

The Role of Macrophage Migration Inhibitory Factor (MIF) in the Pathogenesis of murine Chronic Lymphocytic Leukemia (CLL)

Analysis based on the TCL1 mouse model

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Ever tried. Ever failed. No matter.
Try again. Fail again. Fail better.
(Samuel Beckett, 1983)

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1 Zusammenfassung

Die Interaktion zwischen Tumorzelle und Mikromilieu spielt in der Leukämogenese der chronisch lymphatischen Leukämie (CLL) neben den genetischen Schäden eine bedeutende Rolle. Eine Stimulation durch extrazelluläre Faktoren ist wichtig für das Überleben der CLL-Zelle, denn ohne diese wird die CLL-Zelle außerhalb ihres Mikromilieus in den programmierten Zelltod getrieben.

Vorausgehende Untersuchungen zeigten, dass Macrophage Migration Inhibitory Factor (MIF), ein Chemokin mit proinflammatorischer Wirkung, in der CLL überexprimiert ist. Da MIF regulatorische Einflüsse auf Zellwachstum, -differenzierung und Apoptose besitzt untersucht diese Arbeit die Hypothese, ob MIF eine Rolle bei der Pathogenese der CLL zukommt.

Wir benutzten zu diesem Zweck das Modell der E μ -T-cell leukemia 1 (TCL1)-transgenen Maus (Bichi et al., 2002), bei der eine B-Zell-spezifische Überexpression von TCL1 zu einer Akkumulation und Organinfiltration von CD5⁺IgM⁺ reifen B-Zellen führt. Die transgenen Mäuse entwickeln eine CLL-ähnliche Erkrankung, die sich in einer Leukämie, einer Lymphadenopathie, einer Hepatosplenomegalie, Knochenmarksinfiltration sowie frühzeitigem Tod nach 8 bis 15 Monaten manifestiert. Der Einfluss von MIF wird durch Verpaarung der TCL1^{+/+} Maus mit der MIF^{-/-} Maus (Fingerle-Rowson et al., 2003) *in vivo* getestet. TCL1^{+/wt} MIF^{+/+} und TCL1^{+/wt} MIF^{-/-} Mäuse wurden in dieser Arbeit hinsichtlich Geschwindigkeit der Leukämieentstehung, Tumorlast, hämatopathologischen Veränderungen sowie Proliferations- und Apoptoseverhalten miteinander verglichen.

Die bekannten MIF-Oberflächenrezeptoren CD74/CD44 und CXCR2 sind auch auf den murinen B-Zellen exprimiert. Die TCL1^{+/wt} MIF^{+/+} Mäuse zeigen schon in der prä-leukämischen Phase eine erhöhte Anzahl an CD5⁺IgM⁺ Zellen und TCL1^{+/wt} MIF^{+/+} Mäuse entwickeln auch ca. 3 Monate vor den TCL1^{+/wt} MIF^{-/-} Mäusen eine periphere Leukämie (p = 0.02). Im Alter von 12 Monaten sind Leber und Milz durch Infiltration der Organe durch reife, kleinzellige maligne B-Lymphozyten in TCL1^{+/wt} MIF^{+/+} Mäusen deutlich stärker betroffen als die Organe der MIF-defizienten Tiere. Ursächlich hierfür könnte eine vermehrte Akkumulation von CD68⁺ Makrophagen in den leukämisch infiltrierten Milzen der TCL1^{+/wt} MIF^{+/+} Mäuse sein (p < 0.0001), die als sogenannte „nurse-like cells“ (NLCs) die Leukämiezellen vor der Apoptose schützen.

Dazu passt, dass die Rate an spontaner Apoptose in MIF-defizienten leukämisch infiltrierten Milzen *in vivo* erhöht ist und ein Trend zu vermehrter Apoptose *ex vivo* bei den TCL1^{+/wt}

MIF^{-/-} Splenozyten zu beobachten ist. Diese MIF-bedingten Unterschiede wirken sich sogar auf das Gesamtüberleben aus, denn in den MIF^{-/-} Mäusen beobachten wir eine Verlängerung des medianen Überlebens von 80 Tagen (TCL1^{+wt} MIF^{+/+} 380 Tage vs. TCL1^{+wt} MIF^{-/-} 460 Tage, p = 0.04). Eine Analyse der erworbenen Genomschäden mittels Spektralem Karyotyping (SKY) von Metaphasen leukämischer Splenozyten zeigt verschiedene genetische Aberrationen mit Trisomie, Tetraploidie, Translokationen und Deletionen in beiden Genotypen. Eine Überexpression des Tumorsuppressors p53 durch eine vermutlich inaktivierende Genmutation wurde häufiger in den TCL1^{+wt} MIF^{+/+} als in den TCL1^{+wt} MIF^{-/-} Mäusen gefunden.

Unsere Untersuchungen am Eμ-TCL1 Mausmodell zeigen, dass es sich hier um ein geeignetes Modell für die Validierung von potentiellen Targetmolekülen bei der Pathogenese der CLL handelt. Wie zeigen, dass MIF sowohl in der frühen als auch in der späten Phase der CLL-Entstehung als Tumor-Promoter agiert und im Wesentlichen über Einflüsse des Mikromilieus auf die Leukämie seine Wirkung ausübt. Weitere Untersuchungen schliessen sich noch an, die die Rolle von MIF für die Funktion von nurse-like cells bei der CLL und anderen Tumorentitäten sowie den Einfluss von MIF auf den Akt-Pathway zum Fokus haben.

2 Abstract

Besides the genetic aberrations the interaction between the tumor cell and its microenvironment plays an important role in the leukemogenesis of chronic lymphocytic leukemia (CLL). Stimulation via extracellular factors is important for the survival of the CLL cell, since without this the CLL cell is driven into apoptosis when taken out of its micromilieu.

Preliminary analyses showed that Macrophage Migration Inhibitory Factor (MIF), a chemokine with pro-inflammatory properties, is overexpressed in CLL. As MIF exerts regulatory control on cellular growth, -differentiation and apoptosis, this work tests the hypothesis whether MIF has a contribution in the pathomechanism of CLL development.

To this end, we used the E μ -T-cell leukemia 1 (TCL1) mouse model (Bichi et al., 2002) in which B-cell specific overexpression of TCL1 leads to accumulation and organ infiltration by CD5⁺IgM⁺ mature B cells. The transgenic mice develop a CLL-like disease, which is manifested by leukemia, lymphadenopathy, hepatosplenomegaly, bone marrow infiltration as well as premature death after 8 to 15 months. The impact of MIF is tested *in vivo* by breeding the TCL1^{+/+} mouse to the MIF^{-/-} mouse (Fingerle-Rowson et al., 2003).

In this work, TCL1^{+wt} MIF^{+/+} and TCL1^{+wt} MIF^{-/-} mice were compared with each other regarding the rate of leukemia development, tumor burden, hematopathological alterations, overall survival, cytogenetic changes as well as proliferative capacity and apoptosis.

The known MIF cell surface receptors CD74/CD44 and CXCR2 are also expressed on the murine B cells. TCL1^{+wt} MIF^{+/+} mice show already in the pre-leukemic phase a higher number of CD5⁺IgM⁺ cells, and the development of a peripheral leukemia starts about three months earlier in TCL1^{+wt} MIF^{+/+} mice compared to TCL1^{+wt} MIF^{-/-} animals ($p = 0.02$). At the age of 12 months, organs like liver and spleen are infiltrated to a higher degree by small, mature and malignant B lymphocytes in the TCL1^{+wt} MIF^{+/+} than in the MIF-deficient mice. One reason for this could be the increased accumulation of CD68⁺ macrophages in the leukemia infiltrated spleens and bone marrow of the MIF wildtype mice ($p < 0.0001$). “nurse-like” cells (NLCs) act to protect the leukemic cells from apoptosis.

This fits the observation that the rate of spontaneous apoptosis is elevated in the MIF-deficient leukemia infiltrated spleens *in vivo*, and that a trend towards higher rates of apoptosis can be seen in splenocytes of the TCL1^{+wt} MIF^{-/-} mice *ex vivo*. These MIF-dependent effects even affect overall survival since in the MIF^{-/-} mice, the overall median

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survival is prolonged by 80 days (TCL1^{+wt} MIF^{+/+} 380 days vs. TCL1^{+wt} MIF^{-/-} 460 days, $p = 0.04$). An analysis of genomic aberrations by spectral karyotyping (SKY) of metaphases from leukemic splenocytes shows distinct genetic damages like trisomy, tetraploidy, translocations and deletions in both genotypes. An overexpression of the tumor suppressor p53 presumably due to an inactivating gene mutation was found in TCL1^{+wt} MIF^{+/+} mice at a higher frequency than in TCL1^{+wt} MIF^{-/-} mice.

Our analysis of the E μ -TCL1 mouse model shows that this is a valuable model for the validation of potential target molecules in the pathogenesis of CLL. We are able to demonstrate that MIF acts as a tumor promoter primarily by influencing the micromilieu in the early as well as in the late phase of leukemkia development. Further analyses are on the way which focus on the role of MIF in the function of the nurse-like cells in CLL and other tumor entities and which test the interaction of MIF and Akt.

3 Introduction

3.1 Macrophage Migration Inhibitory Factor (MIF)

Macrophage migration inhibitory factor (MIF) was first described by the scientists Barry Bloom and John David in 1966 when they both independently from each other isolated a protein from cultures of antigen-sensitized lymphocytes that could act at a distance to inhibit the random movement or migration of phagocytes (Bloom and Bennett 1966; David 1966). It was one of the first soluble, non-immunoglobulin cytokines which could be analysed *in vitro*. This molecule was named macrophage migration inhibitory factor (MIF) and its activity was reported over the next 20 years to alter phagocytosis, macrophage adherence, spreading and to increase phagocyte tumoricidal potential (Nathan et al., 1971; Nathan et al., 1973; Churchill et al., 1975).

In 1989, human MIF was cloned and a molecular analysis of the biological, biochemical and biophysical properties was approached (Weiser et al., 1989).

3.1.1 Expression pattern of MIF

MIF protein and MIF mRNA have been detected in a wide variety of tissues and cell types. Since there is no cell line or tissue known to be negative for MIF, one can assume that MIF is an ubiquitously expressed protein. It seems to be expressed at baseline levels in nearly all tissues; however, it can also be induced by a variety of stimuli depending on the cellular context.

Expression of MIF can already be detected at the beginning of life. Kobayashi et al. studied the expression of mRNA during embryogenesis of mice and showed co-expression of MIF in organogenesis parallel to tissue specification. MIF mRNA was detected in somites, precartilaginous primordia in ribs and vertebrae, branchial arches, limb buds, neural tissues, all muscle cell types and during organogenesis of liver, kidney, testis, skin, intestine, adrenal gland and pancreas (Suzuki et al., 1996; Kobayashi et al., 1999). This broad expression pattern of MIF in embryogenesis continues during adult life. MIF protein can be detected constitutively in serum and plasma. This soluble MIF could arise from a great variety of tissues in which MIF is synthesised. Historically, MIF was considered to be a product of activated T-lymphocytes (David 1966), but immunohistochemical analysis of various tissues indicates a broad expression pattern of MIF (Bacher et al., 1997). MIF is expressed in cells of the immune system such as monocytes/macrophages (Calandra et al.,

1994), T- and B-lymphocytes (Bacher et al., 1996; Takahashi et al., 1999), NK-cells (Apte et al., 1998), basophiles/mast cells (Chen et al., 1998) and eosinophils (Rossi et al., 1998). Endocrine organs such as the anterior pituitary gland (Bernhagen et al., 1993), the adrenal cortex, the Leydig-cells of the testis, the epithelial cells of the epididymis and pancreatic β -cells (Waeber et al., 1997) have been reported to be a source of MIF production (Bacher et al., 1997). Other MIF synthesizing cells are smooth and skeletal muscle cells (Benigni et al., 2000), gastric parietal cells (Kudo 1998), keratinocytes and fibroblasts (Abe et al., 2000), hepatocytes and peripheral and central neurons (Bacher et al., 1998). Even erythrocytes have been reported to contain MIF (Mizue et al., 2000), although these data could be confounded by leukocyte contamination or membrane-bound MIF.

