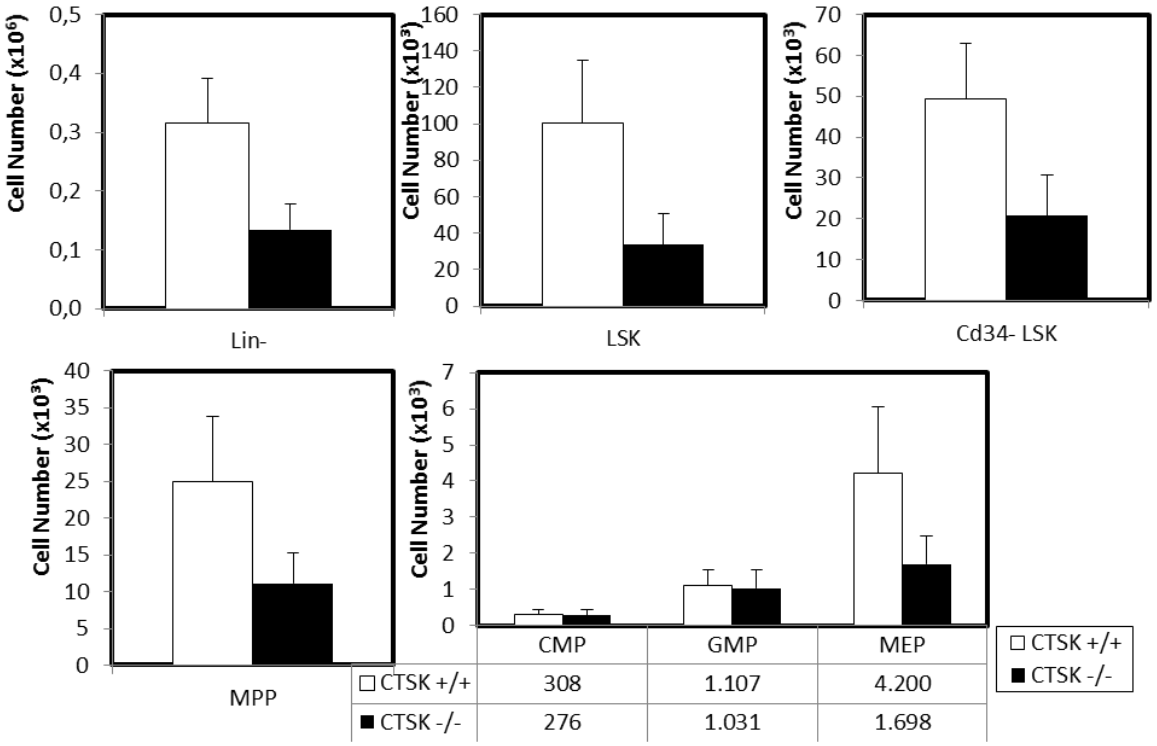

Figure 3.44. Stem cells and progenitors in spleens from secondary transplantation experiments. For gating strategy see Figure 3.26. Means + SEM, $n_{(+/+)}=12$, $n_{(-/-)}=11$.

In the bone marrow, donor B cells were not significantly lowered in the *Ctsk*-/- graft group. Donor T cells, on the other hand, were lower compared to mice transplanted with bone marrow from WT mice (Figure 3.45). This is a confirmation of the results of characterization, where CD8+ cells had been lower in the bone marrow. It also indicates that a loss of cathepsin K influences T cells not only because of an impaired migration as assumed before, but that other mechanisms with an enduring effect have to be involved as well.

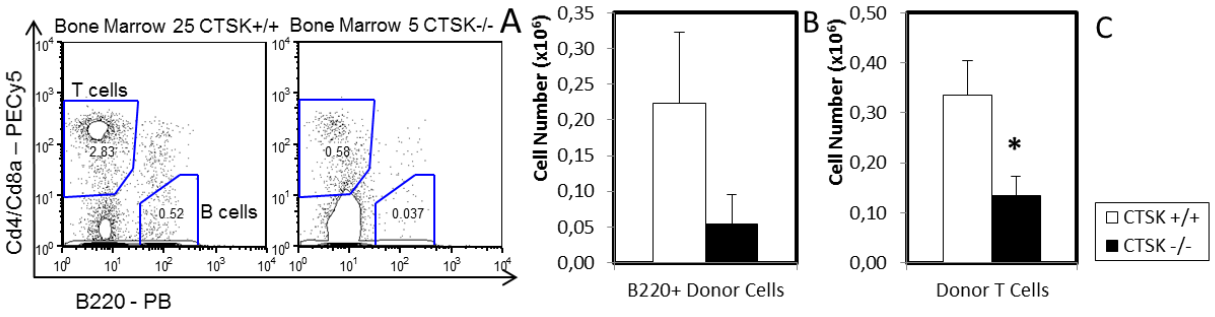

Figure 3.45. Donor B and T cells in bone marrow from secondary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells) C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=10, *p<0.05.

In the spleens, there were found significantly less donor B and T cells in case the grafts came from *Ctsk* null mice (Figure 3.46). This confirms the result from primary transplantation experiments and the assumption that cathepsin K influences mechanisms that affect the number lymphocytes and are different from just facilitating their migration.

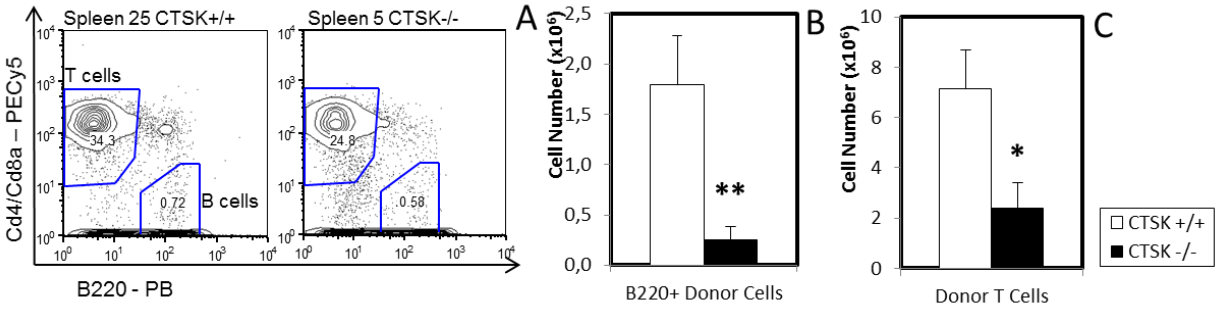

Figure 3.46. Donor B and T cells in spleens from secondary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells) C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=10, *p<0.05, **p<0.01.

In the blood samples, contrary to findings in characterization and primary transplantation experiments, T cells were not elevated in mice that had received grafts transferred from *Ctsk* KOs. Donor B cells also were found to be unaltered (Figure 3.47).

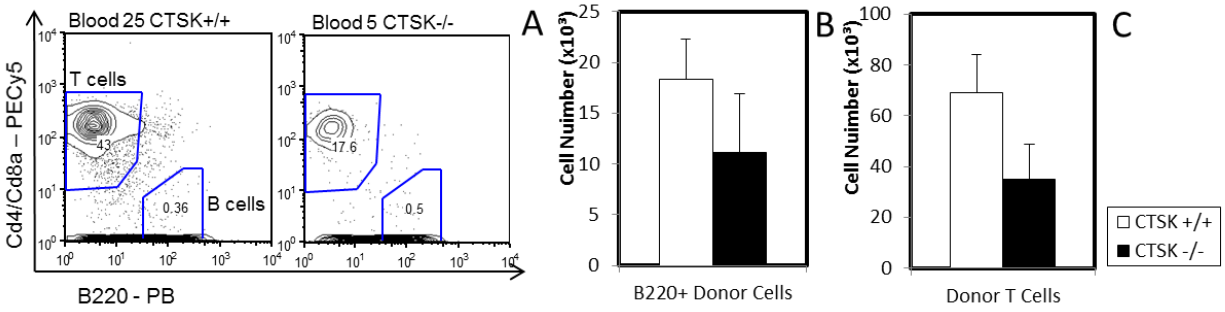

Figure 3.47. Donor B and T cells in peripheral blood (samples of 300µl) from secondary transplantation experiments. A. Flow cytometry plots (subsets of CD45.1+ donor cells, for gating see Figure 3.26). B. Number of donor B cells (B220+ donor cells) C. Number of donor T cells (CD4 and CD8+ donor cells). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=10.

Like in primary transplantations, donor NK1.1+ cells were diminished in the *Ctsk* KO graft group in the bone marrow samples. And also like in primary transplantations, monocytes and granulocytes of donor origin were not different among the groups (Figure 3.48).

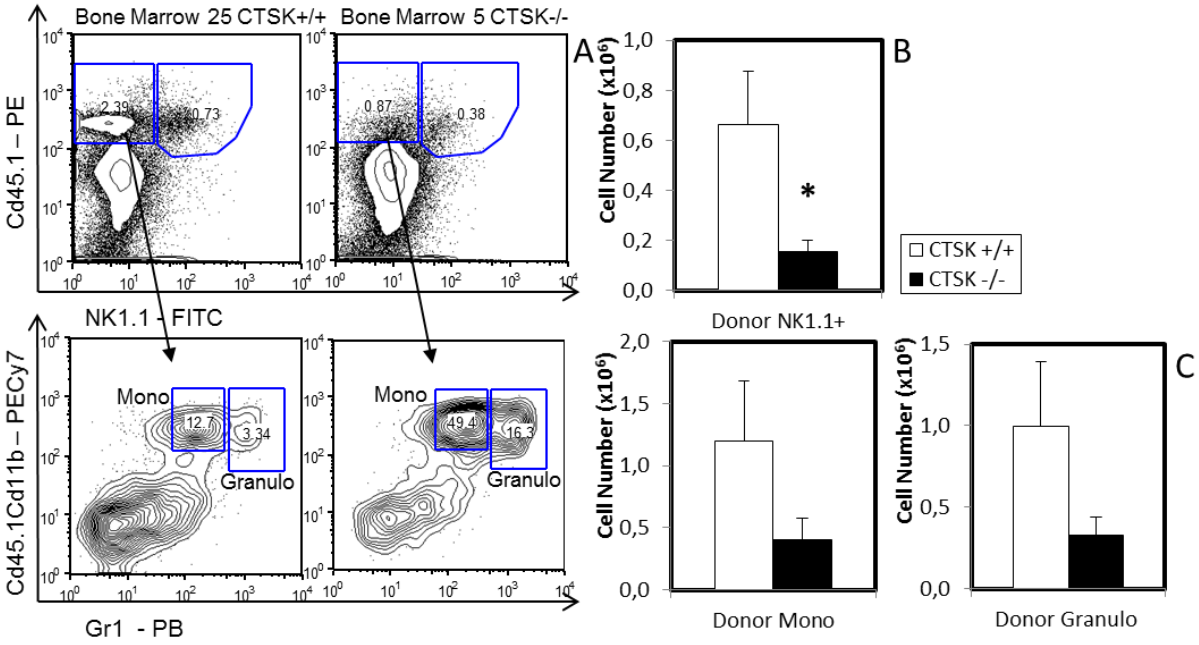

Figure 3.48. Donor NK1.1+ cells, monocytes and granulocytes in bone marrow from secondary transplantation experiments. A. Flow cytometry plots and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes ($Gr1^{mid}$ and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=11, *$p<0.05$.