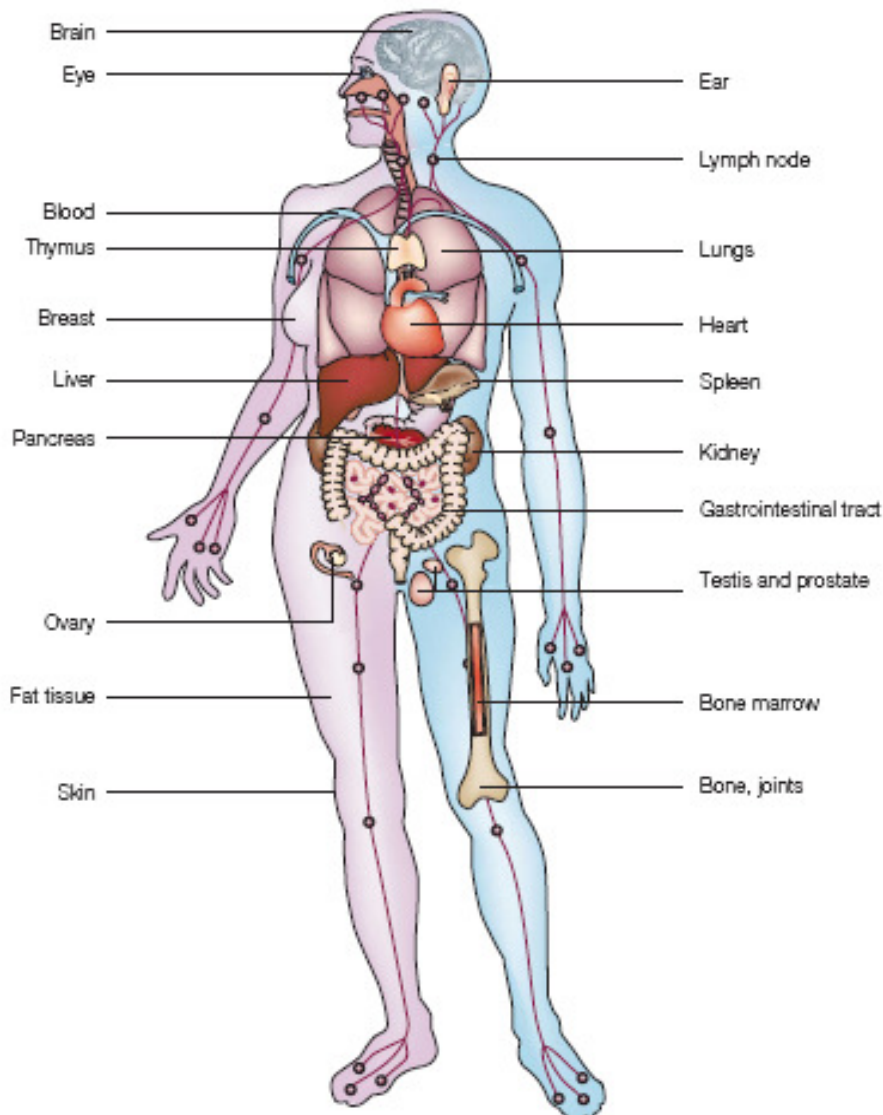


Figure 1: Expression pattern of MIF in humans. MIF is expressed in brain, eye, ear, in the immune system (blood, lymphnode, thymus, spleen, bone marrow), in endothelial cells and epithelial cells, in the skin and in bone and joints (modified from Calandra and Roger 2003).

3.1.2 Organization of the MIF gene locus

The gene for murine MIF is located on chromosome 10 (Bozza et al., 1995; Mitchell et al., 1995) and the human gene maps to chromosome 22q11.2 (Budarf et al., 1997). Both genes are very small (approximately 1.7 kb in size) and show a similar intron-exon organization. The gene consists of three exons of 107, 162 and 66 basepairs and two introns of 188 and 94 basepairs. The sequence-identity within MIF RNA transcripts between mouse and human is very high (70.4%, 86.4% and 67.5% for exons 1, 2, and 3). Consensus sequences which indicate the presence of transcription factor binding sites, have been found in the murine as well as in the human MIF promoter (Mitchell et al., 1995). The promoter region contains enhancer and regulatory binding domains which are implicated to respond to the proto-oncogene *c-fos* (Paralkar and Wistow 1994; Maleszka et al., 1998). Other domains are a SP-1 sequence, a cAMP responsive element (CREB) and an AP-1 sequence. Further upstream there are a cytokine-1 (CK-1) site and a nuclear factor- κ B site (NF- κ B) (Fig. 2).

Two polymorphisms of the MIF gene have been found and are associated with inflammatory diseases: a G to C transition at position -173 is associated to systemic-onset juvenile arthritis (Donn et al., 2004) and a CATT-tetranucleotide repeat of five to eight times at position -794 is linked to rheumatoid arthritis (Baugh et al., 2002; Martinez et al., 2007) (Fig. 2).

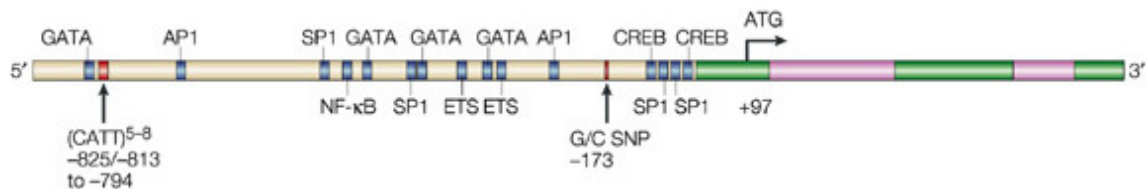


Figure 2: Structure of the MIF gene. It is composed of three short exons (green) and two introns (pink).

3.1.3 Structure of the MIF protein

Human and mouse MIF cDNA were cloned from the Jurkat T cell line (Metz and Bucala 1997), the brain (Paralkar and Wistow 1994), the AtT-20 pituitary cell line (Bernhagen et al., 1994) and the recombinant proteins were expressed. In both species the primary protein has a length of 115 amino acids and a molecular mass of 12.5 kDa. The initiating

methionine is post-translationally removed and the mature protein starts with a proline in position 1.

Determination of the three-dimensional structure of mouse and human MIF was done by X-ray crystallography (Kato et al., 1996; Sugimoto et al., 1996; Sun et al., 1996) and by NMR (nuclear magnet resonance) -spectroscopy (Muhlhahn et al., 1996) and led to the discovery of the protein fold. All X-ray studies showed that MIF is a trimer of identical subunits with the overall dimensions of 35 x 50 x 50 Å. The MIF monomer contains two antiparallel α -helices and six β -strands, four of which form a mixed β -sheet. Three monomers assemble to an α/β structure consisting of six α -helices surrounding three β -sheets that form a barrel with a solvent accessible channel. This orientation is unusual for an eukaryotic protein. Stabilization of the trimer is guaranteed by several hydrogen bonds between the β -sheets and between one of the α -helices and the carboxyl-terminus (C-terminus). One leucine-rich hydrophobic region is also stabilized by hydrophobic bonds.

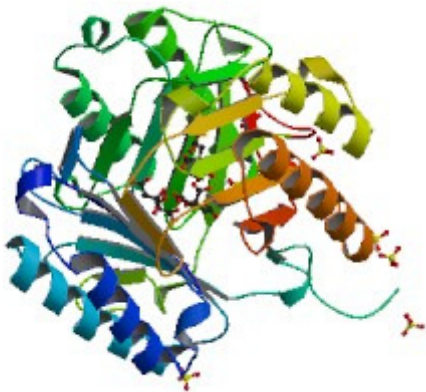


Figure 3: The active form of MIF as a homotrimer.

Looking at the three-dimensional architecture of MIF and other enzymes, a similarity between bacterial tautomerase and human D-dopachrome tautomerase (DDT) can be found. In the genome of mice and men, the gene for DDT lies only at 80 kb distance from MIF and we know that the MIF/DDT locus had evolved as a duplication event during evolution. DDT shares amino acid sequence homology with MIF to about 30%, whereas the crystal structure, its overall topology and trimeric formation is almost identical to MIF (Sugimoto et al., 1999). The protein GIF (glycosylation inhibition factor) which was investigated in the 1990s by Japanese scientists was found to be identical to MIF since its

sequence and structure are identical to MIF (Galat et al., 1994; Nishibori et al., 1996; Watarai et al., 2000).

3.1.4 Extracellular and intracellular MIF

Until today two different ways of signaling have been described for MIF; an extracellular and an intracellular signaling pathway.

The first receptor found to bind extracellular MIF was CD74 (Leng et al. 2003). CD74 is a nonpolymorphic type II integral membrane protein, which was initially considered to function as an MHC class II chaperone only (Stumptner-Cuvelette and Benaroch 2002). With the discovery of MIF as a ligand for CD74, its additional role as an accessory signaling molecule started to become clearer. In macrophages, MIF binds with a high affinity to the extracellular domain of CD74, activating the mitogen activated protein kinase (MAPK) pathway and cell proliferation (Leng et al. 2003). CD74 involves other receptors for signal transduction and another trans-membrane co-receptor, the adhesion molecule CD44, appears to be required for the phosphorylation of the ERK1/2 kinases by MIF (Shi et al., 2006). The activation of both receptors is also required for MIF-mediated protection of the cell from apoptosis. In B lymphocytes, MIF initiates a signaling cascade activating NF- κ B via the Src kinase Syk and Akt. This activation is mediated by translocation of the CD74-intracellular domain which gets released by intramembrane regulated proteolysis (RIP) upon MIF stimulation (Gore et al., 2008)

MIF was also identified to be a non-cognate ligand for the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). By interaction with those two receptors, MIF is able to promote the recruitment of monocytes as well as T cells into inflamed tissue. It is also known that CXCR2 co-localizes with CD74 which suggest that MIF may signal via a combined CD74/CD44/CXCR2 complex.

MIF is also abundantly expressed in the cytosol and intracellular MIF involves another pathway by direct binding and inhibitory interaction with Jab-1/CSN5 (Jab = c-jun activating binding protein). One known function of Jab-1/CSN5 is the coactivation of AP-1 transcription (Bernhagen et al., 1993; Rosengren et al., 1996; Bernhagen et al., 1998; Kleemann et al., 1998). Jab-1/CSN5 is also the subunit 5 of the COP9 signalosome (CSN) which is located in the nucleus and the cytoplasm (Kleemann et al., 2000; Bech-Otschir et al., 2001; Chamovitz and Segal 2001). The CSN consists of eight subunits which are highly similar to the subunits of the 26S proteasome (Glickman et al., 1998; Henke et al.,

1999), which is the major proteolytic system of the cell (Hershko and Ciechanover 1998). Jab-1 specifically binds to p27^{Kip1}, a cyclin-dependent kinase inhibitor (CDKI), and promotes its degradation via the proteasome (Tomoda et al., 1999). Inhibition of Jab-1 by MIF antagonizes Jab-1-dependent regulation of p27^{Kip1} and stabilizes this CDKI (Kleemann et al. 2000). Other known targets of Jab-1/CSN5 are the tumor suppressor p53 or hypoxia-inducing factor-1 α (HIF-1 α) (Bech-Otschir et al., 2001; Bae et al., 2002).

3.1.5 MIF and tumorigenesis

In many different tumor entities, MIF is described to be correlated with the cancer prognosis. Specifically, this correlation was done for hepatocellular carcinomas, colon cancers and prostate cancers (Meyer-Siegler et al., 2002; Legendre et al., 2003; Hira et al., 2005). Interestingly, MIF seems to affect the two ways of the adaptive immune system, namely the Th1 and the Th2 routes. The first route activates macrophages and neutrophils by secretion of IL-2, IL-12m IFN γ and TNF α . The Th2 route, which acts as a counterpart to Th1 activates cytokines including IL-4, IL-5, IL-13 and IL-10. MIF affects these pathways in differently, on the one hand by sustaining the macrophage viability and thereby leading to a sustained inflammatory reaction (Mitchell et al., 2002) and on the other hand MIF activates the ERK-MAPK pathway and increases thereby cell proliferation (Mitchell et al., 2002). MIF is also capable of inducing angiogenesis. In a recent study it was shown that MIF enhances the differentiation of endothelial cells to blood vessels (Chesney et al., 1999; Amin et al., 2003), and there is evidence that MIF is able to modulate VEGF functioning.