The samples from spleens and peripheral blood as well reflected the findings from primary transplantations: In the spleens, the number of donor NK1.1+ cells was lower in the group with a transplant from a microenvironment lacking cathepsin K, and their number was unchanged in the peripheral blood. And there were no significant changes in the numbers of monocytes and granulocytes in both the organs (Figures 3.49 und 3.50)

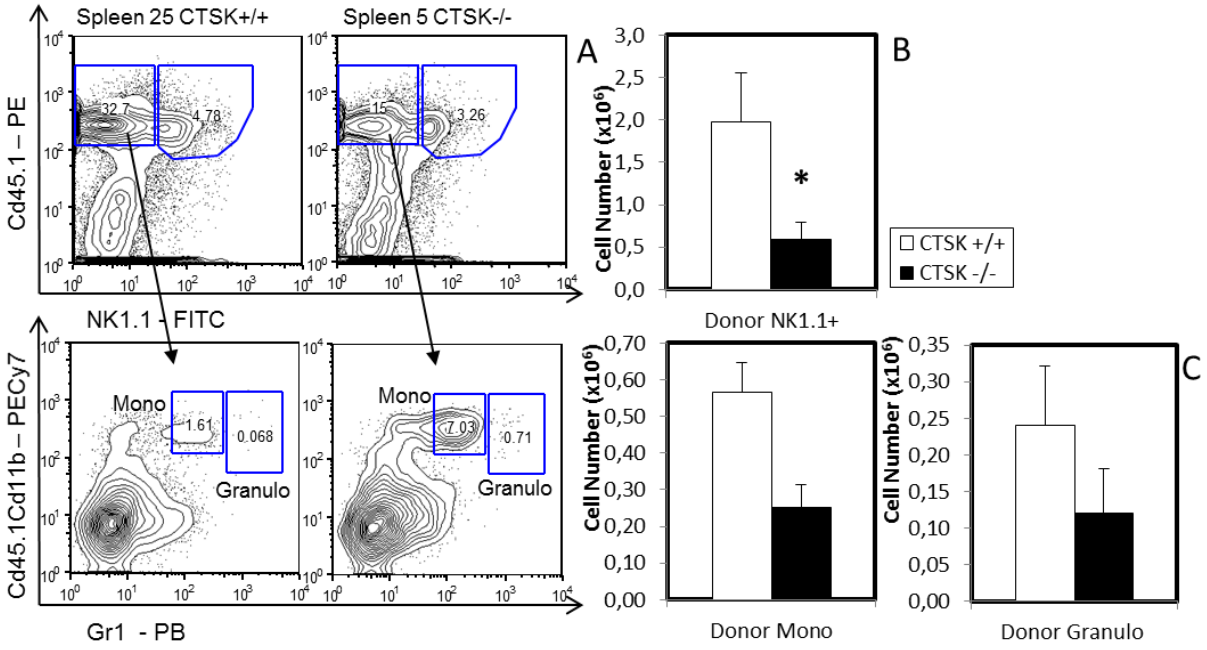

Figure 3.49. Donor NK1.1+ cells, monocytes and granulocytes in spleens from secondary transplantation experiments. A. Flow cytometry plots and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes (Gr1^{mid} and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=11, *p<0.05.

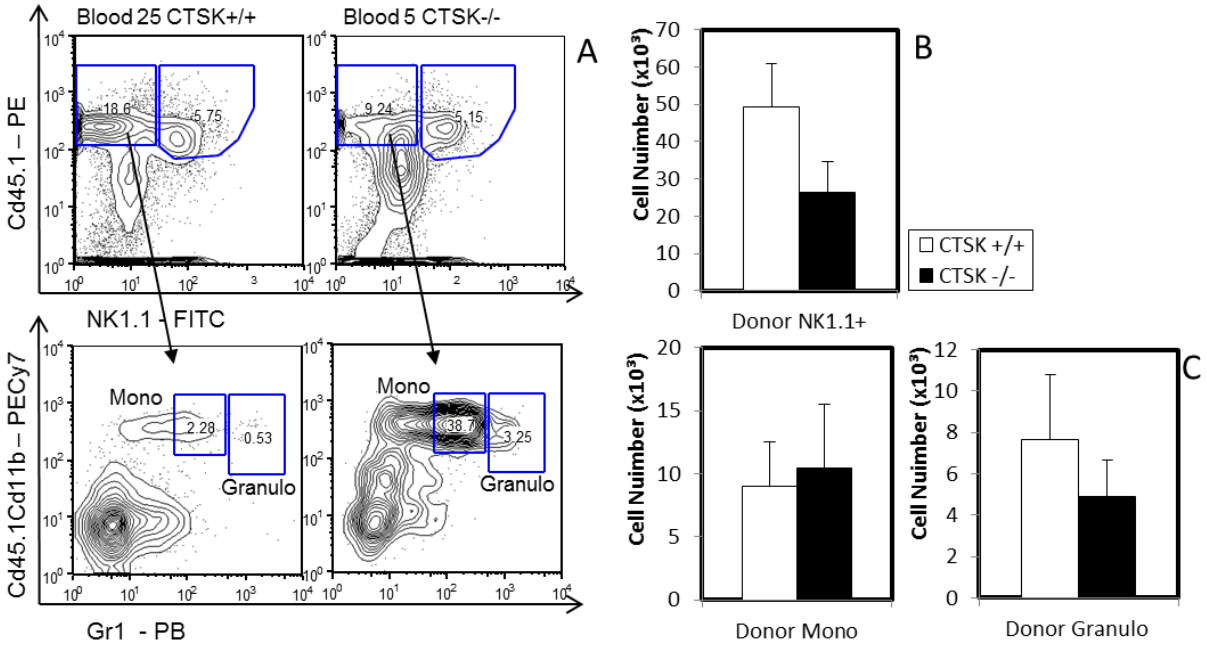

Figure 3.50. Donor NK1.1+ cells, monocytes and granulocytes in peripheral blood (samples of 300µl) from secondary transplantation experiments. A. Flow cytometry plots and gating strategy. B. Number of donor natural killer cells (NK1.1+ CD45.1+). C. Number of donor monocytes (Gr1^{mid} and CD11b+, NK1.1- CD45.1+) and granulocytes (Gr1+ and CD11b+, NK1.1- CD45.1+). Means + SEM, $n_{(+/+)}$=12, $n_{(-/-)}$=11.

3.4 Homing of lineage negative bone marrow cells

3.4.1 Blood count and cell numbers

The results of the blood counts form the mice in the homing experiments, which had been irradiated and received lineage negative bone marrow cells as a transplant to the tail veins the day before, can of course not be seen as a result of the homing process as the transplant did not have the chance to repopulate the bone marrow or differentiate during this short time. The changes that were observed, however, can be interpreted as reactions to radiation. In both groups there was a much lower number of white blood cells than measured in other experiments with a lower number of eosinophil granulocytes in the *Ctsk* KO than in the wild type group (Table 3.4, Figure 3.51). Eosinophilia is a known reaction of the organism to radiation (Muggia FM, 1973), that *Ctsk* KO mice did not show to the same extent as wild type mice.

Again, alterations in the parameters for megakaryocyte-erythrocyte-progenitor derived cells were observed. Though not anemic, the KO mice had a lower MCHC as in secondary transplantations and a higher RDW as observed beforehand in the characterization experiments. And like in primary transplantation experiments, the MPV was found to be lower in the KO group. (Figure 3.51)

Table 3.4. Complete blood count after homing.
Results of two independent experiments; arithmetic means. ±SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11, *p<0.05, **p<0.01.

	CTSK +/+	CTSK -/-
WBC [10³/µl]	1.78 ± 0.07	1.65 ± 0.15
Lymph. [10³/µl]	0.85 ± 0.04	0.76 ± 0.10
Mono. [10³/µl]	0.10 ± 0.01	0.08 ± 0.02
Gran. [10³/µl]	0.82 ± 0.05	0.75 ± 0.11
Eos. [10³/µl]	0.14 ± 0.02	0.09 ± 0.01*
RBC [10⁶/µl]	8.39 ± 0.15	8.58 ± 0.36
HGB [g/dl]	15.29 ± 0.23	15.05 ± 0.45
HCT [%]	43.14 ± 0.60	44.73 ± 1.89
MCV [µm³]	51.82 ± 0.19	52.37 ± 0.37
MCH [pg]	19.12 ± 0.18	18.47 ± 0.30
MCHC [g/dl]	36.92 ± 0.34	35.21 ± 0.51*
RDW [%]	13.24 ± 0.16	13.96 ± 0.17**
PLT [10³/µl]	1114 ± 112	1098 ± 160
MPV [µm³]	5.71 ± 0.06	5.32 ± 0.10**

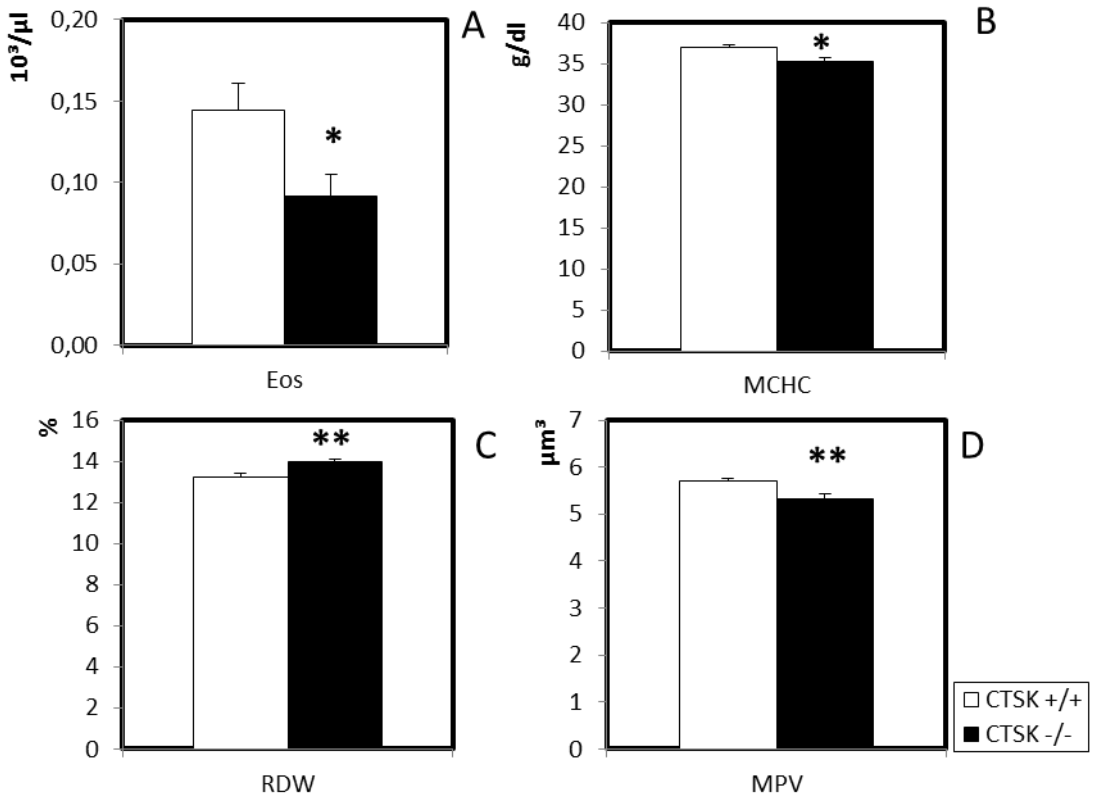

Figure 3.51. Eosinophils (A), Mean corpuscular hemoglobin concentration (B), red cell distribution width (C) and mean platelet volume (D) in blood counts after homing. Significant difference between wild type and *Ctsk* KOs: Eosinophils, MCHC, RDW and MPV. +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11, *p<0.05, **p<0.01.