Another mechanism for MIF acting as a tumor promoter could be the inhibition of p53-dependent apoptosis. The interaction of MIF with p53 was first shown by Hudson et al. in 1999 (Hudson et al., 1999). They demonstrated the abolishment of p53 activity by treating the cells with MIF: Investigations on the MIF knockout mouse model (Fingerle-Rowson et al., 2003) showed p53-dependent growth alterations in fibroblasts.

Evidence of MIF being involved in cell cycle regulation showed an involvement via the SCF complex. MIF co-regulates the activity of the skip-cullin-F-box protein complex (SCF complex) by inhibition of Jab-1 and might affect proteasomal control of intracellular protein degradation (Fingerle-Rowson and Petrenko 2007; Nemajerova et al., 2007).

3.1.6 MIF and p53

Regulation of cell proliferation is necessary for normal cell development and prevention of malignant transformation. DNA damage, due to oxidative stress, ionizing or ultraviolet irradiation or mutagenic substances enhances the rate of tumor development (Rich et al., 2000). To ensure efficient DNA repair in the cell before mitosis, the cell has developed p53-dependent and p53-independent checkpoint pathways which delay cell cycle progression if the genome is not intact. The p53 tumor-suppressor has at least two roles in preventing proliferation of the cell: induction of cell cycle arrest and induction of apoptosis (Attardi et al., 1996).

In earlier studies, MIF has been implicated to play a role in the regulation of cell growth and apoptosis (Wistow et al., 1993; Kobayashi et al., 1999). The expression levels of MIF alter during growth arrest (Fingerle-Rowson et al., 2003) and can be correlated with tumor grading and clinical prognosis in several human cancers (Meyer-Siegler and Hudson 1996; del Vecchio et al., 2000; Meyer-Siegler 2000; Markert et al., 2001; Tomiyasu et al., 2002). In 1999, MIF was found as a functional inhibitor of p53-dependent transcription in several cell-based genetic screens (Hudson et al., 1999). This proposed interaction was subsequently confirmed by *in vivo* studies with MIF knockout mice which showed that genetic deletion of MIF enhances p53-dependent activation of the p53 target p21^{CIP} and inhibited malignant transformation *in vitro* as well as tumor formation *in vivo* (Fingerle-Rowson et al., 2003).

The proinflammatory action of MIF together with its inhibitory role in the p53 pathway has led to the concept that MIF may act as a molecular link between inflammation and tumorigenesis. MIF at sites of chronic inflammation may impair the normal response to genetic damage, enhances cell proliferation and promotes the accumulation of oncogenic mutations (Cordon-Cardo and Prives 1999).

3.2 Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is a malignant disease of B cells which is characterized by the accumulation of CD5⁺ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (Harris et al., 1994; Rozman and Montserrat 1995; Chiorazzi et al., 2005; Chiorazzi et al., 2005)

CLL is the most frequent leukemia in the western world, representing about 25 to 30 % of all leukemias. It is more common in men than in females, with a sex ratio of 1.5 – 2 : 1

(Rozman and Montserrat 1995). The occurrence of CLL is not due to any known environmental factors such as ionizing radiation or chemical compounds. Most cases appear sporadically and only 5 % of CLL are known to be familial (Houlston et al., 2003).

3.2.1 Biology and Pathogenesis of CLL

The morphological analysis of CLL blood smears shows an accumulation of small mature lymphocytes. The membrane of these lymphocytes is more fragile than normally and this leads to the typical picture of smudge cells in peripheral blood smears (Matutes and Polliack 2000).

Immunophenotype analysis is necessary to establish the diagnosis of CLL. Its phenotype is very distinctive with leukemic B cells expressing mature B cell markers as CD19, low levels of CD20 and high levels of CD5 together with the expression of CD23 which helps to distinguish it from other CD5⁺ malignancies. CLL lymphocytes typically express low levels of surface immunoglobulin, most frequently IgM (Matutes et al., 1994; Moreau et al., 1997).

Most of the CLL cells are arrested in the G0 phase of the cell cycle (O'Brien et al., 1995; Korsmeyer 1999). Only little is known about the control mechanism, but a recent work on lymphocytes suggests that the G0 state represents an active program that requires signals from the outside of the cells (Takeda et al., 1996; Lam et al., 1997; Rooke et al., 1997) phenomenon of the resting B cells needs further investigations for the analysis of the CLL pathogenesis.

CLL cells carry different genomic aberrations which are seen by conventional cytogenetics and fluorescence in situ-hybridization (FISH). Abnormalities can be found in up to 82 % of patients with CLL. In 18 %, no genetic abnormalities are found, but still these cases may carry submicroscopic aberrations (Dohner et al., 2000). The most frequent chromosomal abnormalities are listed in the table 1.

Chromosome	Aberration	Frequency	Genes affected	Prognosis
13	del 13q14	55%	miR15, miR16	good
11	del 11q22-23	18%	ATM,	poor
17	del 17p13	7%	p53	poor
12	+12	16%	CDK2, CDK4, MDM-2	poor
6	del 6q	5 %	unknown	poor
14	t(14;19)(q32;q13)	10 %	unknown	

Table 1: Prognostic values of the different chromosomal aberrations.

Some of those abnormalities can occur alone or in combination, e.g. structural changes on chromosomes 11, 13 and 14 may occur together with deletions and translocations on chromosome 13q14 (Juliussen and Gahrton 1990; Juliussen et al., 1990; Dohner et al., 1999). Using conventional cytogenetic analysis, studies identified trisomy 12, 11q-deletion (11q-) and 17p-deletion (17p-) as markers of poor prognosis, whereas other patients with normal karyotype or 13q-deletion (13q-) had a better survival (Crossen 1997; Garcia-Marco et al., 1997; Dohner et al., 1999).

CLL cells express the B cell receptor (BCR) on the cell surface, as mature B lymphocytes do. The BCR is encoded by the immunoglobulin (Ig) genes which undergo somatic hypermutation during B cell differentiation in the germinal follicle in order to increase BCR affinity for the antigens. Sequencing of the variable genes of the heavy chain locus (IgV_H) allows therefore telling whether a B cell has undergone somatic hypermutation. The mutated form of the B-CLL carries in those cases a good prognostic value for the disease while the unmutated form is associated with progressive disease (Damle et al., 1999; Hamblin et al., 1999).

3.2.2 Staging

Two staging systems are currently used to define disease stage, prognosis and especially whether and when to start treatment, the Rai staging and the Binet staging system. In Europe, the Binet staging system is used which is based on the number of lymphoid sites involved and hematopoietic parameters (hemoglobin (Hgb) and platelet (Plts)) (Binet et al., 1981):

- (1) *Stage A*: includes patients with Hgb > 10 g/dL, Plts > 100.000 mm⁻³ and up to two lymphatic sites involved
- (2) *Stage B*: includes patients with Hgb > 10 g/dL, Plts > 100.000 mm⁻³ and more than two lymphatic sites involved
- (3) *Stage C*: includes patients with compromised bone marrow function (Hgb < 10 g/dL and/or Plts < 100.000 mm⁻³) regardless the number of lymphatic sites involved.

3.2.3 Prognosis

From a clinicians perspective, CLL is known to be a very heterogenous disease. The survival of the patients after the first diagnosis ranges from months to decades, with a median of 7.5 years (Dighiero and Binet 2000). Therefore, when the diagnosis is CLL, it is important to determine the patient's individual risk for progression.

There are three traditional prognostic factors which are easily determined: The clinical stage according to Binet or Rai in which low stage patients have a better prognosis (Cheson et al., 1996). The lymphocyte doubling time indicates disease progression if it is < 6 months (Montillo et al., 2005). The third parameter is bone marrow infiltration which indicates a poorer prognosis with diffuse infiltration than with nodular infiltration (Molica et al., 1990; Mauro et al., 1994; Montserrat et al., 1996).

On the other hand there are biological prognostic factors which are indicators for the extension of the malignant clone (Melo et al., 1986; Melo et al., 1986; Melo et al., 1986). If the percentage of prolymphocytes in the peripheral blood exceeds 10 %, it implies a worse prognosis for the patient. On the other hand, serum thymidine kinase (TK) levels, a cellular enzyme involved in a salvage pathway for DNA synthesis, correlates with survival (Hallek et al., 1999; Dickinson et al., 2006). Another serum marker is the β 2-microtubulin (β 2M)-level which inversely correlates with the response to chemotherapy and overall survival (Keating 1999). Soluble CD23 (sCD23) is a powerful predictor of disease activity and progression (Reinisch et al., 1994; Sarfati et al., 1996; Molica et al., 1999; Saka et al., 2006). sCD23 level can be used as predictor for clinical outcome, particularly in early stages (Molica et al., 1996; Schwarzmeier et al., 2002). p53, a transcription factor, known to act as tumor suppressor and regulator of the cell cycle, is another prognostic marker of CLL. Its inactivation (17p-) correlates with the transformation of CLL to an aggressive lymphoma, the so-called Richter's transformation (Gaidano et al., 1991).

At the end of the last millennium, new biological markers have been identified, that allow a more precise risk-stratification of CLL patients already at the moment of the initial diagnosis.

IgV_H mutation is one of those new prognostic markers. Unmutated CLL cases (meaning ≥ 98 % similarity to the corresponding germline sequence) are associated with an aggressive disease and a shorter median survival time (~ 8 years vs. 24 years) as compared to mutated cases (Damle et al., 1999; Hamblin et al., 1999). CD38 expression on CLL cells was first considered to be a surrogate marker for the mutational status, but studies

confirmed CD38 to have an independent prognostic value (Damle et al., 1999). ZAP-70 was initially considered to be expressed by T and natural killer (NK) cells, but it was also found to correlate with the clinical prognosis. An expansion of the ZAP70-positive cells to more than 20 % of the leukemic cells carries a negative prognosis (Crespo et al., 2003; Wiestner et al., 2003).

Chromosomal abnormalities are the most important prognostic parameters (Dohner et al., 1999). Single chromosomal aberrations are quite characteristic for CLL, but they also occur in combination especially during the clonal evolution of the disease (see also table 1). The most frequent aberration is the deletion 13q- which implies the best prognosis for the CLL patients. Deletions of chromosomes 11q and 17p confer much shortened median survival. Some of these abnormalities may already be present at the time of diagnosis, others (e.g. 17p and 11q) may occur during the progression of the disease and especially after chemotherapy.

3.2.4 Current therapy and treatment

Today there are different treatment options ranging from single-agent treatment over combinatorial chemotherapy to the use of monoclonal antibodies in combination with chemotherapy (immunochemotherapy). Until today, unfortunately these therapies do not cure CLL, only the use of hematopoietic stem cell transplantation can result in complete and durable remission of the leukemia.

Prior to the year 2000, single-agent treatment was based on alkylating reagents such as chlorambucil, alone or together with corticosteroids. Most of the patients eventually relapsed, and the complete remission (CR) rate was below 10 %. The use of a combinatorial chemotherapy (e.g. CHOP (cyclophosphamide, doxorubicine, vincristine, originally known as Oncovine and prednisolone) regimen) resulted in higher response rates, but toxicity also increased and it remained unclear whether patients had a benefit in terms of overall survival.

The advent of purine analogs, such as fludarabine, brought a clear improvement in therapy, reaching at least a CR rate of approximately 15 % with a significant improvement of progression-free survival (Rai et al., 2000). Combination of fludarabine with cyclophosphamide, the FC regimen (Eichhorst et al., 2006) and recently with the anti-CD20 antibody Rituximab, the FCR regimen (Hallek et al. 2008, ASH abstract) achieved an impressive increase of CR rates to almost 60 %. Alemtuzumab, a humanized antibody

against CD52, which is expressed on all human lymphocytes, monocytes and macrophages, is another antibody successfully used for the treatment of refractory CLL patients.

The concept of high-dose chemotherapy supported by autologous stem cell transplantation has produced also high remission rates, but given the increased toxicity and risk, the benefit in terms of progression-free survival was too little and the long-term side effects (secondary cancers, compromise of bone marrow function) too frequent to support this concept any longer, especially since no definitive cure can be reached (Pavletic et al., 1998; Sutton et al., 1998).

Therefore, allogenic hematopoietic stem cell transplantation is the only therapy offering a potential cure of the disease (Michallet et al., 1996; Rondon et al., 1996; Doney et al., 2002; Moreno et al., 2005). But this comes at a substantial risk of mortality from infection and relapse.

3.3 T cell leukemia-1 (TCL1)

The T cell leukemia-1 family of oncoproteins was first discovered because of its involvement in characteristic chromosomal rearrangements in mature T-cell tumors. Those translocations and inversions juxtaposed in the coding regions at 14q32 or Xq28. They interact with the T cell receptor (TCR) at 14q11 or 7q35. There are three human members of the family, mature T-cell proliferation 1 (MTCP1), TCL1 (also called TCL1A) and TCL1B (also known as TML1) (Stern et al., 1993; Virgilio et al., 1994; Pekarsky et al., 1999; Sugimoto et al., 1999). In the mouse *Mtcp1* and *Tcl1* were identified by homology to human TCL1 (Madani et al., 1996; Narducci et al., 1997) and five additional mouse genes (*Tcl1b1*, *Tcl1b2*, *Tcl1b3*, *Tcl1b4* and *Tcl1b5*) were cloned by their homology to the human TCL1B (Hallas et al., 1999).

3.3.1 Expression pattern of TCL1

TCL1-family genes in mice are mainly expressed during early embryogenesis, in fetal and adult tissues and precursor lymphocytes. *Tcl1* is also expressed during B-cell development from the pro-B cell through germinal center B cells and also in T-cell development, mainly in CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes (Narducci et al., 1997; Kang et al., 2005).

During human embryogenesis, the expression of TCL1 family genes has not been evaluated. It is known that TCL1A expression begins in pre-B cells, is downregulated in germinal center B cells and silenced in memory B and plasma cells (Virgilio et al., 1994; Teitell et al., 1999; Muschen et al., 2002). In T-cell development, TCL1A is only expressed in early T cells before the appearance of the surface T cell receptor (Narducci et al., 1997; Hoyer et al., 2005). Other, non-lymphatic tissues do not express TCL1, with the exception of ovaries (Virgilio et al., 1994).

3.3.2 Organization of the TCL1 gene locus

The TCL1 family of genes span between 4 and 8 exons and transcribe mRNAs which have a length of 1.2 to 2.0 kb. They are located on chromosome 14q32 and on Xq28 in humans. In mice, the *Tcl1* genes are located on chromosome 12 and the X chromosome.

It is known that rearrangements of the TCL1-gene locus are associated with T-cell leukemias of the mature phenotype, e.g. T-cell prolymphocytic leukemia (T-PLL) (Taylor et al., 1996), adult T-cell leukemia (ATL) (Narducci et al., 1997) and with T-cell leukemias that develop in patients with the Ataxia telangiectasia syndrome (AT) (Brito-Babapulle and Catovsky 1991). In most cases, the TCL1 locus on chromosome 14q32.1 rearranges with the T-cell receptor (TCR) α/δ locus on chromosome 14q11 (Russo et al., 1989; Virgilio et al., 1993). These rearrangements are either reciprocal translocations or inversions. By chromosomal aberrations, the TCL1 oncogene is placed under aberrant enhancer/promoter stimulation which leads to its overexpression.

Introduction of the human TCL1 cDNA under the control of the *lck* promoter leads to transgenic mice developing premalignant T-cell proliferations (Virgilio et al., 1998). Pekarsky et al. could show that TCL1 is a real oncogene and that the deregulation of TCL1 initiates the process of malignant transformation (Pekarsky et al., 2001).

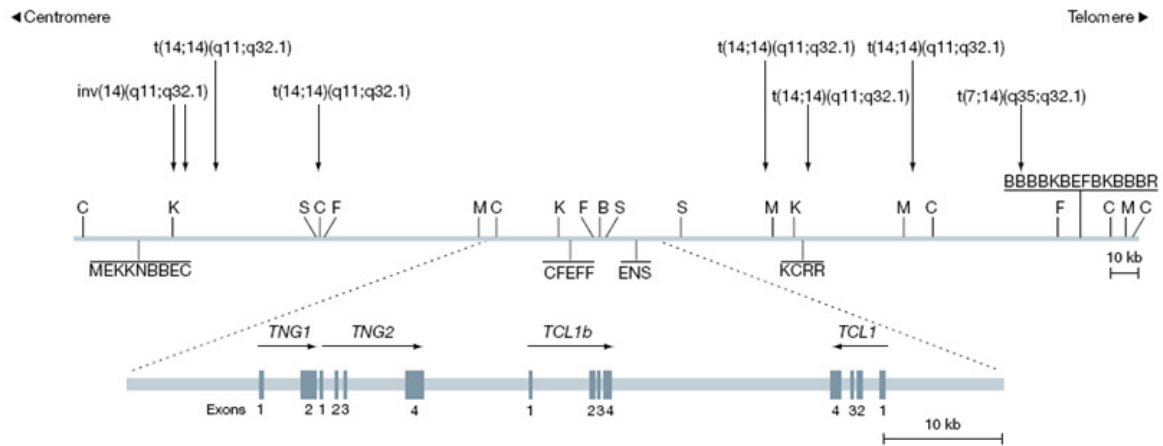


Figure 4: Genomic organization of the human TCL1 gene locus (modified from(Pekarsky et al., 2001).

3.3.3 Structure of the TCL1 protein

The TCL1 family proteins range in size from 13 to 15 kDa with a moderate sequence conservation between the family members (Fu et al., 1994). The proteins are non-enzymatic and their structures, expression patterns and subcellular locations dictate their physiological and tumorigenic properties. Murine Tcl1 and human TCL1A show a tightly packed internal hydrophobic core and share a novel β -barrel structure which includes two four-stranded β -chain sheets connected by a long looping strand (Fu et al., 1998; Hoh et al., 1998; Petock et al., 2001). Purification studies of human TCL1 provide evidence for dimerization (Hoh et al. 1998, structure).

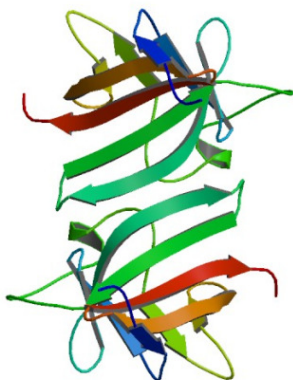


Figure 5: The TCL1 oncoprotein as a homodimer.

3.3.4 Functional aspects of TCL1

TCL1 interacts with the N-terminal pleckstrin homology (PH) domain of the serine/threonine kinase Akt (Laine et al., 2000; Pekarsky et al., 2000). Like TCL1, Akt belongs to a multiprotein family that includes Akt1, Akt2 and Akt3 (also called PKB α , PKB β and PKB γ) (Bellacosa et al., 1991; Datta et al., 1999; Brazil and Hemmings 2001). Akt activation is initiated by the binding of an appropriate growth- or survival-stimulating ligand to the cell surface receptor, e.g. epidermal growth factor (EGF) to EGF receptor or stimulation of the BCR by soluble antigens. The bound cell surface receptor recruits a receptor tyrosine kinase which triggers phosphatidylinositol 3-kinase (PI3K) activation at the membrane which produces the Akt docking phospholipids. Akt is recruited to the inner leaflet of the cytoplasmic membrane, facilitating the assembly of a signaling complex. Akt needs its PH domain for membrane relocalization and direct binding to the docking phospholipids and activation by phosphorylation. (Burgering and Coffey 1995; Delcommenne et al., 1998; Chan et al., 1999; Auguin et al., 2004; Bayascas and Alessi 2005). Studies showed that Akt is able to cause T-cell lymphoma in mice (Staal et al., 1977).

In yeast-two hybrid screens Laine et al. found that all TCL1 isoforms bind to the PH domain of Akt. Substrate phosphorylation and the kinase activity of Akt are enhanced by the binding of TCL1 which induces conformational changes of Akt within an oligomeric TCL1-Akt protein complex (Laine et al., 2000). Akt plays a central role in cell survival and proliferation. As TCL1 is located in the cytoplasm as well as in the nucleus (Pekarsky et al., 2000) it can promote the nuclear transition of Akt (Pekarsky et al., 2001) which is normally located in the cytoplasm (Chan et al., 1999).

3.3.5 TCL1 in Chronic Lymphocytic Leukemia

As it is known that TCL1 is highly expressed in most B-cell tumors, the TCL1 levels in CLL were analysed by immunohistochemical stainings, western blotting and flow cytometry (Herling et al., 2006). TCL1 is not uniformly expressed, but appears to be only upregulated in a subset of CLL patients. Herling et al. found a correlation between high TCL1 levels and the subset of CLL which is characterized by an unmutated V_H gene status as well as high ZAP-70 expression. Low levels of TCL1 were found in actively proliferating centers of the tumor components (Herling et al., 2006).

3.3.6 The TCL1 mouse model

Research in CLL was limited by the lack of a suitable mouse model for CLL until 2002 when an American group overexpressed human TCL1 in B cells of mice and found that these mice developed a lymphoproliferative disease very reminiscent of CLL. The TCL1 mouse model was further investigated by several independent groups and found to be a valuable model for the analysis of CLL pathogenesis.

In these mice, a 350 bp fragment of the human TCL1 coding region is expressed under the control of a mouse IgV_H promoter and the E μ enhancer whose activity specially targets immature and mature B cells. The animals were bred on a B6C3 background (a cross between a female C57Bl/6NCrl mouse and a male C3H/HeNCrl mouse).

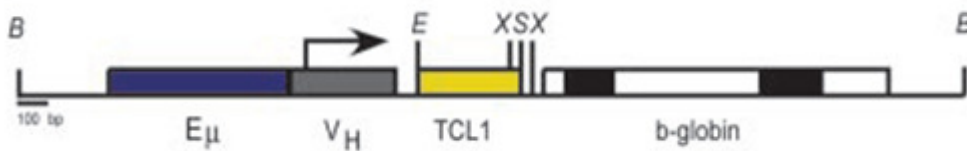


Figure 6: Transgenic construct used to generate the TCL1 transgenic mouse. The human TCL1 gene is expressed under the control of the B cell specific E μ -enhancer and the IgV_H promoter.

These animals spontaneously develop B cell hyperplasia, initially in the peritoneal cavity and then in lymph nodes, spleen, bone marrow and blood, resulting in a massive B cell clonal expansion (Bichi et al., 2002). Accumulation of CD5-positive B cells leads to enlarged spleens, livers and lymph nodes along with high blood lymphocyte counts.

Analysis of the IgV_L and IgV_H genes show no significant somatic hypermutation, indicating that the molecular features of the B cell receptor of the transgenic mice resemble those from human CLL patients with the unmutated and more aggressive form of CLL (Yan et al., 2006). The model has also been successfully used for the analysis of new therapeutic drugs for the treatment of human CLL (Johnson et al., 2006).

3.4 Aim of the Work

MIF is a potential molecular link between inflammation and tumorigenesis. In the pathogenesis of CLL, the microenvironment plays a prominent role since the disease would not exist without continuous protection of the CLL cells by pro-survival signals from the micromilieu. In this work we would like to analyse the role of MIF in the pathogenesis of CLL.

Introduction

To understand the relevance of MIF for the pathogenesis of CLL, we used the above described E μ -TCL1 mouse model for human CLL (Bichi et al., 2002). We have crossed the E μ -TCL-1-transgenic mouse model with MIF knockout (MIF^{-/-}) mice. TCL1^{+wt} MIF^{-/-} and TCL1^{+wt} MIF^{+/+} mice were compared with respect to leukemia development, tumor burden, cytogenetic aberrations and survival. Further studies should reveal the mechanism by which MIF affects CLL pathogenesis.