As a result of irradiation, cell numbers in bone marrow and spleens were in both groups reduced when compared to findings in characterization. The spleens of *Ctsk* KOs contained a higher number of cells remaining than those of irradiated wild type mice (Figure 3.52). The spleen weights were not altered (Figure 3.53).

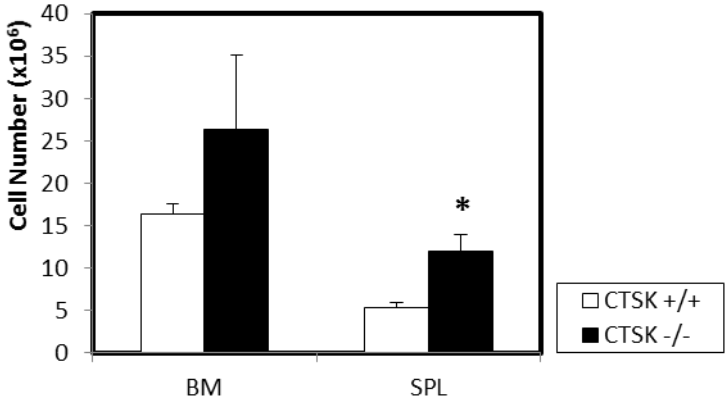

Figure 3.52. Means of cell numbers in bone marrow and spleens from homing experiments. +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11, *p<0.05.

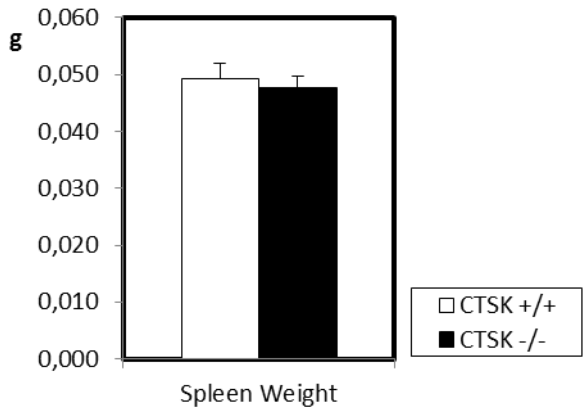

Figure 3.53. Spleen weight, means of wild type and *Ctsk* KO samples after homing. +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11.

3.4.2 Flow cytometry

The homing experiments were set up in order to find out if the alterations concerning engraftment and numbers of HSCs, especially the lowered number of LT-HSCs in secondary transplantations, may originate in a disturbance of the migration of BM stem cells. Disabled entry to the bone marrow could have an impact on the engraftment after a transplantation but might also affect the homeostasis of HSCs, as a small portion of them constantly migrates from and to the bone marrow. It was found, however, that the donor cells were able to get to the bone marrow during the first hours after transplantation in KO as well as in WT mice (Figure 3.54).

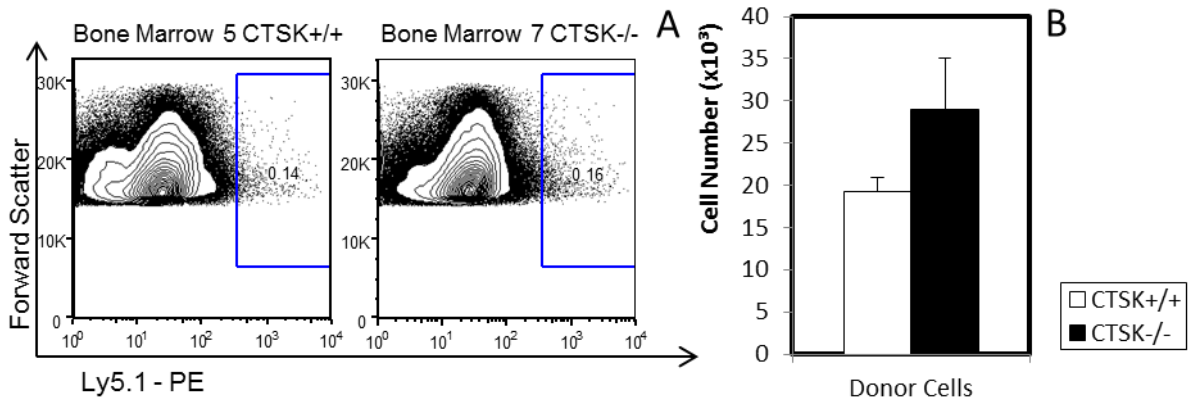

Figure 3.54. Donor cells found in bone marrow samples 17 hours after transplantation. A. Gating. B. Number of donor cells (CD45.1+ cells) in wild type and *Ctsk* KO mice. Means +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11.

It is difficult to draw more conclusions than this from the experiment, as not many donor HSCs and progenitors were found in any of the organs (Figures 3.55, 3.57 and 3.58C). Due to a restricted number of donor mice available it was not possible to inject more than a maximum of 10^6 cells per mouse.

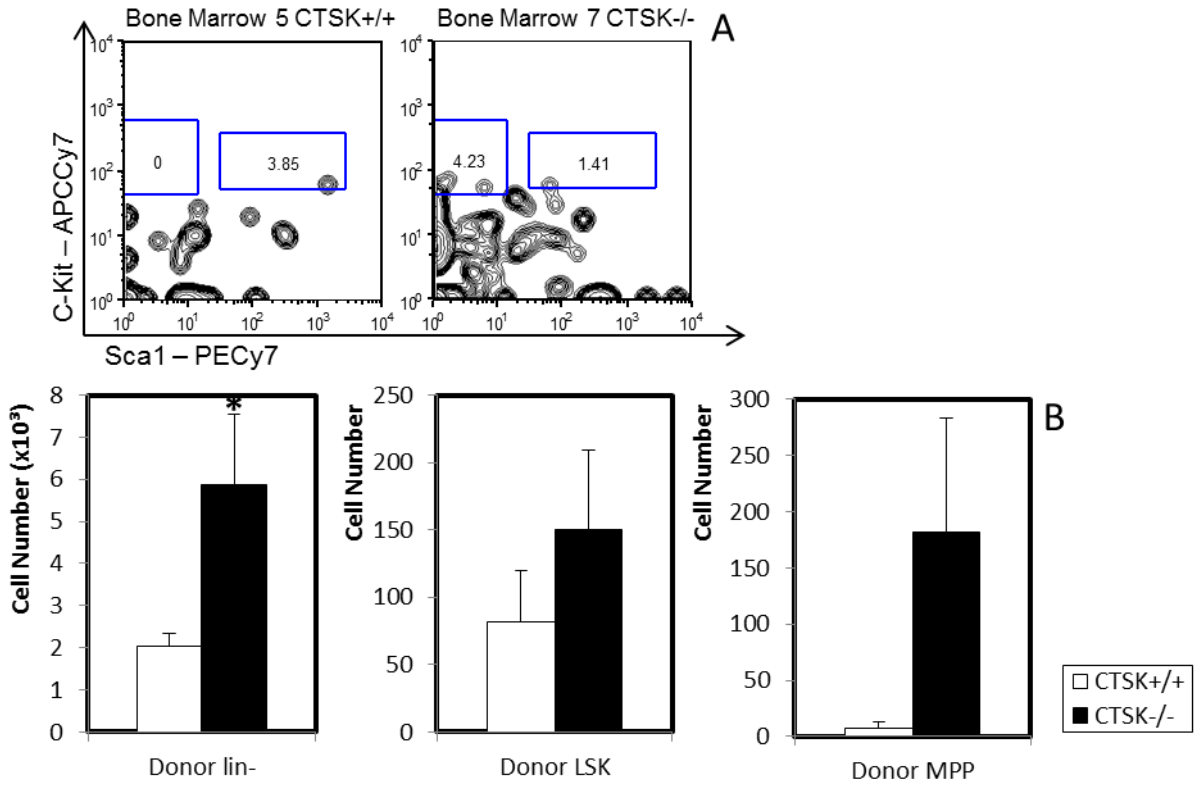

Figure 3.55. Hematopoietic stem cells found in bone marrow samples 17 hours after transplantation. A. Gating. B. Number of lineage negative donor cells, donor LSK (c-Kit+ Sca-1+ lin- CD45.1+) and donor MPP (c-Kit+ Sca-1- lin- CD45.1+) in wild type and *Ctsk* KO mice. Means +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11, *p<0.05.

In the spleen and blood samples the number of donor cells was unchanged as well (Figure 3.56, Figure 3.58)

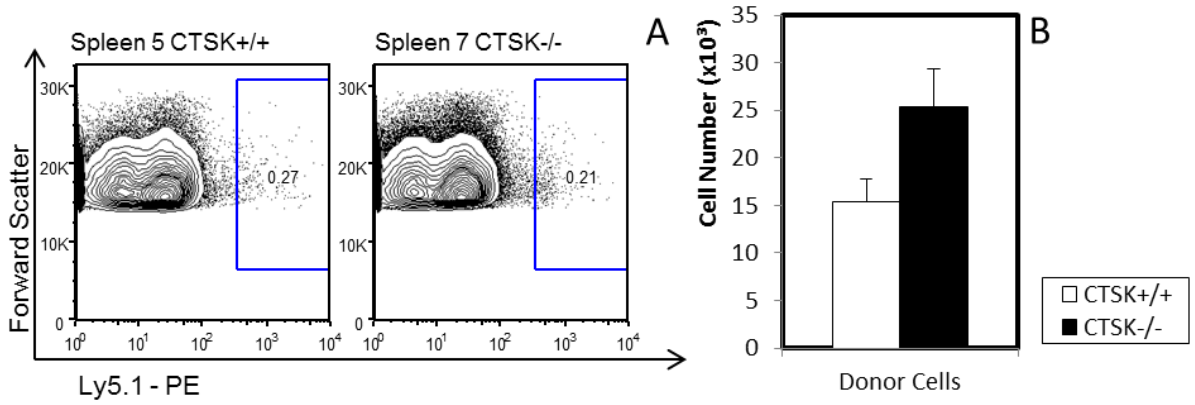

Figure 3.56. Donor cells found in spleens 17 hours after transplantation. A. Gating. B. Number of donor cells (CD45.1+ cells) in wild type and *Ctsk* KO mice. Means +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11.