4 Results

4.1 Macrophage Migration Inhibitory Factor (MIF) is overexpressed in malignant B cells and human chronic lymphocytic leukemia (CLL)

Macrophage Migration Inhibitory Factor (MIF) is a known mediator in inflammatory diseases. It is known to have an influence in the innate immune system (Denkinger et al., 2004). In infections, like e.g. sepsis or malaria, MIF is rapidly released by the immune cells and has autocrine and paracrine effects promoting cell growth and survival (Calandra and Roger 2003). In contrast to MIF acting as a cytokine, MIF secretion is also induced due to glucocorticoid hormones (Calandra and Bucala 1995). MIF's role in the adaptive immune system is still under investigation, though it is known that MIF is released by activated lymphocytes. Possibly acting through an autocrine loop, MIF is known to activate T lymphocytes and to support their proliferation (Bacher et al., 1996).

Research into the biological mechanisms of MIF action have resulted in several possibilities how MIF could influence and regulate cellular growth, differentiation, malignant transformation or apoptosis ((Takahashi et al., 1998; Chesney et al., 1999; Yang et al., 2000; Mitchell et al., 2002; White et al., 2003). In different cancer entities like metastatic prostate cancer or breast carcinoma, MIF expression is upregulated. Different studies described its involvement in the development of lymphomas as well as tumor-associated angiogenesis (Chesney et al., 1999; Nishihira 2000). As inflammation is a critical risk factor for tumor development (Karin et al., 2006), MIF is potentially one link between chronic inflammation and tumor formation or tumor progression (Hardman et al., 2005; Bucala and Donnelly 2007). The MIF expression levels seem to correlate with the tumor aggressiveness (here tested in human melanoma) and also with its metastatic potential (Shimizu et al., 1999).

In CLL, inflammatory processes play an important role since the disease has features of one that is driven by antigenic stimuli (Ghia et al., 2008). Also, survival of the leukemic clone crucially depends on environmental stimuli provided by cytokines, chemokines, angiogenic factors or the extracellular matrix since the leukemic cells spontaneously become apoptotic when the micromilieu is taken away (Burger et al., 2000).

To investigate whether MIF protein expression is upregulated in human CLL, we selected 10 patients with CLL and a white blood count (WBC) > 50 G/l. The protein content of Ficoll-isolated peripheral blood mononuclear cells (PBMC's) of these CLL patients was compared with the protein content of sorted CD19⁺ B cells from the peripheral blood of

10 healthy donors. In CLL patients, by densitometric analysis MIF was found to be upregulated approximately four-fold compared to healthy B cells. A representative example of this analysis is given in Fig. 7.

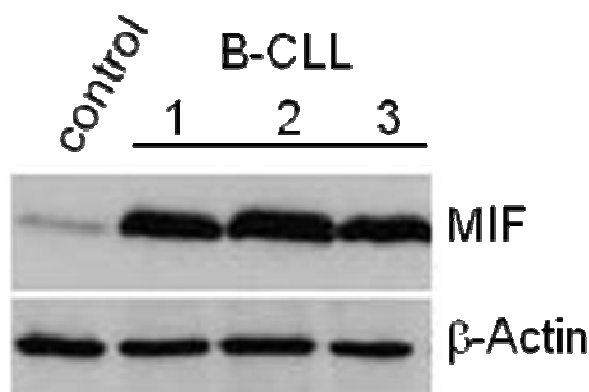


Figure 7: MIF protein is overexpressed in human B-CLL. Representative western blot analysis of MIF protein expression in human CLL PBMC's in comparison to sorted CD19⁺ B cells of a healthy control. MIF is 4-fold upregulated in CLL patient samples. 50 µg of protein were loaded onto the gel, membrane was incubated o/n with anti-MIF antibody (dilution 1:1000). β-Actin (1:3000) was used as loading control.

In order to find out whether overexpression of MIF contributes to the development of CLL or has only bystander significance, we took advantage of the Eµ-TCL1 transgenic mouse (Bichi et al., 2002) to analyse the role of MIF in the pathogenesis of CLL *in vivo*.

4.2 Characterization of the Eµ-TCL1 model for murine CLL

The Eµ-TCL1 mouse model (Bichi et al., 2002) expresses a 350 bp fragment containing the entire coding region of the human TCL1 gene under the Eµ-enhancer and an IgV_H promoter. The animals were generated on a B6C3H mixed background. The resulting phenotype of TCL1 overexpression is a lymphoproliferative disease which arises after 8 to 15 months of age and strongly resembles human CLL. Later investigations showed that this CLL is of the unmutated form, thus representing more or less the more aggressive form of human CLL (Yan et al., 2006).



Figure 8: Appearance of a $TCL1^{+/+}$ mouse at the age of 10 months. This mouse shows a distended abdomen due to hepatosplenomegaly and the fur is scruffy. At this stage of disease the animals are still active and agile. When the disease progresses and the general performance of the mice declines and they need to be euthanized.

With increasing age, the animals show clear signs of disease which are manifested by scruffy fur, increase in size of the abdomen due to splenomegaly and a reduction in the general performance. Macroscopic examination of euthanized mice at an advanced disease stage shows enlarged lymphoid organs (spleen, liver and lymph nodes) (Fig. 8). Some animals also developed ascites, an accumulation of fluid in the peritoneal cavity.



Figure 9: Example of a leukemic splenomegaly in a $TCL1^{+/+}$ mouse in comparison to the normal spleen size in a C57Bl/6 wildtype control mouse. Both animals were sacrificed at the age of 12 months. The spleen of the $TCL1^{+/+}$ mouse has a weight of 4.25 g whereas the spleen of the wildtype animal only weighs 0.25 g.

To determine whether the disease is really a B cell lymphoma, lymphoid organs such as spleen, lymph nodes, liver and bone marrow were collected and fixed in 4 % formalin solution. After 1 week of fixation, the organs were processed and embedded into paraffin.

Results

Sections of 3 μm width were cut and stained with Giemsa. Furthermore, staining for the CLL marker CD5 and the B cell marker CD79a were done in cooperation with PD Dr. H. Kvasnicka at the Institute of Pathology, Cologne University.

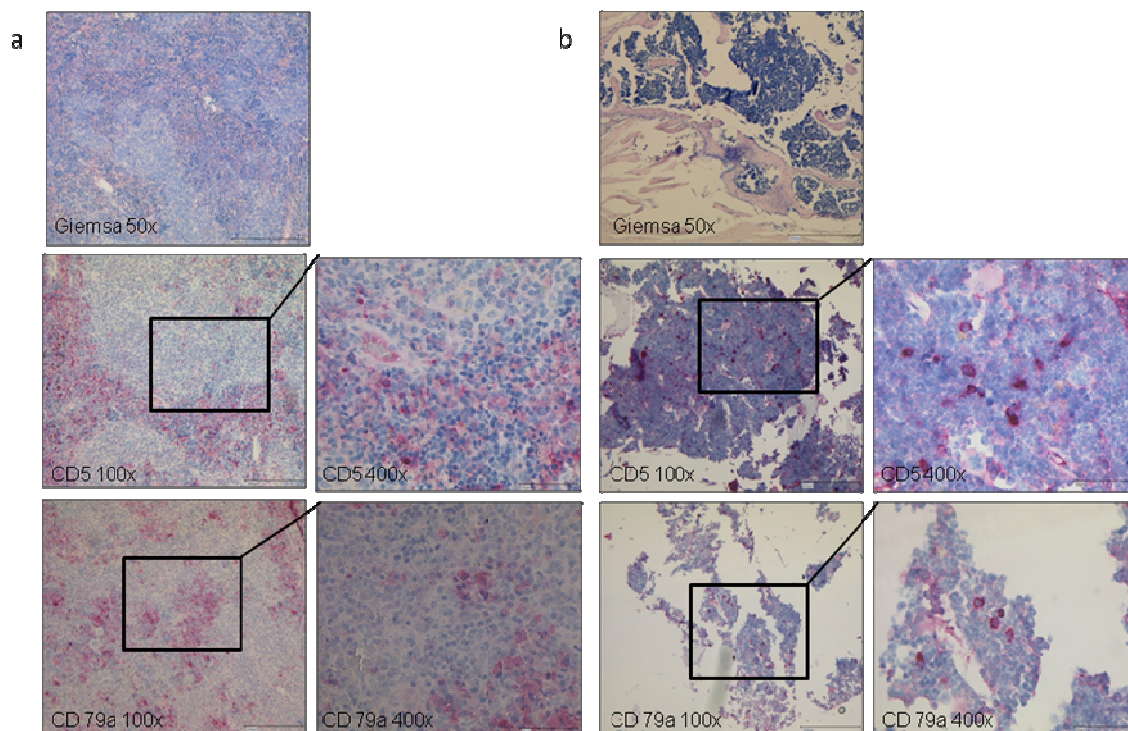


Figure 10: TCL1^{+/+} mice develop a CD5-positive B cell lymphoma. Representative Giemsa stain and immunohistochemical staining for CD5 and CD79a in spleen (a) and bone marrow (b) from a leukemic TCL1^{+/+} mouse. The leukemic infiltrates are positive for CD5 and CD79a.

Histopathological examination of the affected spleens showed that the splenic architecture was totally destroyed by many small lymphocytes which infiltrated the organ (Fig. 10a). These small, mature looking lymphocytes are positive stained by IHC for the B cell marker CD79a and CD5. Similar leukemic infiltrates can be found in the liver or in the bone marrow (Fig. 10b).

Next we wanted to know whether the known MIF receptors CD74/CD44 and CXCR2 are expressed on B cells in this disease model. Peripheral blood from the tail vein of pre-leukemic (age 3 months) and leukemic TCL1^{+/+} mice was incubated with specific fluorescence-labelled monoclonal antibodies binding to CD74, CD44 and CXCR2 and the surface expression was measured with the use of a FACS Canto flow cytometer.

As shown in Fig. 11, the MIF receptors are expressed both in pre-leukemic as well as in leukemic animals. CD74, the first known high affinity receptor for MIF is expressed at low

levels in both groups, but expression is not significantly altered by the development of overt leukemia. In contrast, the CD44 receptor, one of the signaling modules for CD74 activated by MIF is significantly higher on the B cells of the leukemic mice compared to the pre-leukemic B cells (25975.8 ± 11072.2 for leukemic vs. 6378.7 ± 1797.3 for pre-leukemic, $p = 0.0003$). The chemokine receptor CXCR2 is also expressed in both groups and its expression is also significantly elevated on the B cells of the leukemic mice (2275.7 ± 598.2 for leukemic vs. 624.2 ± 337.8 for the pre-leukemic, $p = 0.03$) (Fig. 11).

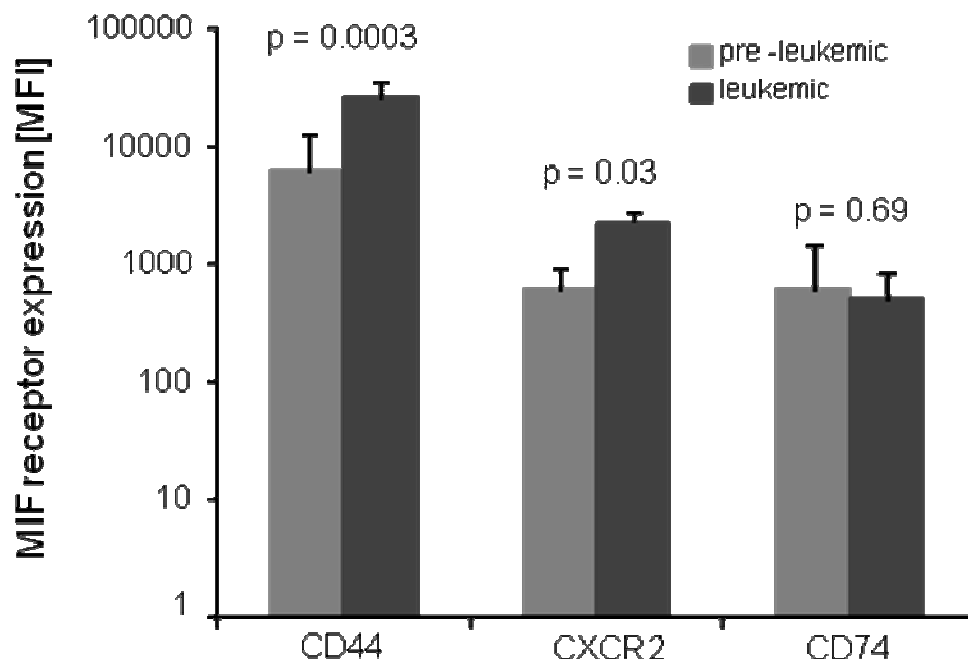


Figure 11: Expression of the MIF receptors CD74, CD44 and the chemokine receptor CXCR2 in $TCL1^{+/+}$ mice. Peripheral blood of pre-leukemic and leukemic animals ($n = 5$ each) was analysed by FACS. The CD44 signaling complex is significantly higher expressed in the leukemic mice ($p = 0.0003$) as well as the CXCR2 receptor ($p = 0.03$). CD74 is expressed to nearly the same extend in both groups.

Taken together, these preliminary insights into the $E\mu$ - $TCL1$ mouse model suggested to us that it was a suitable model for the analysis of MIF's role in CLL pathogenesis.

4.3 Analysis of the role of Macrophage Migration Inhibitory Factor (MIF) in the development of murine CLL

In order to obtain a clear picture of what MIF's role might be in the pathogenesis of CLL we bred the $TCL1^{+/+}$ transgenic mouse to the $MIF^{-/-}$ mouse which had been generated by deleting the entire *mif* gene using a loxP-Cre based deletion system (Fingerle-Rowson

Results

et al., 2003). The $MIF^{-/-}$ animals do not show any developmental abnormalities, are fertile and produce litters of normal size. Most importantly, their rate of spontaneous tumor or leukemic formation is not elevated compared to normal wildtype littermates (Fingerle-Rowson, personal communication).

Breeding of the $MIF^{-/-}$ mouse to the $TCL1^{+/+}$ transgenic mouse was done in the animal facility of the experimental surgery, currently run by the Clinic I of Internal Medicine at the University Hospital Cologne. Since a homozygous state of the transgene might cause artificial phenotypes, special care in the breeding strategy was taken to avoid a homozygous state of $TCL1$ in the F2 generation. The animals of the F2 generation with the genotypes $TCL1^{+/wt} MIF^{-/-}$ and $TCL1^{+/wt} MIF^{+/+}$ were used for the analysis of the leukemic development and survival.

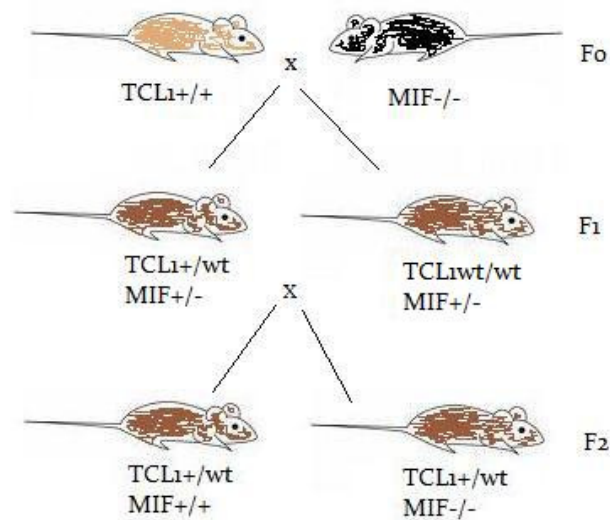


Figure 12: Breeding strategy of the $TCL1^{+/+}$ mouse to the $MIF^{-/-}$ mouse. Animals of the F1 generation with the genotype $TCL1^{+/wt} MIF^{+/-}$ were bred to animals with the genotype $TCL1^{wt/wt} MIF^{+/-}$ to obtain the animals of the F2 generation. The animals with the desired genotypes $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ were used for the experiments.

Animals with the genotypes $TCL1^{wt/wt} MIF^{+/+}$ and $TCL1^{wt/wt} MIF^{-/-}$ from the F2 generation are used as controls. The genotypes of the animals were determined by polymerase chain reaction (PCR). A piece of tail was taken from each mouse while weaning it from the parents. DNA was extracted from the tail tissue and a genomic PCR with *mif*-gene-specific and *tcl1*-gene-specific primers was run and analysed on an agarose gel.

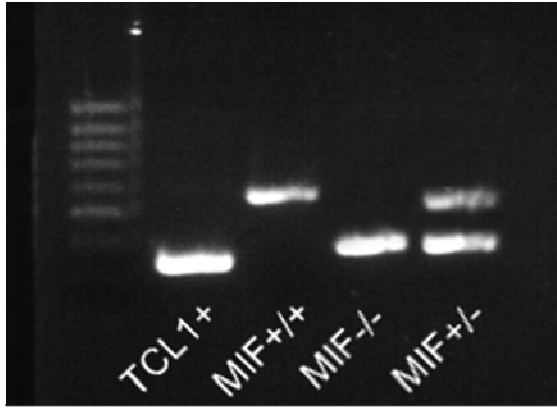


Figure 13: Genotyping of the TCL and MIF status. Gel of a genotyping PCR. Lane 1 shows the amplification product of TCL1 specific primers (300 bp), lane 2 the product of the MIF wildtype allele (544 bp), and lane 3 the product of the MIF knockout allele (383 bp). In lane 4 the expected bands of a MIF heterozygous mouse is given (544 bp and 383 bp).

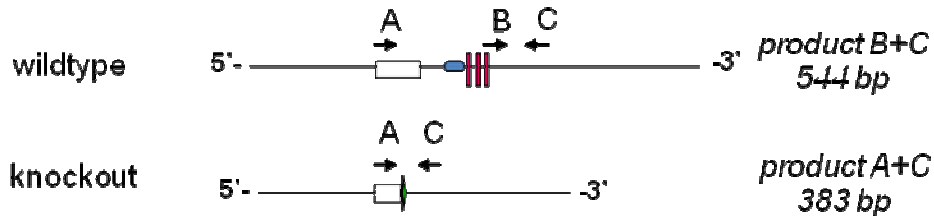


Figure 14: Schematic view of genotyping strategy for MIF. The wildtype *mif* gene is determined with the specific primers B and C, B binds in exon 3 (product 544 bp). In the knockout, the primers A and C bind to the MIF gene, leading to a 383 bp product.

After determination of the correct genotypes, animals were either put into the different experimental groups or used as control animals.

4.4 Macrophage Migration Inhibitory Factor receptors are expressed in murine CLL

As I could show before in the pre-leukemic as well as in the leukemic $TCL1^{+/+}$ mice, the MIF receptors CD74 and its signaling complex CD44 and the MIF chemokine receptor CXCR2 are expressed. To check whether there is a difference due to the MIF knockout, the analysis was repeated for the $TCL1^{+/wt}$ $MIF^{+/+}$ and $TCL1^{+/wt}$ $MIF^{-/-}$ mice. Five animals of each group were analysed by FACS for their receptor expression. Peripheral blood from the tail vein of pre-leukemic animals of both groups was taken for the analysis. The animals had the age of 6 months.

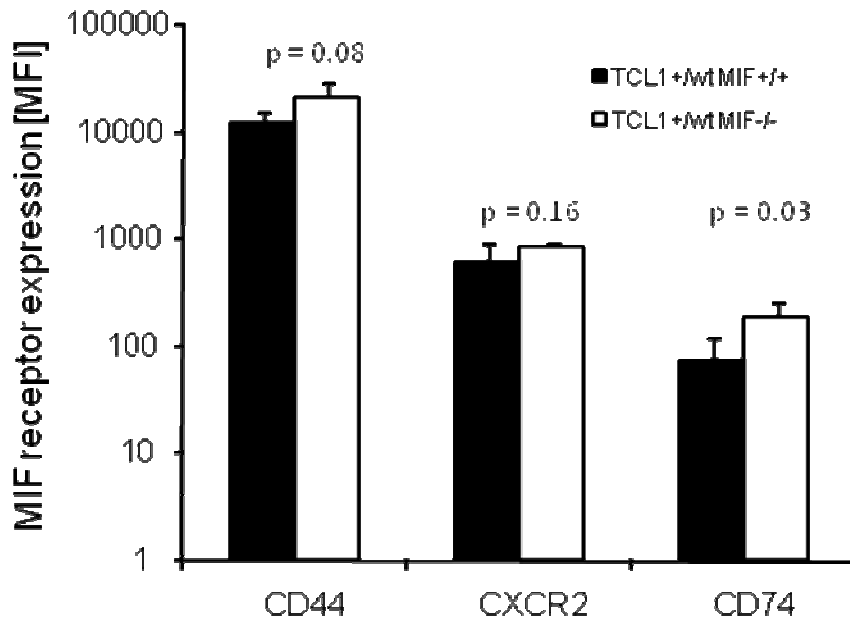


Figure 15: Expression of the MIF receptors CD74, CD44 and the chemokine receptor CXCR2 in $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice. Peripheral blood of pre-leukemic animals ($n = 5$ per genotype) was analysed by FACS. The CD44 signaling complex as well as the CXCR2 receptor are expressed to nearly the same extent in both groups. CD74, the extracellular MIF receptor is also expressed in both groups, but it is significantly higher in the $MIF^{-/-}$ animals ($p = 0.03$).

Fig. 15 shows the expression of the different receptors in pre-leukemic $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice ($n = 5$ per group). Each receptor is expressed on the surface of the blood cells, all of them higher in the $TCL1^{+/wt} MIF^{-/-}$ mice. CD44, the signaling complex is expressed highest again in both genotypes. CXCR2 is also expressed in both groups, a little bit higher in the $TCL1^{+/wt} MIF^{-/-}$ mice. Only CD74, the MIF receptor is significantly higher expressed on the $TCL1^{+/wt} MIF^{-/-}$ mice (90.5 ± 38.1 for $MIF^{+/+}$ vs. 286.5 ± 135.2 for the $MIF^{-/-}$ mice, $p = 0.03$).

4.5 Analysis of the leukemia development

The analysis of the development of the leukemia in the peripheral blood was done from tail vein blood taken in intervals of three months (i.e. months 3, 6, 9 and 12) and analyzed for leukocyte numbers, differential blood stain and the presence of $CD5^+IgM^+$ cells. Fig. 16 shows a representative example of a blood smear from a $TCL1^{+/wt} MIF^{+/+}$ mouse at the age of 9 months with evident lymphocytic leukemia. Most prominent is the

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strongly increased number of small, mature lymphocytes. Besides those intact cells, many cells with ruptured membranes can be seen. These cells, so called smudge cells or “Gumprecht’s nuclear shadows” are due to an increased fragility of the cellular membrane, however, the cellular rupture occurs only during the preparation of the slides. These smudge cells are a diagnostic hallmark of human CLL.

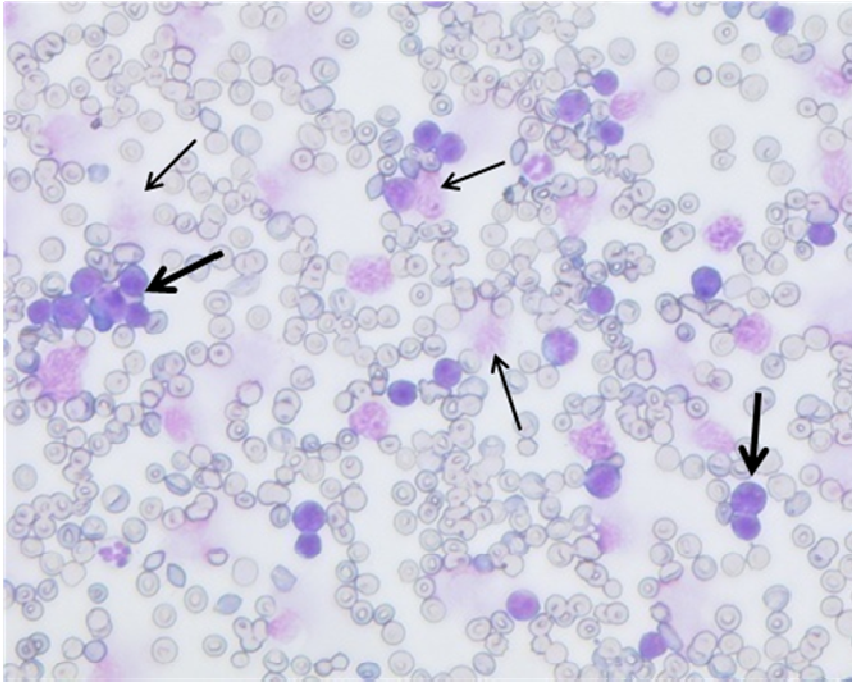


Figure 16: Giemsa stain of a peripheral blood smear of a leukemic $TCL1^{+/wt}$ $MIF^{+/+}$ mouse. There is an accumulation of small lymphocytes with thin cytoplasm and dense nuclei (thick arrows). Thin arrows point towards smudge cells (also called “Gumprecht’s nuclear shadows”), the characteristic hallmark feature of chronic lymphocytic leukemia.