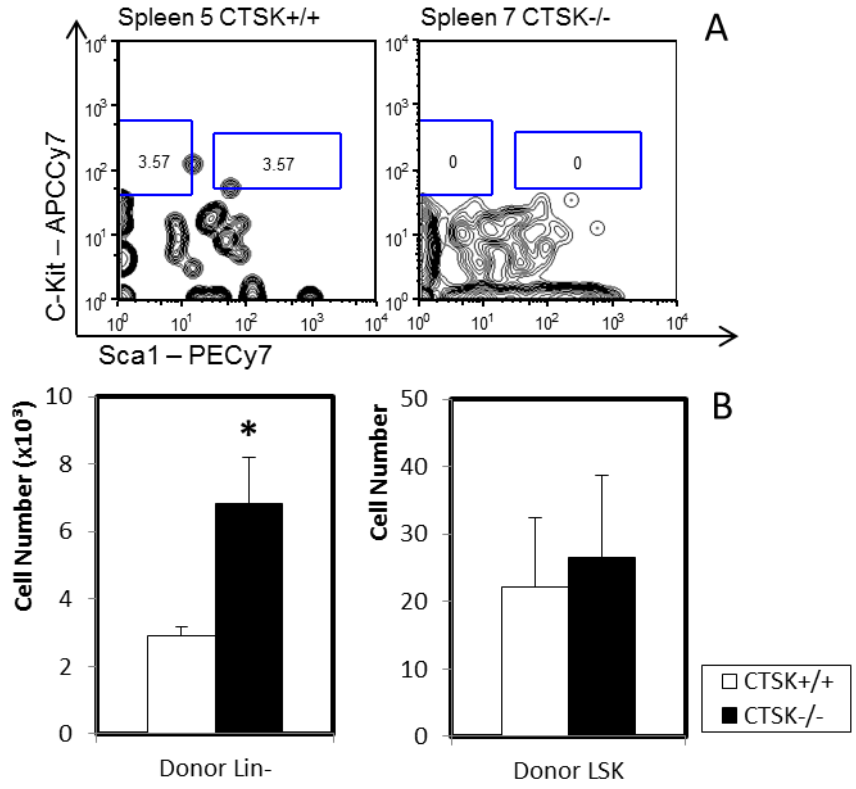

Figure 3.57. Hematopoietic stem cells found in spleens 17 hours after transplantation. A. Gating. B. Number of lineage negative donor cells and donor LSK (c-Kit+ Sca-1+ lin- CD45.1+) in wild type and *Ctsk* KO mice (no MPPs found). Means +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11, *p<0.05.

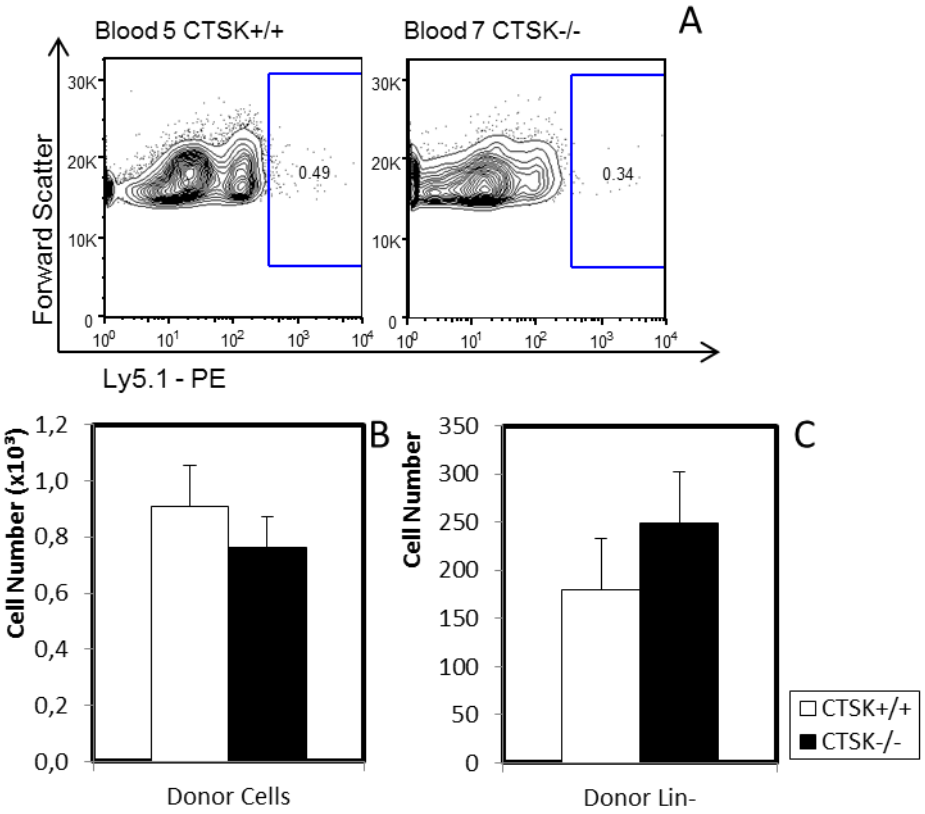

Figure 3.58. Donor cells and lineage negative donor cells found in peripheral blood samples (300µl) 17 hours after transplantation. A. Gating of donor cells. B. Number of donor cells (CD45.1+ cells) and lineage negative donor cells in wild type and *Ctsk* KO mice (no MPPs or LSKs found). Means +SEM, $n_{(+/+)}$=11, $n_{(-/-)}$=11.

3.5 Mobilization of hematopoietic stem cells

3.5.1 Blood count and cell numbers

After having been treated with filgrastim for four days (see 2.2.3), the KO and wild type mice were analyzed together with mice that had not received filgrastim. The experiment was performed three times. The blood count and spleen weights, however, could not be determined at one time for hygienic reasons, as an infection with mouse parvovirus had occurred in a different rack in the same room at the time of the experiment.

Generally, the blood counts show elevated numbers of white blood cells in both groups that had received G-CSF treatment. When comparing the untreated mice, lymphocytes were found to be lower in the KO group. This is a new finding, as it did not occur when characterizing the mice. But like in characterization, the MCH was lower in the KO mice. RDW, however, was unchanged (Table 3.5).The only difference between the treated groups was a significantly lower number of platelets in the KO group (Figure 3.59). One explanation could be a very active coagulation in the mobilized mice, especially in the KO samples. Blood clots (and very low numbers of platelets in the same mice; especially KOs) were found in seven out of nine KO samples and in four out of nine wild type mice.

Table 3.5. Complete blood count of after sacrifice of mice from mobilization experiments.
Results of two independent experiments, groups *Ctsk*+/+ (G-CSF) and *Ctsk*-/- (G-CSF) treated with recombinant granulocyte colony-stimulating factor (filgrastim). Arithmetic means ±SEM, $n_{(+/+)}=7$, $n_{(-/-)}=7$, $n_{(+/+,\ G\text{-}CSF)}=9$, $n_{(-/-,\ G\text{-}CSF)}=9$, *$p<0.05$.

	CTSK +/+	CTSK -/-	CTSK +/+ (G-CSF)	CTSK -/- (G-CSF)
WBC [10^3/µl]	5.54 ± 0.63	4.46 ± 0.70	11.86 ± 1.75	14.05 ± 3.03
Lymph. [10^3/µl]	4.16 ± 0.44	2.86 ± 0.35*	5.82 ± 0.63	6.00 ± 1.19
Mono. [10^3/µl]	0.17 ± 0.03	0.15 ± 0.03	0.40 ± 0.08	0.51 ± 0.14
Gran. [10^3/µl]	1.20 ± 0.18	1.35 ± 0.33	5.63 ± 1.08	7.53 ± 1.72
Eos. [10^3/µl]	0.07 ± 0.02	0.10 ± 0.03	0.75 ± 0.16	1.10 ± 0.26
RBC [10^6/µl]	9.67 ± 0.61	9.41 ± 0.61	9.83 ± 0.29	8.87 ± 0.72
HGB [g/dl]	14.46 ± 0.91	14.79 ± 0.95	15.75 ± 0.34	14.27 ± 1.15
HCT [%]	50.91 ± 3.86	54.95 ± 3.89	56.97 ± 1.47	51.19 ± 4.18
MCV [µm³]	58.43 ± 0.42	58.31 ± 0.72	58.00 ± 0.49	57.66 ± 0.58
MCH [pg]	16.72 ± 0.21	15.74 ± 0.34*	16.03 ± 0.16	16.06 ± 0.16
MCHC [g/dl]	28.69 ± 0.54	27.10 ± 0.63	27.69 ± 0.29	27.92 ± 0.16
RDW [%]	13.64 ± 0.18	14.19 ± 0.21	13.65 ± 0.14	14.07 ± 0.35
PLT [10^3/µl]	1356 ± 212	897 ± 344	1124 ± 180	536 ± 157 *
MPV [µm³]	5.75 ± 0.06	5.76 ± 0.19	5.56 ± 0.07	5.57 ± 0.10

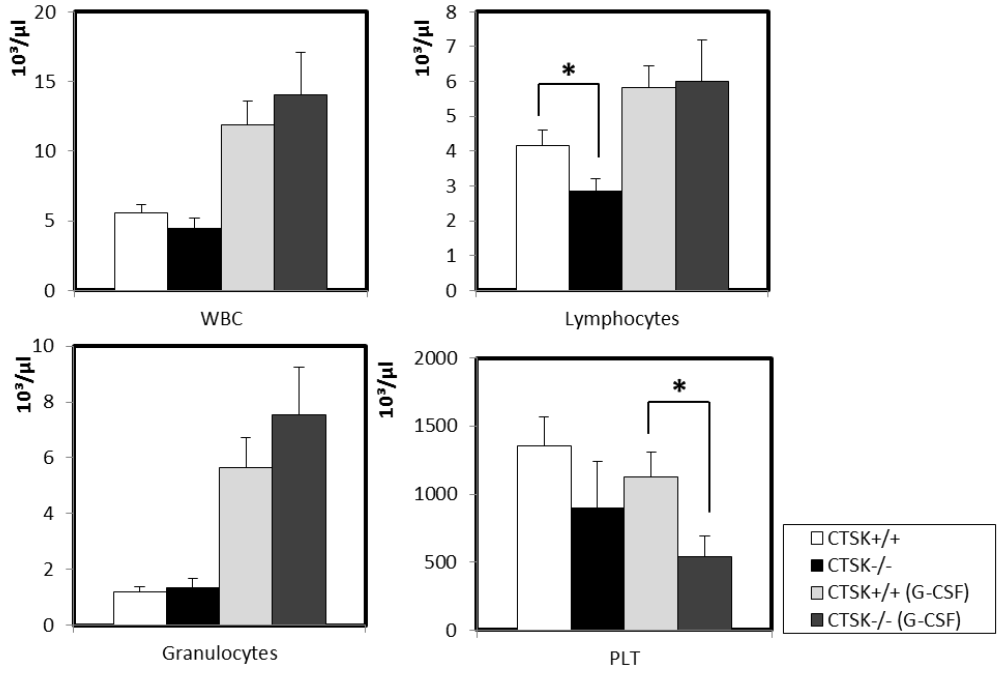

Figure 3.59. Number of white blood cells (WBC), lymphocytes, granulocytes and platelets in blood counts of samples from mobilization experiments. Means +SEM, $n_{(+/+)}$=7, $n_{(-/-)}$=7, $n_{(+/+,\ G\text{-}CSF)}$=9, $n_{(-/-,\ G\text{-}CSF)}$=9, *p<0.05.

In both treated and untreated groups, the absolute cell numbers in bone marrow were lower in the cathepsin K deficient mice. Also spleen cell numbers in the untreated KOs were lower (Figure 3.60). This as well did not occur when first characterizing the mice, but at least spleen cell numbers can be explained as two of the four KO mouse spleens in one experiment were found to be partially necrotic.

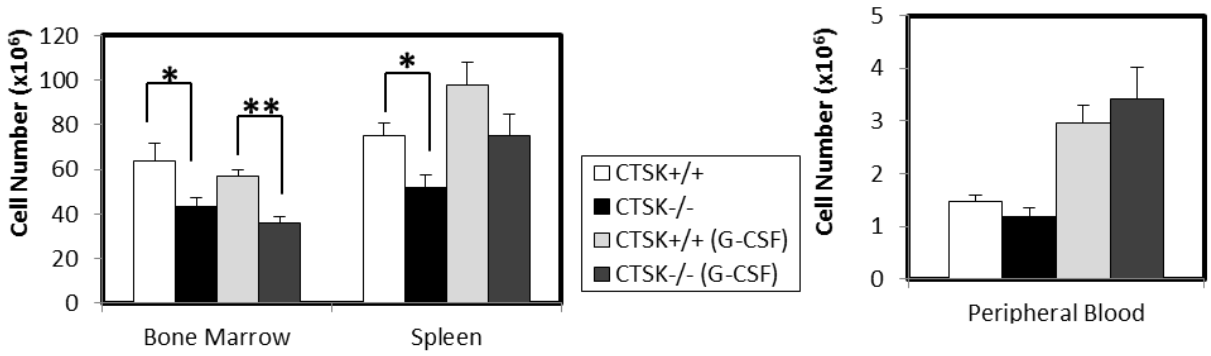

Figure 3.60. Means of cell numbers in bone marrow samples, spleens and peripheral blood samples (300µl) in mice from mobilization experiments. +SEM, $n_{(+/+)}$=10, $n_{(-/-)}$=10, $n_{(+/+,\ G\text{-}CSF)}$=13, $n_{(-/-,\ G\text{-}CSF)}$=11, *p<0.05, **p<0.01.

There were no differences between the spleen weights of treated or untreated mice. (Figure 3.61)

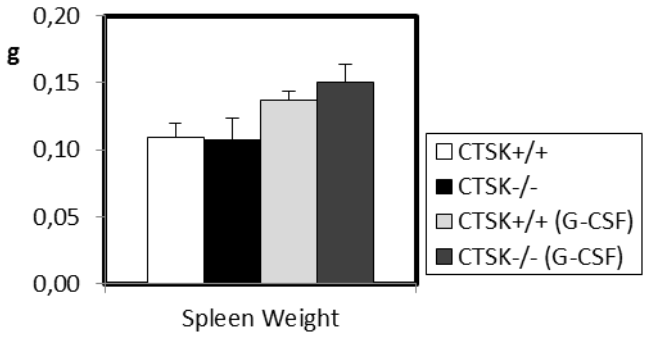

Figure 3.61. Average weights of mouse spleens from mobilization experiments. +SEM, $n_{(+/+)}=7$, $n_{(-/-)}=7$, $n_{(+/+,\ G\text{-}CSF)}=9$, $n_{(-/-,\ G\text{-}CSF)}=9$.

3.5.2 Flow cytometry

It has been shown that inhibition of cathepsin K impairs in the mobilization of HSCs and progenitors from the bone marrow. It is able to cleave and degrade SDF-1, which works as a strong chemotactic agent in the niche and can herby facilitate mobilization (Kollet O, 2006). That is why it was decided to examine mobilization with G-CSF in *Ctsk* KO mice.

Bone marrow, spleen and peripheral blood cell samples were analyzed by flow cytometry. The gating strategy is shown in figures 3.62, 3.64 and 3.66.

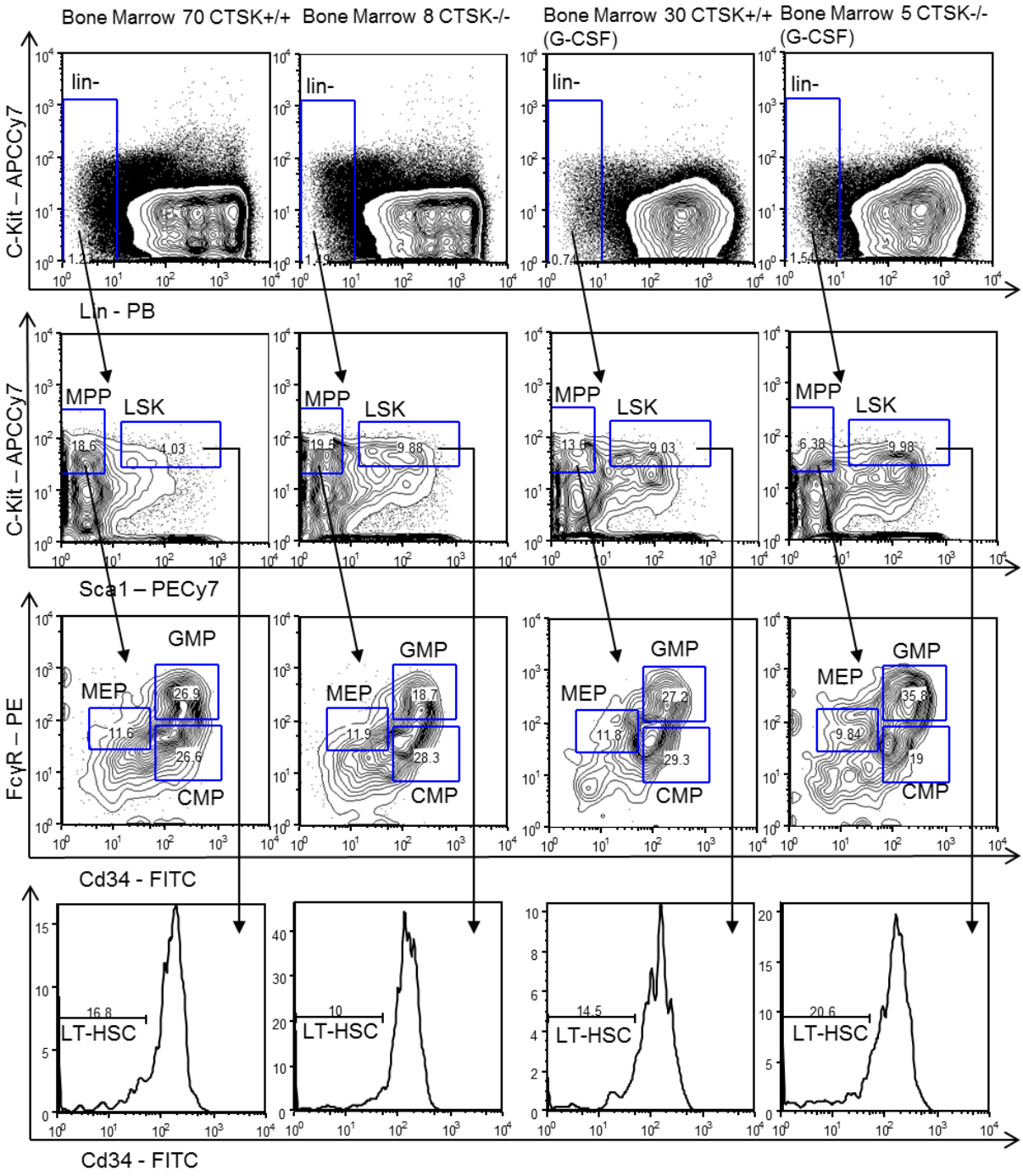

Figure 3.62. Gating strategy for hematopoietic stem cells, bone marrow of untreated wild type and knockout mice (*Ctsk*+/+, *Ctsk*-/-) and mice treated with filgrastim (*Ctsk*+/+ (G-CSF), *Ctsk*-/- (G-CSF)). Lineage negative cells (CD3- B220- Ter119- Gr-1- CD11b-), MPP (c-Kit+ Sca-1- lin-), LSK (c-Kit+ Sca-1+ lin-), MEP (CD34- FcγR^{low} MPP), GMP (CD34+ FcγR+ MPP), CMP (CD34+ FcγR- MPP) and LT-HSC (CD34- LSK).

In the bone marrow, there were no alterations found concerning the KO and WT mice treated with G-CSF (Figure 3.63).

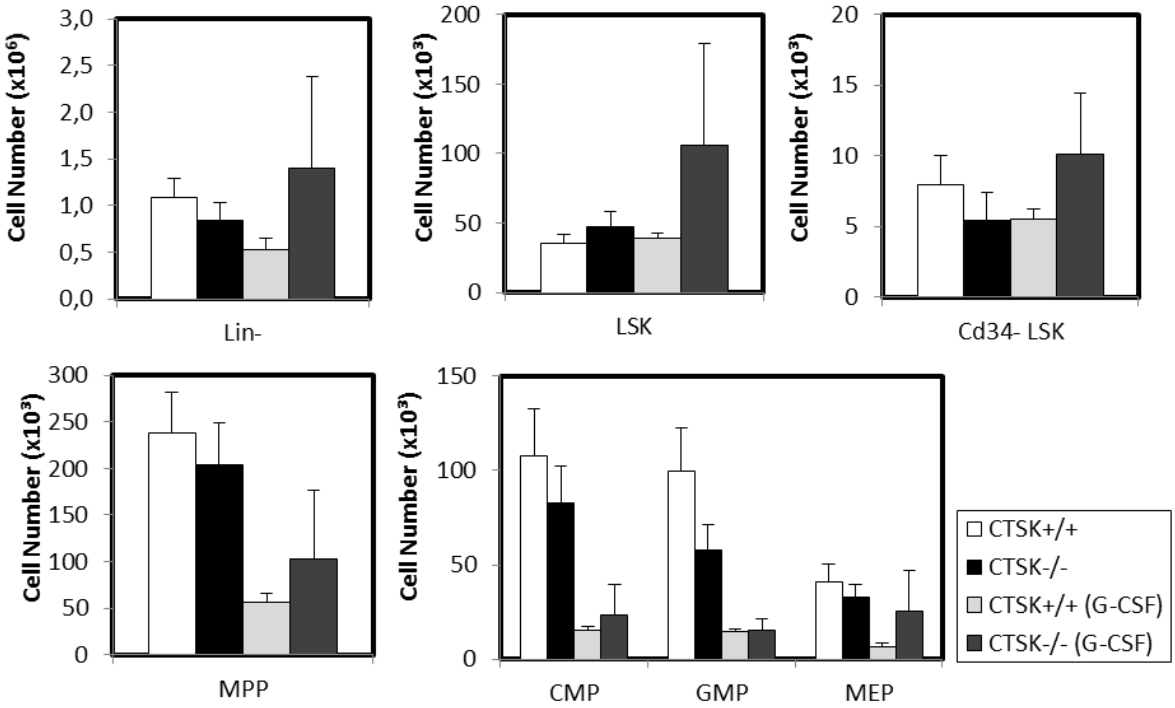

Figure 3.63. Number of stem cells and progenitors in bone marrow of untreated and treated *Ctsk* KO mice and wild type controls. Results of three independent experiments. Long bones of both hind legs. Means + SEM, $n_{(+/+)}$=10, $n_{(-/-)}$=10, $n_{(+/+,\ G\text{-}CSF)}$=13, $n_{(-/-,\ G\text{-}CSF)}$=11.