White blood count (WBC), the red blood cell counts (RBC) and platelet counts (PLT) are measured by laser flow cytometry from the tail vein blood of the mice. These parameters give a global overview on the state of the hematopoietic system and provide a first hint to the potential presence of leukemia.

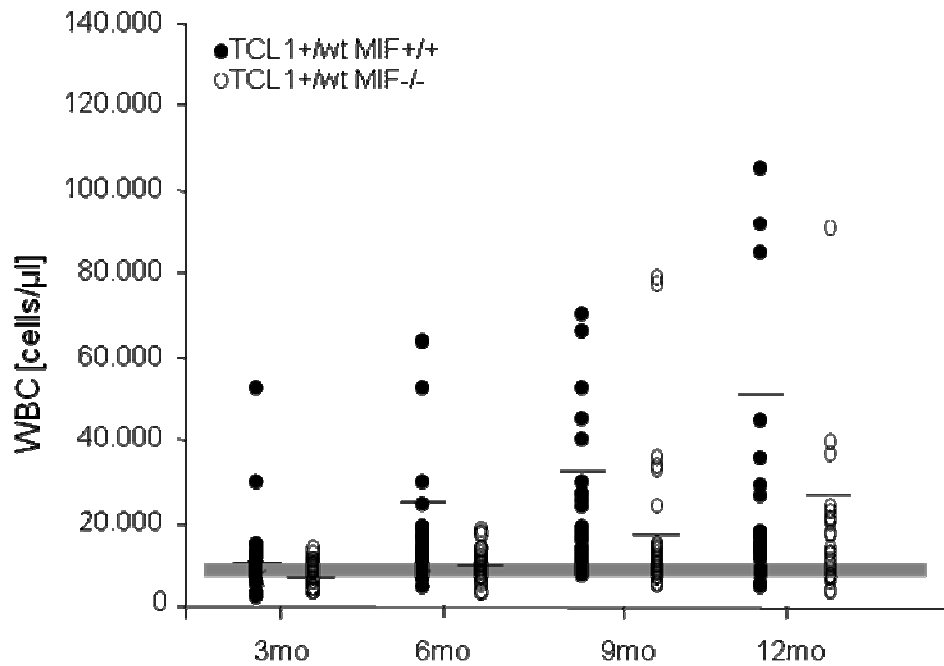


Figure 17: Analysis of the white blood count (WBC) from tail vein blood of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice over time. The grey bar shows the normal range of WBC of C57Bl/6 control animals (between 7.000 and 12.000 cells/ μ l). Animals of the $TCL1^{+/wt} MIF^{+/+}$ group develop an increased WBC more frequently than $TCL1^{+/wt} MIF^{-/-}$ animals, with a higher median in $TCL1^{+/wt} MIF^{+/+}$ mice.

At the age of 3 months, the animals of the $TCL1^{+/wt} MIF^{+/+}$ group already show a significantly higher WBC ($p = 0.049$) in comparison to the $TCL1^{+/wt} MIF^{-/-}$ animals, a trend which continues during the later time points despite the dying of leukemic animals (Fig. 17).

As the normal range of leukocytes in the mouse is between 7.000 and 12.000 cells/ μ l of blood, we set an arbitrary threshold at 20.000 cells/ μ l of blood in order to differentiate leukemia from non-malignant leukocytosis. Fig. 18 displays the Kaplan-Meier incidence curve of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice developing a leukocyte count $> 20.000/\mu$ l. The $TCL1^{+/wt} MIF^{+/+}$ animals exceeded this threshold approximately three months earlier than the $TCL1^{+/wt} MIF^{-/-}$ animals ($p = 0.02$) (Fig. 18).

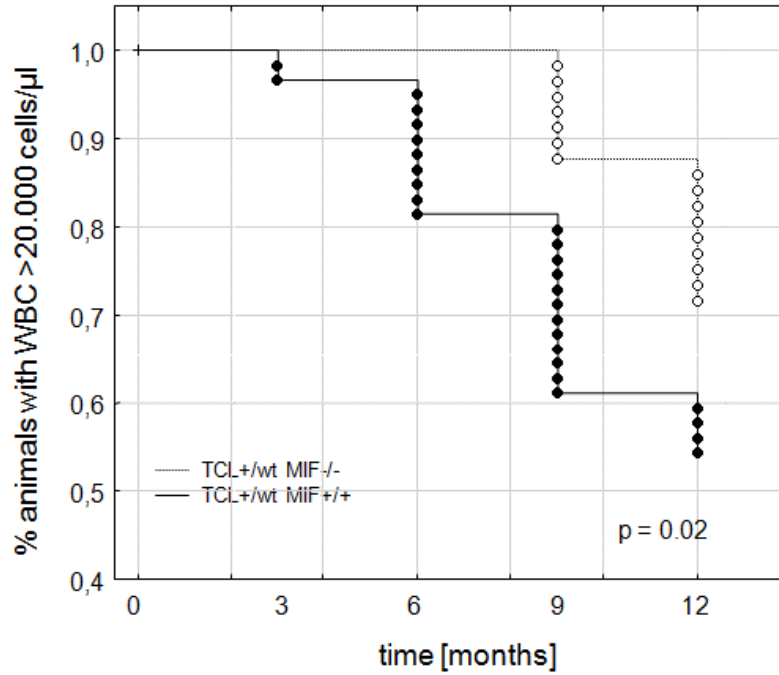


Figure 18: Kaplan-Meier incidence curve of mice developing a WBC > 20.000 cells/μl. TCL1^{+wt} MIF^{+/-} (black dots) and TCL1^{+wt} MIF^{+/+} (white dots) mice which developed peripheral leukemia with a WBC > 20.000 cells/μl were counted as events. The TCL1^{+wt} MIF^{+/-} mice develop the leukemia about 3 months in advance to the TCL1^{+wt} MIF^{+/+} mice. Statistical significance was tested using the logrank test (p = 0.02).

Determination of haemoglobin, red blood counts (RBC) and platelet counts provide key parameters of bone marrow function which could be influenced by infiltrating CLL cells. Of these, the RBC showed that there was a slight, but progressive decrease in the production of red blood cells over time which is most likely due to an increasing bone marrow infiltration by CLL cells. Animals develop progressive anemia, however the presence of absence of MIF did not influence the degree of anemia in a consistent way (Fig. 19). Histological analysis of bone marrow revealed that TCL1-induced leukemia also infiltrated the bone marrow (Fig. 26). Platelet counts and hemoglobin showed no significant differences between the two genotypes, though here a slight decrease in the haemoglobin was seen. The platelets declined at month 12 in both groups, probably due to the compromised bone marrow by lymphocyte infiltration (Fig. 20).

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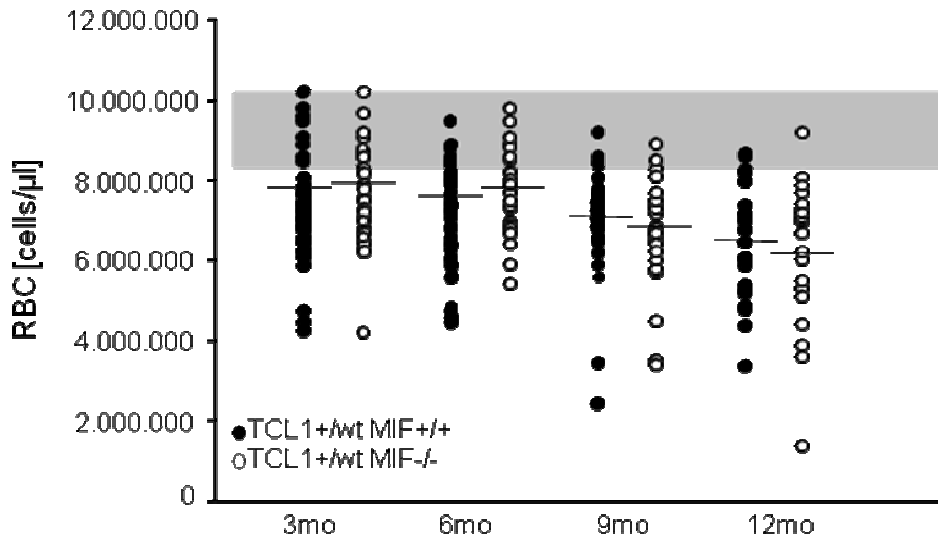


Figure 19: Red blood count (RBC) in $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice over time. Analysis was done on the Sysmex XE-2100. The group of the $TCL1^{+/wt} MIF^{-/-}$ mice develop a much severe anemia in contrast to the $TCL1^{+/wt} MIF^{+/+}$ animals. The grey bar indicates the normal RBC count of C57Bl/6 animals which ranges from 8.200.000 to 10.500.000 cells/μl of blood.

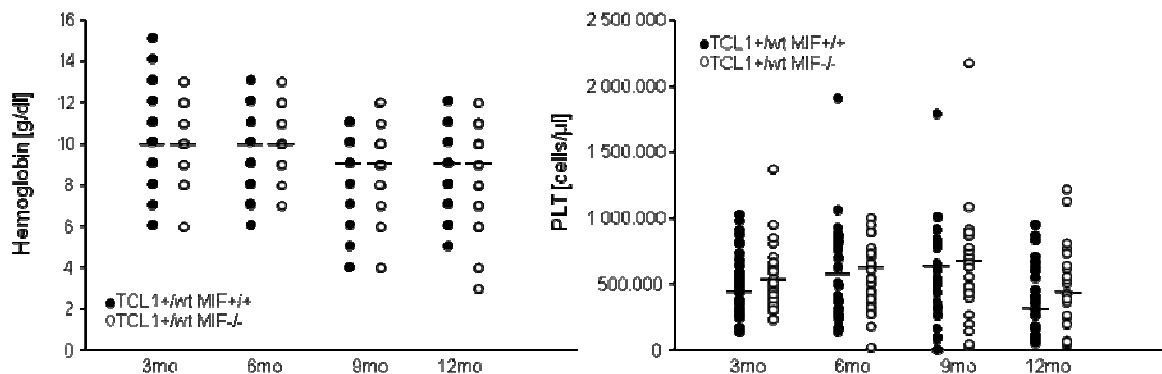


Figure 20: Hemoglobin and platelet analysis in $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice. The hemoglobin and the platelet analysis over time shows no differences between the two groups. In $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$, hemoglobin goes down in the same way. Also the platelet count behaves the same in both groups, it

In addition, we performed an accurate count of the malignant cells by FACS analysis for $CD5^+IgM^+$ cells. CD5 is normally only expressed on NK cells, but not on B cells. Aberrant expression of CD5 on B cells is a pathogenomic feature of human and murine CLL.

Fig. 21 a - d shows a representative example for each genotype. In the wildtype animals, the malignant, $CD5^+IgM^+$ clone develops significantly faster than in the knock out animals.

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This is indicated by the percentage of CD5⁺IgM⁺ cells based on the total number of lymphocytes. In the TCL1^{+wt} MIF^{+/+} animal, the malignant clone in month 3 is about 4.73 %, growing to 5.41 % in month 6 and 7.13 % in month 9. It expands to 72.86 % at the last time point of 12 months of age.

In contrast, the TCL1^{+wt} MIF^{-/-} (Fig. 21 e – h) mice show less severe signs of disease, the malignant clone is nearly not detectable in the peripheral blood. The clone develops from 1.49 % of the total lymphocytes in month 3 to 4.39 % in month 6. After 9 months of age the mouse still only has a malignant clone of 4.64 % of total lymphocytes which does not exceed 5.95 % after 12 months of age. This mouse did not show any signs of disease until the age of 18 months, when it finally developed a CLL-like disease (data not shown).

When mice develop frank leukemia, they show visible signs of disease, like scrubby fur, apathy and flat breathing.