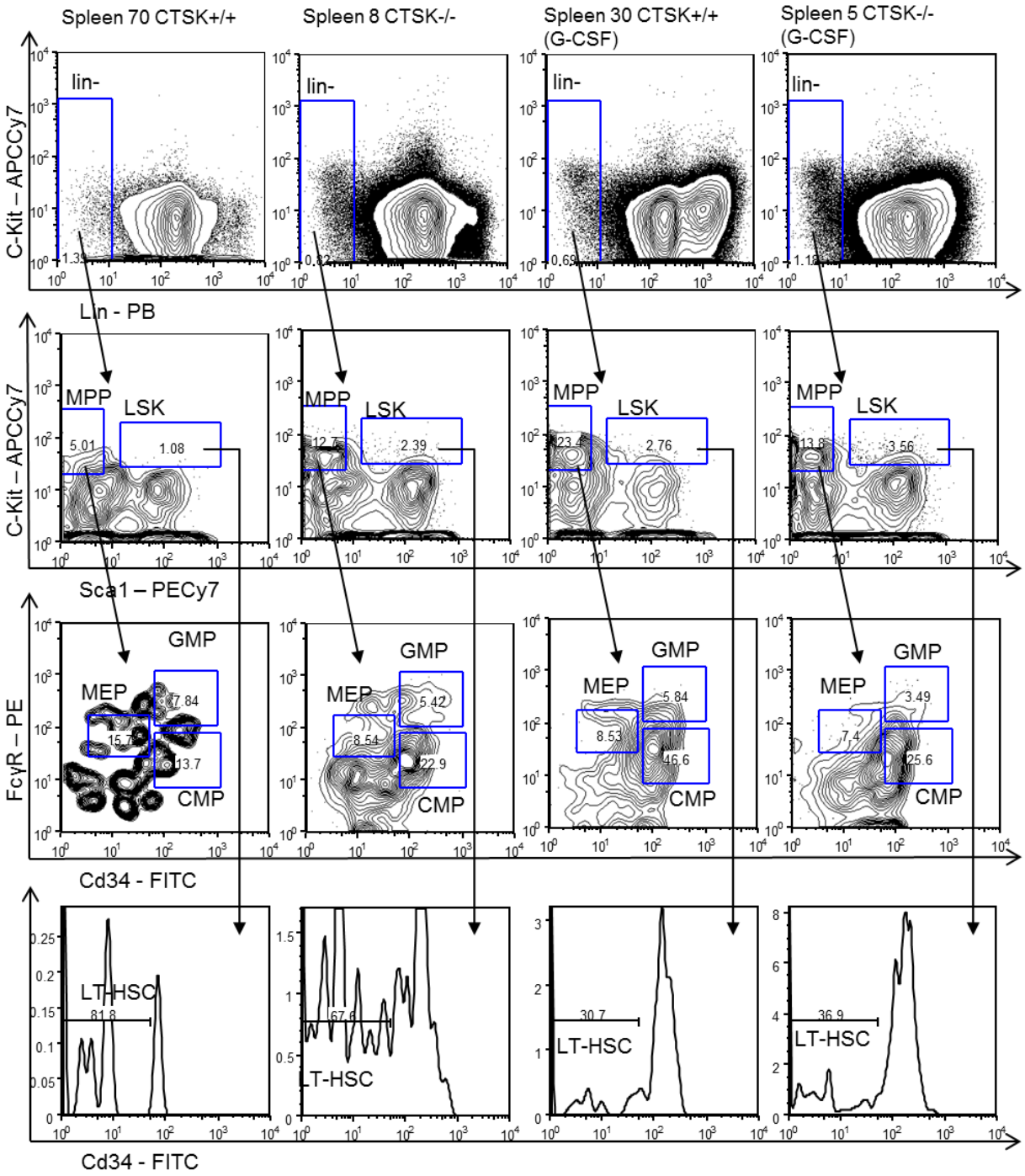

Figure 3.64. Gating strategy for hematopoietic stem cells, spleens of untreated wild type and knockout mice (*Ctsk*+/+, *Ctsk*-/-) and mice treated with filgrastim (*Ctsk*+/+ (G-CSF), *Ctsk*-/- (G-CSF)). Lineage negative cells (CD3- B220- Ter119- Gr-1- CD11b-), MPP (c-Kit+ Sca-1- lin-), LSK (c-Kit+ Sca-1+ lin-), MEP (CD34- FcγR^{low} MPP), GMP (CD34+ FcγR+ MPP), CMP (CD34+ FcγR- MPP) and LT-HSC (CD34- LSK).

In the spleens, there were no significant differences between the treated groups. Surprisingly, the mice that had not received G-CSF had a lower number of lin- cells (Figure 3.65). This was a new finding compared to characterization (see 3.1.2).

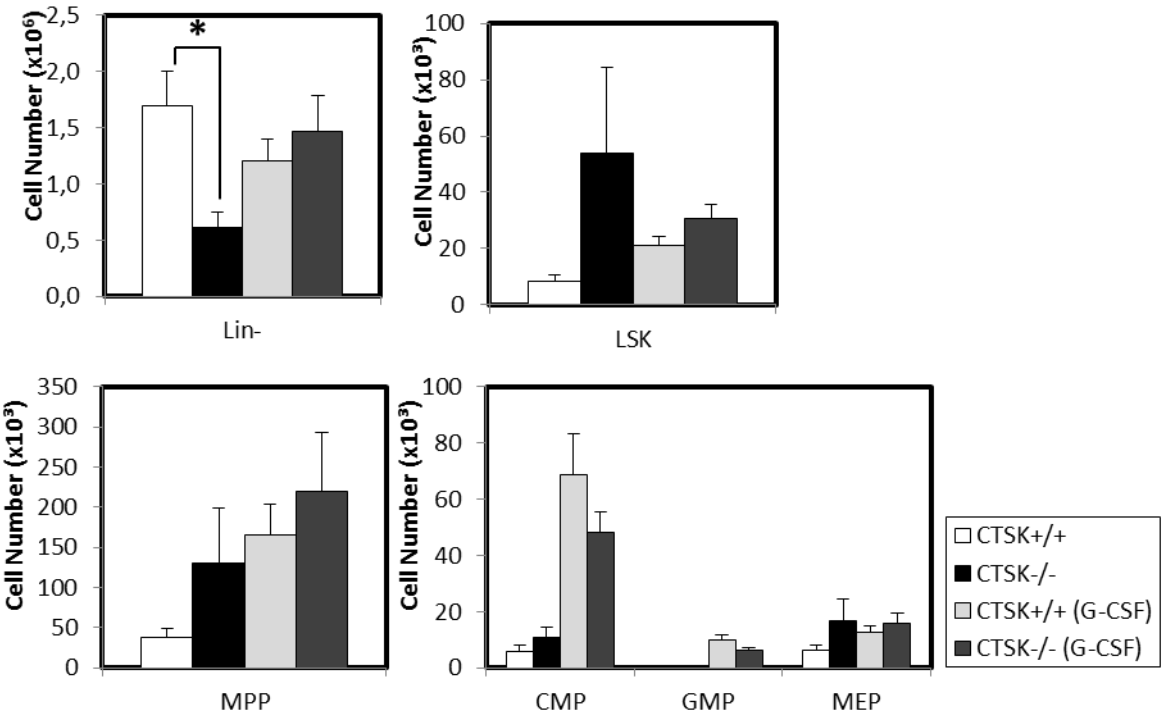

Figure 3.65. Number of stem cells and progenitors in spleens of untreated and treated *Ctsk* KO mice and wild type controls. Results of three independent experiments. Means + SEM, $n_{(+/+)}$=10, $n_{(-/-)}$=10, $n_{(+/+,\ G\text{-}CSF)}$=13, $n_{(-/-,\ G\text{-}CSF)}$=11, *$p<0.05$.

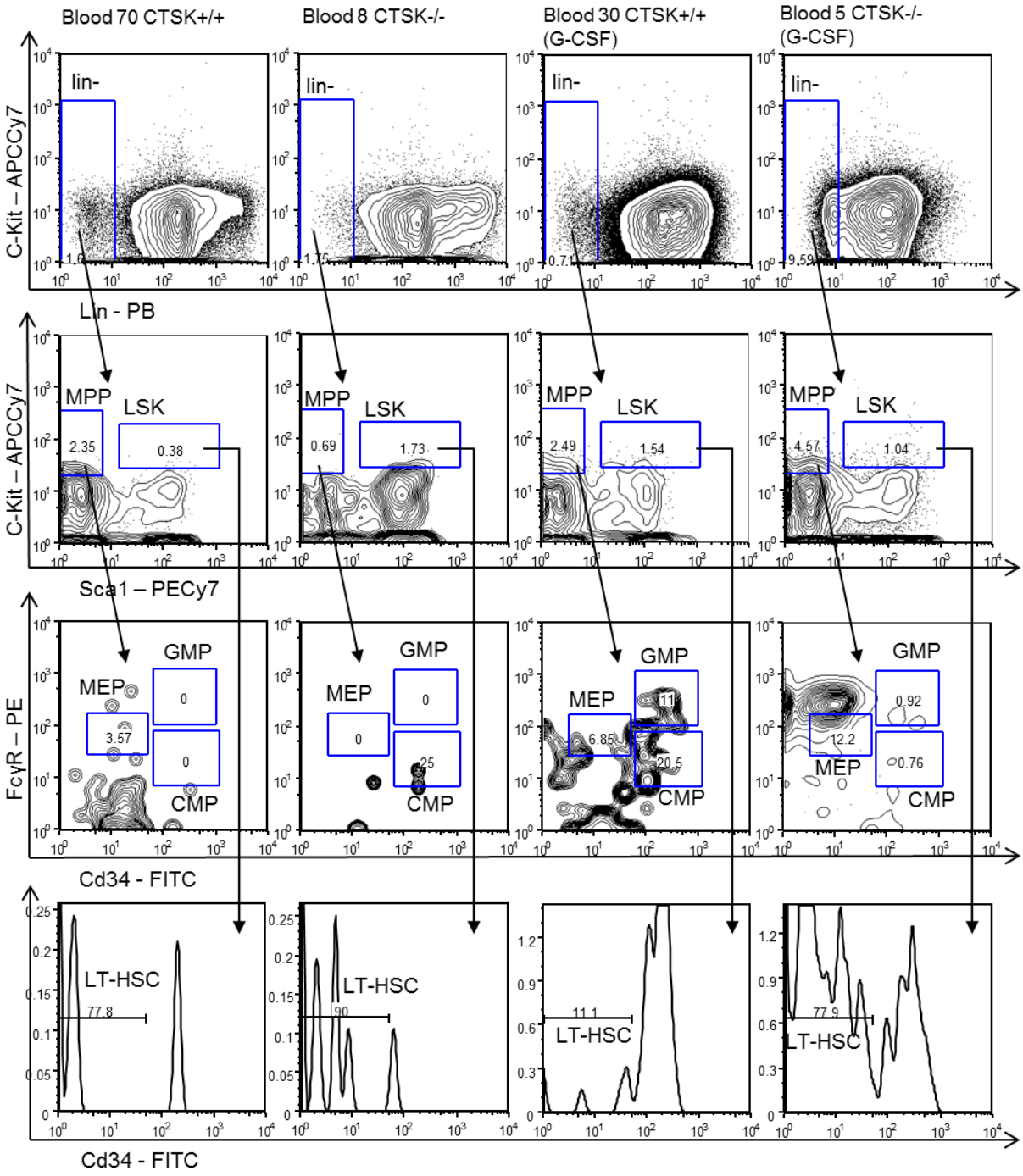

Figure 3.66. Gating strategy for hematopoietic stem cells, peripheral blood of untreated wild type and knockout mice (*Ctsk*+/+, *Ctsk*-/-) and mice treated with filgrastim (*Ctsk*+/+ (G-CSF), *Ctsk*-/- (G-CSF)). Lineage negative cells (CD3- B220- Ter119- Gr-1- CD11b-), MPP (c-Kit+ Sca-1- lin-), LSK (c-Kit+ Sca-1+ lin-), MEP (CD34- FcγR^{low} MPP), GMP (CD34+ FcγR+ MPP), CMP (CD34+ FcγR- MPP) and LT-HSC (CD34- LSK).

In blood samples, finally, there were no hints of an impaired mobilization. No significant differences were found in the number of lin- cells, LSKs, LT-HSCs or myeloid progenitors. (Figure 3.67). In the untreated groups, there was a reduction of MPPs found in blood samples of KO mice.

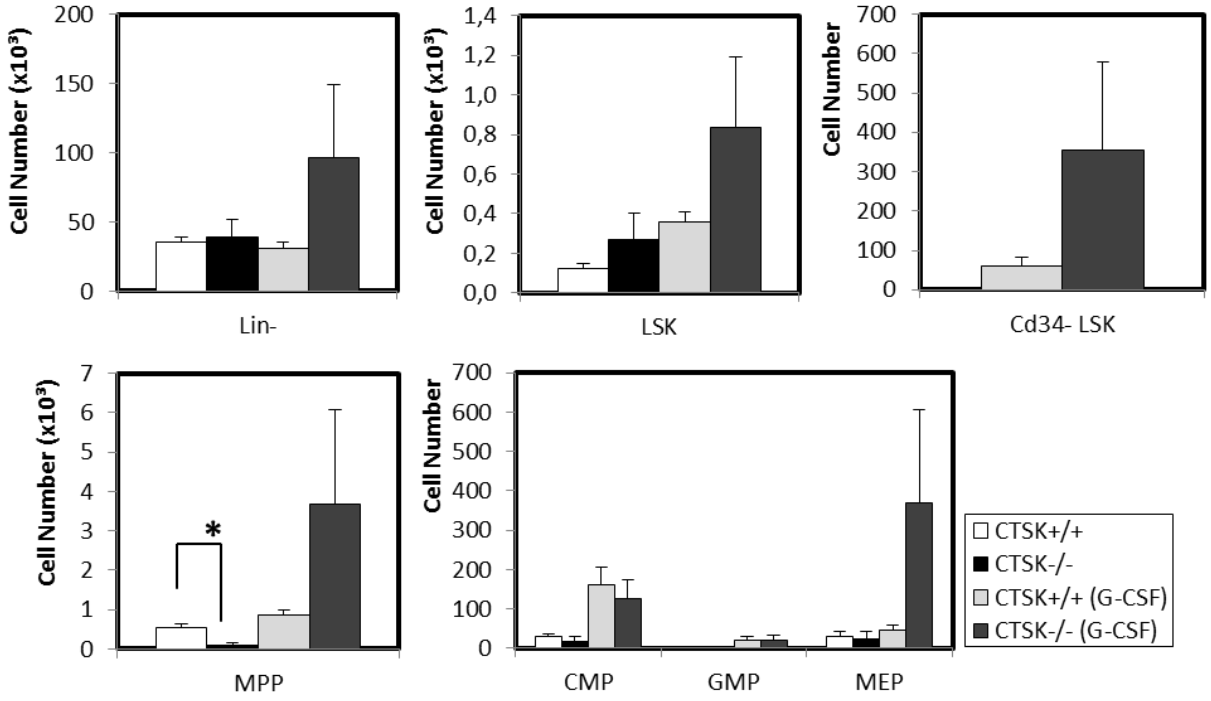

Figure 3.67. Number of stem cells and progenitors in 300µl samples of peripheral blood from untreated and treated *Ctsk* KO mice and wild type controls. Results of three independent experiments. Means + SEM, $n_{(+/+)}$=10, $n_{(-/-)}$=10, $n_{(+/+,\ G\text{-}CSF)}$=13, $n_{(-/-,\ G\text{-}CSF)}$=11, *$p<0.05$.

Surprisingly, by flow cytometry there was no proof found for an impaired mobilization of HSCs and progenitors in cathepsin K deficient mice. For the evaluation of their functional capacity, CFA were set up with peripheral blood cells.

3.5.3 Colony forming unit assay

The only alteration detected by CFA was a lower number of large CFU-GM in the *Ctsk* KO mice which consist of more than 1000 cells (Figure 3.68). This suggests an only small impact of cathepsin K deficiency on the mobilization of functional progenitors in vivo. The appearance of BFU-E and CFU-GEMM and the total number of colonies was unaffected.

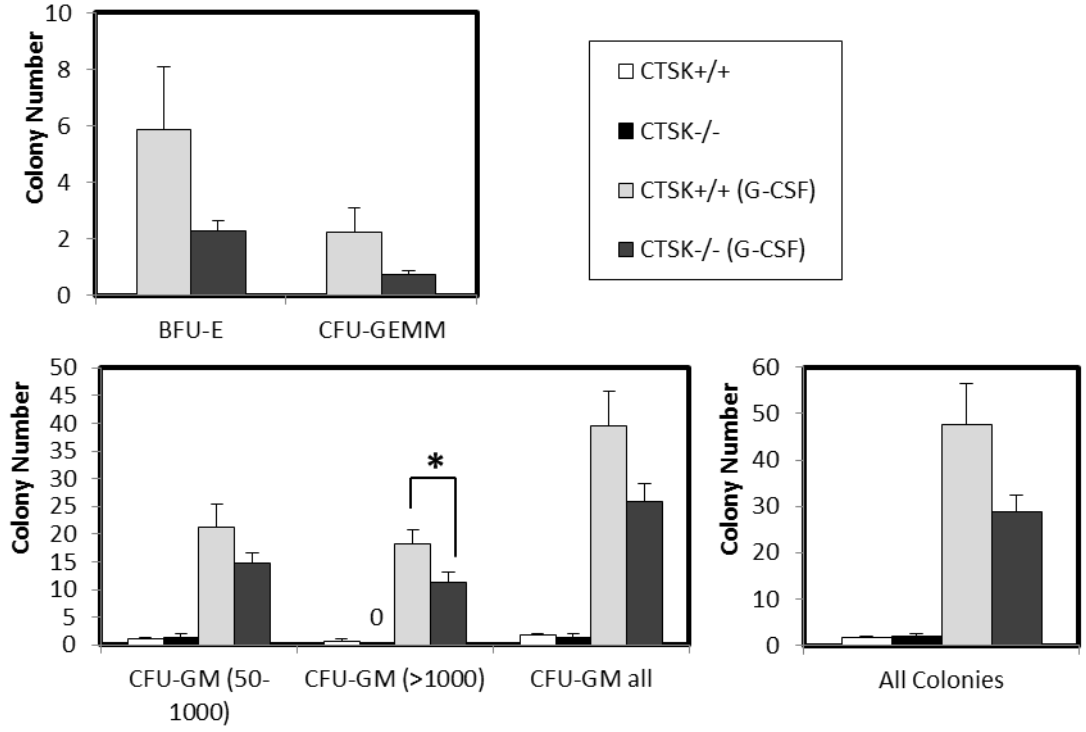

Figure 3.68. Colony forming unit assays prepared with peripheral blood obtained from *Ctsk* KO mice and wild type controls untreated or treated with filgrastim showing colonies of erythroid progenitors, granulocyte-macrophage progenitors and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitors. BFU-E (bust-forming unit - erythroid), CFU-GEMM (colony-forming unit - granulocyte, erythroid, macrophage, megakaryocyte), CFU-GM (colony-forming unit - granulocyte macrophage) and total number of colonies. Means +SEM, $n_{(+/+)}=7$, $n_{(-/-)}=7$, $n_{(+/+,\ G\text{-}CSF)}=13$, $n_{(-/-,\ G\text{-}CSF)}=11$, *$p<0.05$.

4 Discussion

In recent years, a multitude of new insights has been gained about the hematopoietic niche and its components. An increasing amount of data is being collected, the interpretation of which suggests roles for a number of membrane-bound or soluble factors to be involved in maintaining the HSC pool or promoting their differentiation. However, 36 years after the concept of the hematopoietic niche was first proposed (Schofield, 1978), there is still no full understanding of the interactions in the niche. Moreover, there is an ongoing discussion about which cells comprise the stem cell niche. In addition to osteoblasts (Calvi LM, 2003) and vascular endothelial cells (Kiel MJ, 2005), many other cell types like nestin+ mesenchymal stem cells (Méndez-Ferrer S, 2010), adipocytes (Yokota T, 2003; Naveiras O, 2009) bone marrow macrophages (Winkler IG, 2010), arteriolar endothelial cells (Kunisaki Y, 2013), and even sympatric innervation (Katayama Y, 2006) have been proposed as parts of the niche.

A matter of discussion is the question whether osteoclasts, the major source of cathepsin K in the bone marrow, form a part the niche. There is some controversy over contradictory results in this question (see 1.1). However, so far, mainly mobilization of hematopoietic stem cells has been examined in different mouse strains lacking osteoclasts or osteoclast activity. A thorough hematopoietic examination of cathepsin K null mice has never been done so far even though there are hints for its relevance in the context (see 1.2).

It was the aim of this thesis to examine if cathepsin K deficiency has effects on the cellular composition of murine blood as well as on hematopoiesis. We found changes concerning coagulation and lymphocytes. Also, GMP numbers were altered in cathepsin K deficient mice and extrinsically caused stem cell exhaustion was observed after secondary transplantations. Those findings, which will be discussed in this chapter, together with the results of other groups, illustrate that cathepsin K is important in the regulation of hematopoiesis.

4.1 Thrombocytes

When harvesting blood samples from cathepsin K KO mice, the first thing that became obvious was the enhanced tendency of those samples to coagulate. In the blood counts performed with this blood, some of the samples obtained from the KO mice showed extremely high platelet counts. Others, namely the ones that had blood clots in them, showed low counts

due to platelet consumption by the clot. This was observed in steady-state hematopoiesis and after primary transplantation of HSCs into cathepsin K deficient mice. However, the differences concerning the platelet numbers in these experiments were not significant. But by flow cytometry, in the bone marrow of *Ctsk* KOs a significantly higher fraction of platelets was detected.

So far, there is no evidence in literature connecting cathepsin K with megakaryopoiesis. It is known, however, that cathepsin K cleaves SDF-1 in BM (Kollet O, 2006). Thus, the absence of cathepsin K leads to SDF-1 abundance.

SDF-1, on the other hand, does enhance platelet formation, supposedly by enhancing VCAM-1/VLA-4 interaction of vascular endothelial cells and CXCR4 positive megakaryocytes. (Avecilla ST, 2004; Avraham H, 1993). Figure 4.1 illustrates this possible mechanism.

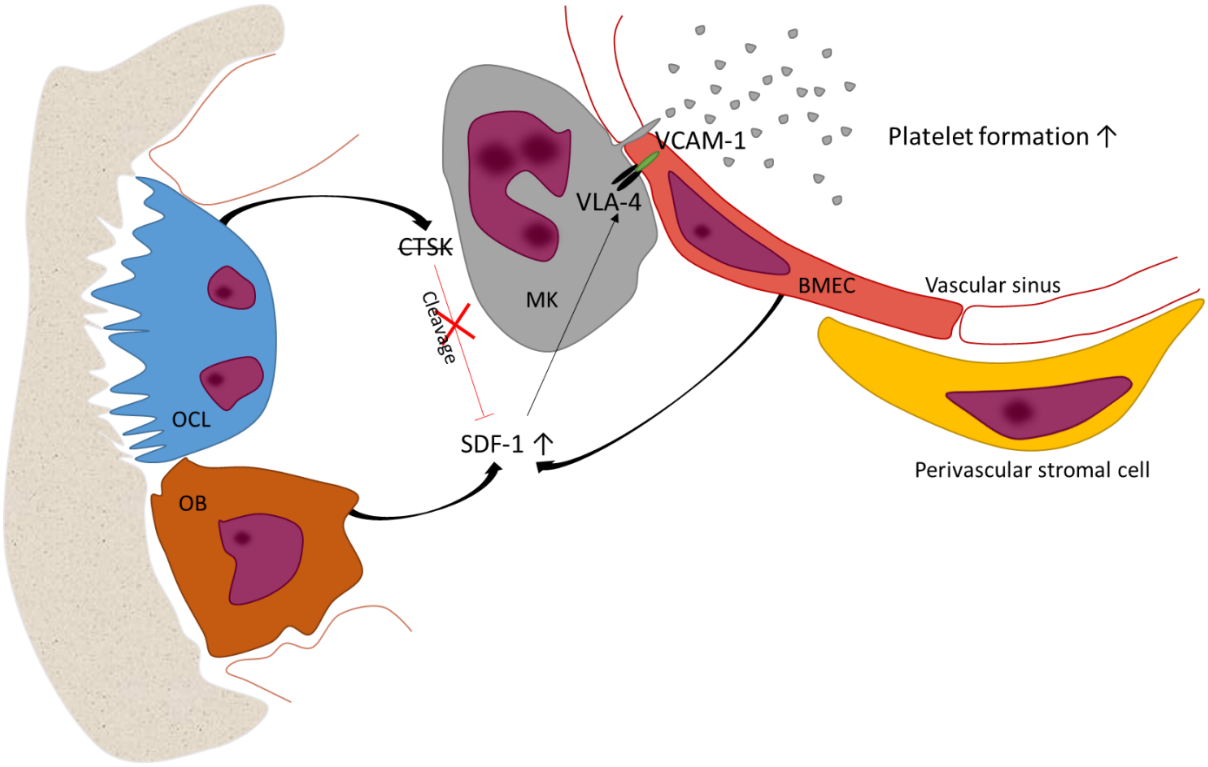

Figure 4.1. SDF-1 abundance in CTSK deficient mice enhances platelet formation. OCL: osteoclast, OB: osteoblast, MK: megakaryocyte, BMEC: bone marrow endothelial cell.

If those assumptions are correct and cathepsin K mediated SDF-1 cleavage is indeed a factor that regulates platelet numbers, our findings would emphasize the impact cathepsin K has on the SDF-1/ CXCR4 pathway. However, not much is known about SDF-1 in cathepsin K

deficient mice and other mechanisms could cause the change of coagulation in CTSK deficient mice as well.

4.2 Lymphocytes

In the white cell line, a number of notable changes was found. There were less CD8+ T cells in bone marrow but more CD8+ T cells in the peripheral blood of KO mice compared to wild type controls.

Similarly, less total T cells were found in spleens after primary transplantations and more in their peripheral blood. Unfortunately, due to a restricted number of detectors in the flow cytometer, CD8 and CD4 expression was not studied separately here. Accordingly, less T cells were found in the bone marrow and spleens of secondarily transplanted mice. However, in those experiments no alterations of the number of T cells in peripheral blood were discovered.

For the findings in steady-state hematopoiesis and in primary transplantations, T cell retention in blood is a thinkable explanation. The protease cathepsin K is able to facilitate the migration of cells as has been shown in the surroundings of a number of tumor metastases (Tsai JY, 2014). In secondary transplantations, there is no retention in the blood. This is consistent with the fact that cathepsin K is present in the recipient mice. However, there are still decreased numbers of T cells in bone marrow and spleens. This indicates that migration cannot be the only factor of influence. Cathepsin K possibly also has an impact on development, or, as there are no changes in thymi, on the survival of T cells. Natural killer cells are also not retained in peripheral blood but decreased in bone marrow, spleens and lymph nodes of primarily transplanted *Ctsk* KO mice as well as in bone marrow and spleens after secondary transplantations. This is consistent with a possible impaired development of NK1.1+ cells as they develop in the bone marrow.

The only published data that links cathepsin K to lymphopoiesis applies to B cells. In our experiments, they were unaltered in steady state hematopoiesis, but had highly significantly reduced cell numbers in spleens of primarily transplanted *Ctsk* KOs and the respective secondary transplantations.

As SDF-1 works as a strong chemoattractant for B cells (Bleul CC, 1996), B cells would migrate to the bone marrow if SDF-1 levels would be influenced to a major extent by CTSK. But while

this might explain the decreased number of B cells in spleens, there is no elevation of B cell numbers in the bone marrow. SDF-1 does not seem to play a major role in this context.

A more speculative model involves the hedgehog pathway. It is known to extrinsically promote B lymphopoiesis when smoothened (SMO), a cellular component of the pathway, is inhibited in stromal cells (Cooper CL, 2012). The intercellular factor that mediates B lymphopoiesis is not known. But it has been demonstrated that Hh, via osteopontin, elevates cathepsin K levels (Das S, 2011). This raises the question whether cathepsin K might act as the downstream mediator in this pathway that directly influences lymphopoiesis. However, B-cell numbers were not (significantly) reduced in bone marrow but only in spleens after primary transplantations.

4.3 Mobilization, Homing and Engraftment

No severe mobilization defects in *Ctsk* KO mice were identified by flow cytometry or CFU assays. Solely large CFU-GM consisting of over 1000 cells were lowered in the blood of KO mice compared to wild type controls. Notably, those larger colonies usually arise from the more primitive progenitors.

The comparatively unchanged mobilization of HSCs to the blood contradicts the findings published in Orit Kollet's paper where mobilization defects were caused by the administration of a cathepsin K inhibitor (Kollet O, 2006). On the other hand, it is consistent with results that suggest that the depletion of osteoclasts has no effect on mobilization (Miyamoto K, 2011). It can be assumed that depletion of osteoclasts is followed by a massive decline of cathepsin K as they are its main producer in the bone marrow, indicating that the effects observed in the bone marrow of cathepsin K null mice should be related to those in osteoclast deficient mice.

No homing defects were detected in cathepsin K deficient mice. Assuming that cathepsin K is a regulator in the CXCL12/CXCR4 axis and knowing that SDF-1 has chemotactic effects on hematopoietic stem cells and progenitors, an even accelerated homing process can be expected in KOs which would in this case have elevated SDF-1 levels. Although we did not find any changes concerning the numbers of donor cells homed, the lineage negative donor cells were significantly higher in KO mice. But the very low absolute numbers of donor cells detected by flow cytometry in this experiment raise doubt about the biological significance of this result.

Further tests are necessary for its confirmation, maybe using alternative cell-tracing techniques like CFSE stain.

Significant results in respect of the engraftment in a cathepsin K deficient environment were detected. Engraftment was worse in thymi and lymph nodes of KO groups and there was a lower portion of donor cells in the peripheral blood of KO mice. In the secondary transplantation experiments, the donor cells engrafted spleens of KO mice less but there were no differences concerning the donor cells in the peripheral blood. Other organs were not examined. There were, however, no alterations of bone marrow engraftment in neither of the experiments. But the impaired engraftment in the other organs are hints for a reduced repopulating activity in the absence of cathepsin K.

4.4 Hematopoietic stem cells and progenitors

In steady-state experiments, the only change of the hematopoietic phenotype was a reduction of GMPs in *Ctsk*-/- bone marrow compared to wild type bone marrow. Also, more CFU-GM formed in CFU assays set up with knockout bone marrow cells. The numbers of HSCs and other progenitors were not altered.

This can be explained with a shift from primitive to more adult and proliferative GMPs in the absence of cathepsin K. The high disposition of GMPs to form colonies by differentiation would then empty the GMP pool.

Very recently, a paper was published where steady-state hematopoiesis in *Ctsk* -/- mice was examined as well. There, LSK numbers in bone marrow were decreased in *Ctsk* KOs and increased in the spleens. However, the fraction of LSKs in the bone marrow was unaltered. Data relating to GMPs was not published. In this paper, the findings were explained with extramedullary hematopoiesis (Jacome-Galarza C, 2014). In contrast, in our experiments, cellularity of bone marrow or spleens in steady-state hematopoiesis, and consequently absolute LSK numbers, were not significantly different.

This might be explained with the fact that the mice were bred on different backgrounds (Jacome-Galarza: mixed C57BL6/L129). There are a few examples for different hematologic phenotypes on different backgrounds, for example in different strains of Cdkn1a KO mice (van

Os R, 2007). However, in this case, the different phenotypes might only be owed to a different extent of osteopetrosis and thus extramedullary hematopoiesis in the respective mouse strains. Further experiments to evaluate stem cell function like serial transplantations were not conducted in that study.

In our study, no changes of stem cell or progenitor numbers were discovered in primary transplantations.

However, in secondary transplantations, significantly less lin- cells and LT-HSCs were detected by flow cytometry. This is a most notable result, as they reveal an important loss of self- renewal capacity among the most primitive hematopoietic stem cells in the absence of cathepsin K and their diminution while primary engraftment.

SDF-1, on the contrary, is important for quiescence and self-renewal (Sugiyama T, 2006). This means that SDF-1 regulation through cleavage by cathepsin K is not a crucial pathway at least in early hematopoiesis.

Stem cell exhaustion in serial transplantation is a phenomenon observed in many mice that are deficient of factors that have proven as crucial for hematopoiesis in the past. One example is the SFRP1-/- mouse where changes of the numbers of LSKs or LT-HSCs first showed in secondary recipients as well. It was then demonstrated that SFRP1, an inhibitor of Wnt signaling, influences cell cycling by regulation of ß-catenin.

4.5 Conclusion

The results of this study demonstrate that cathepsin K has an influence on hematopoiesis. Mainly the results of the secondary transplantations, where exhaustion of lin- cells as well as of LT-HSCs occurred, support this conclusion. The mechanisms involved, however, remain unclear. SDF-1, which is cleaved by cathepsin K and has a major impact on hematopoiesis, cannot serve as the sole target of cathepsin K in this context. SDF-1 abundance caused by cathepsin K deficiency would lead to an increase of quiescent LT-HSCs. Therefore, further research has to be done in order to solve the question by what mechanisms cathepsin K influences hematopoiesis. Crosstalks with pathways such as SCF, hedgehog or canonical and noncanonical Wnt signaling have to be examined and an influence on cell cycling by cathepsin K has yet to be evaluated.

5 Abbreviations

Ang-1	Angiopoietin 1
BM	Bone marrow
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
CFA	Colony forming unit assay
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTSK	Cathepsin K
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C motif chemokine receptor type 4
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
(D)PBS	Dulbecco’s phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
Eos.	Eosinophils
FCS	Fetal calf serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-monocyte progenitor
GP5	Glycoprotein 5
Gran.	Granulocytes

Gy	Gray
HBSS	Hank's buffered salt solution
HCl	Hydrogen chloride
HCT	Hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGB	Hemoglobin
HSC	Hematopoietic stem cell
IL-6	Interleukin 6
KO	Knockout
lin	Lineage
LN	Lymph node
LSK	Lineage-negative, Sca-1+, c-Kit+ cells
LT-HSC	Long-term repopulating hematopoietic stem cell
Lymph.	Lymphocytes
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean cell volume
MEP	Megakaryocyte-erythrocyte progenitor
Mono.	Monocytes
MPP	Multipotent progenitor
MPV	Mean platelet volume
NK cell	Natural killer cell
NKT cell	Natural killer T cell

PB	Peripheral blood
PI	Propidium iodide
PLT	Platelets
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBC	Red blood cells
RDW	Red cell distribution width
Sca-1	Stem-cell antigen 1
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SEM	Standard error of the mean
SFRP1	Secreted frizzled-related protein 1
SLAM	Signaling lymphocyte activation molecule
SPL	Spleen
ST-HSC	Short-term repopulating hematopoietic stem cell
WBC	White blood cells
Wnt	Wingless/int
WT	Wild type

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