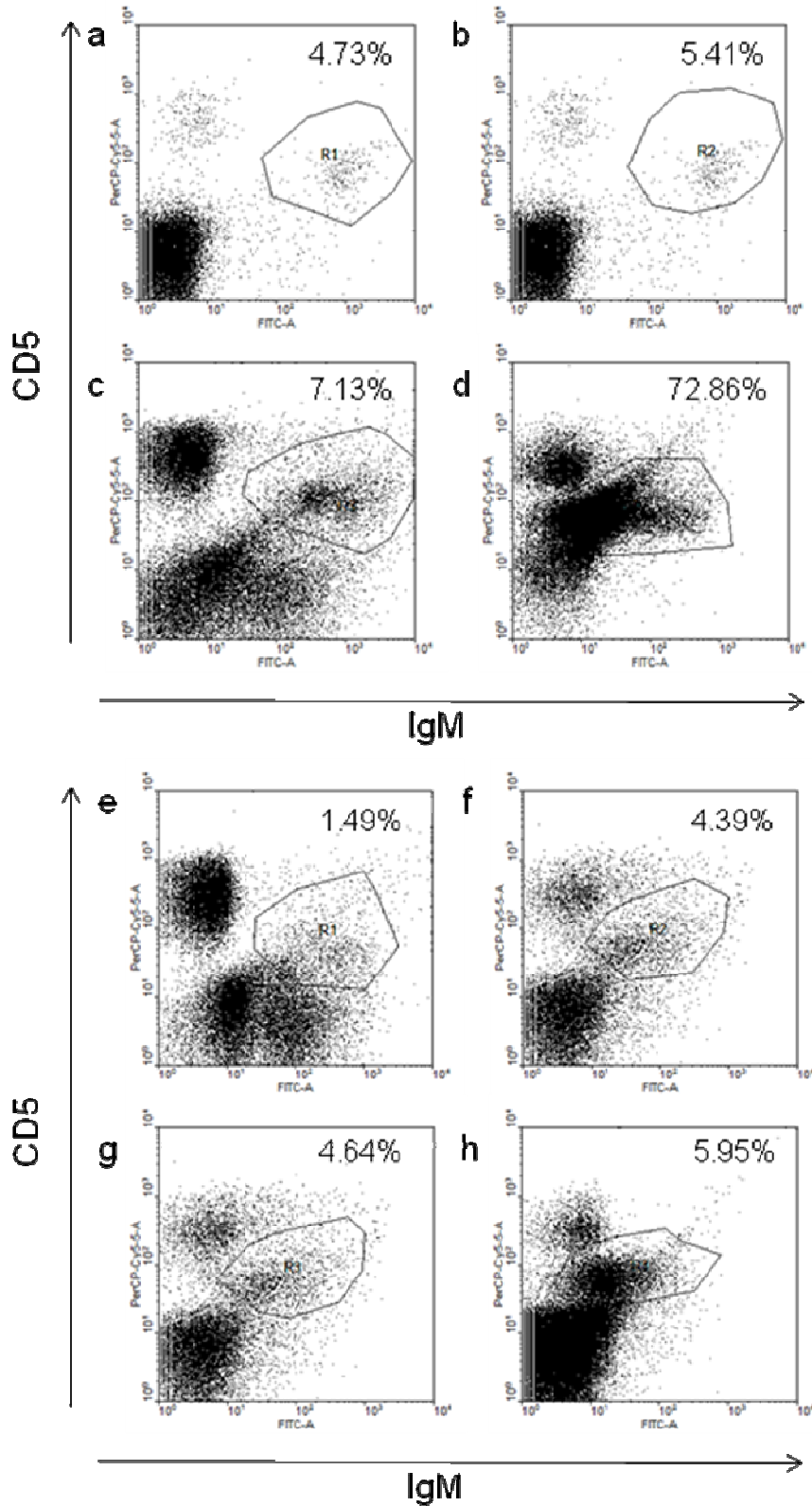


Figure 21: FACS analysis of the malignant clone (CD5⁺IgM⁺ cells). Analysis was performed from peripheral tail vein blood after 3 (a, e), 6 (b, f), 9 (c, g) and 12 months of age (d, h). a-d gives a representative example from the group of TCL1^{+/wt} MIF^{+/+} mice, e-h gives an example for

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TCL1^{+wt} MIF^{-/-} mice. The figures given indicate the percentage of malignant cells of all lymphocytes.

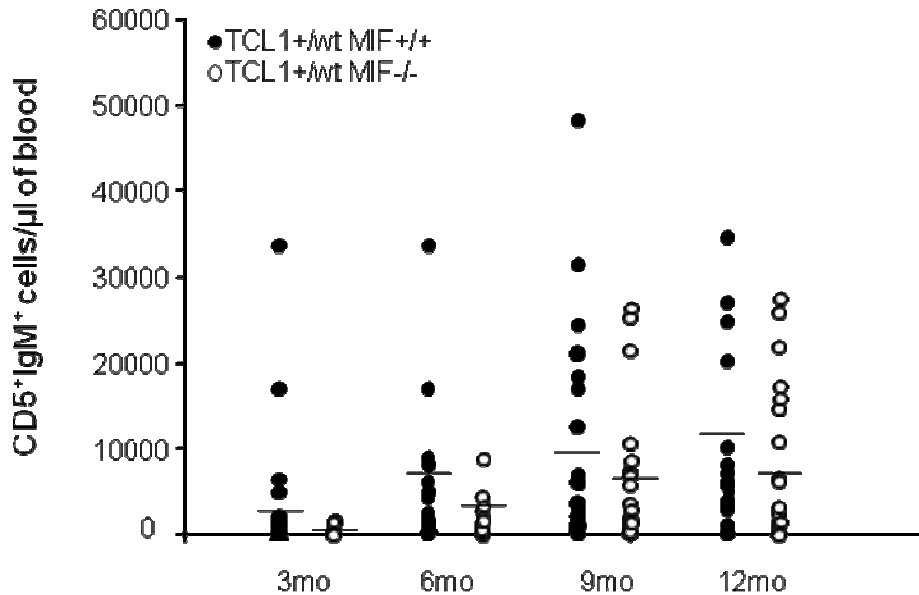


Figure 22: Summary of the absolute number of the malignant CD5⁺IgM⁺ clone. At months 3 and 6 the difference between the two genotypes is significant ($p = 0.002$ and $p = 0.004$).

Fig. 22 shows the summary of the total FACS analysis of $n = 40$ mice per group for the development of the malignant clone. This graph gives the absolute numbers of CD5⁺IgM⁺ cells in the peripheral blood. At month 3, the malignant clone of both groups is still at very low levels, however in the group of the TCL1^{+wt} MIF^{+/+} mice some animals with increased CD5⁺IgM⁺ counts can already be detected. Over the next time points of analysis, the number of mice with an expansion of the malignant clone increases and the difference between the two genotypes is significant at months 3 and 6 ($p = 0.002$ and $p = 0.004$). Significance is lost at later time points since the diseased animals die and are no longer available for analysis.

The animals at the age of 12 months were then further analysed for organ weight and infiltration as well as functional assays were performed, like apoptosis testing and proliferation.

4.6 MIF increases leukemia load in murine CLL

Since our analysis of the peripheral blood of the $TCL1^{+/wt} MIF^{-/-}$ and $TCL1^{+/wt} MIF^{+/+}$ mice had shown that the presence of MIF leads to accelerated and increased leukemia development, we next tested whether this observation could also be extended for other organs such as liver, spleen and bone marrow. Mice ($n = 20$ per group) were euthanized at the age of 12 months and their organs processed for pathological examination. Spleen, liver and enlarged lymph nodes were taken out and weighed. One part of each organ was then fixed in 4 % buffered formalin and processed for further hematopathological analysis. The weight of spleen, the organ which is typically affected by enlargement in human CLL, was also enlarged in the TCL1 mouse model, sometimes to a massive degree with spleens reaching the pelvic end of the abdomen. Overall, the spleen showed a clear trend towards stronger enlargement in the $TCL1^{+/wt} MIF^{+/+}$ mice, but due to the relatively low number of animals used for this analysis the difference was not quite statistically significant ($TCL1^{+/wt} MIF^{+/+}$ $1.3 \text{ g} \pm 0.8$, $TCL1^{+/wt} MIF^{-/-}$ $0.67 \text{ g} \pm 0.5$, $p = 0.07$). On the other hand, the liver, an organ which also may be infiltrated and enlarged in humans by CLL, showed a significant difference ($TCL1^{+/wt} MIF^{+/+}$ $3.27 \text{ g} \pm 1.1$, $TCL1^{+/wt} MIF^{-/-}$ $1.67 \text{ g} \pm 0.68$, $p = 0.0028$) with a higher weight in the $MIF^{+/+}$ animals. Thus, the presence of MIF leads to a clear increase of the leukemic load in murine CLL (Fig. 23).

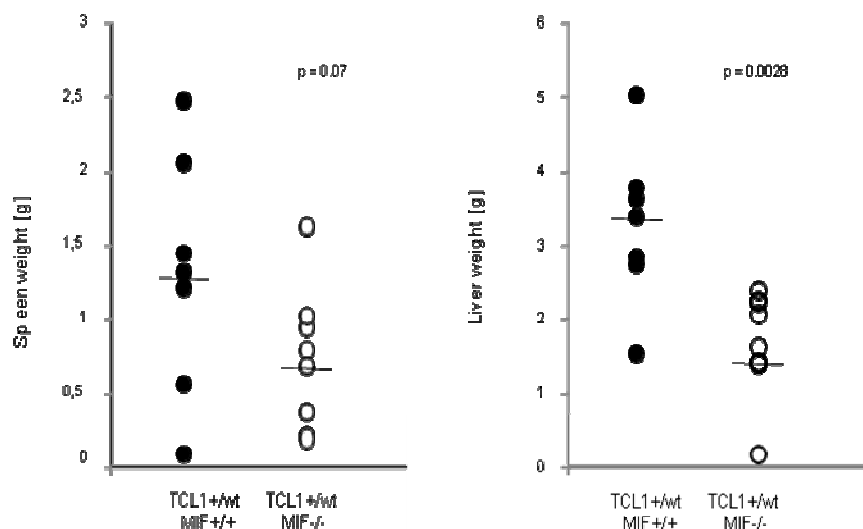


Figure 23: MIF promotes hepatosplenomegaly in CLL. Analysis of spleen and liver weight of $TCL1^{+/wt} MIF^{+/+}$ ($n = 7$) and $TCL1^{+/wt} MIF^{-/-}$ ($n = 9$) mice. The livers of the $TCL1^{+/wt} MIF^{+/+}$ animals were significantly larger than those of the $TCL1^{+/wt} MIF^{-/-}$ animals. Spleens of $TCL1^{+/wt} MIF^{+/+}$

animals were larger than those of $TCL1^{+/wt} MIF^{-/-}$ animals, but this difference did not quite reach statistical significance ($p = 0.07$).

4.7 Immunohistochemical analysis of the $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ animals

As a next step, we wanted to determine the cellular source of the $TCL1$ -induced leukemia. We performed a histopathological screening of the organs and stained the spleen and bone marrow for B- (CD79a), T- (CD3) and macrophage- (CD68) cell markers. Malignant or normal B cells were differentiated by the use of CD5.

Fig. 24 provides representative pictures from Giemsa and immunohistochemical staining of spleens from $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ animals. The Giemsa stain reveals extensive infiltration of the spleen by small, mature looking lymphocytes which clearly point the presence of lymphoma. These lymphatic cells are positive by immunohistochemistry for CD79a and CD5 which proves that this lymphoma is of the CLL phenotype. A T-cell lymphoma could be excluded since these atypical lymphocytes were clearly negative for CD3. $CD3^+$ T cells though were also present in the spleens of both genotypes and there was no significant difference. Interestingly, staining for CD68, a macrophage marker, demonstrates a significantly higher number of macrophages in the infiltrated areas of the spleen of $TCL1^{+/wt} MIF^{+/+}$ animals as compared to $TCL1^{+/wt} MIF^{-/-}$ mice (29.4 ± 46.4 vs. 9.7 ± 38.5 , $p < 0.0001$) (Fig. 25).

Results

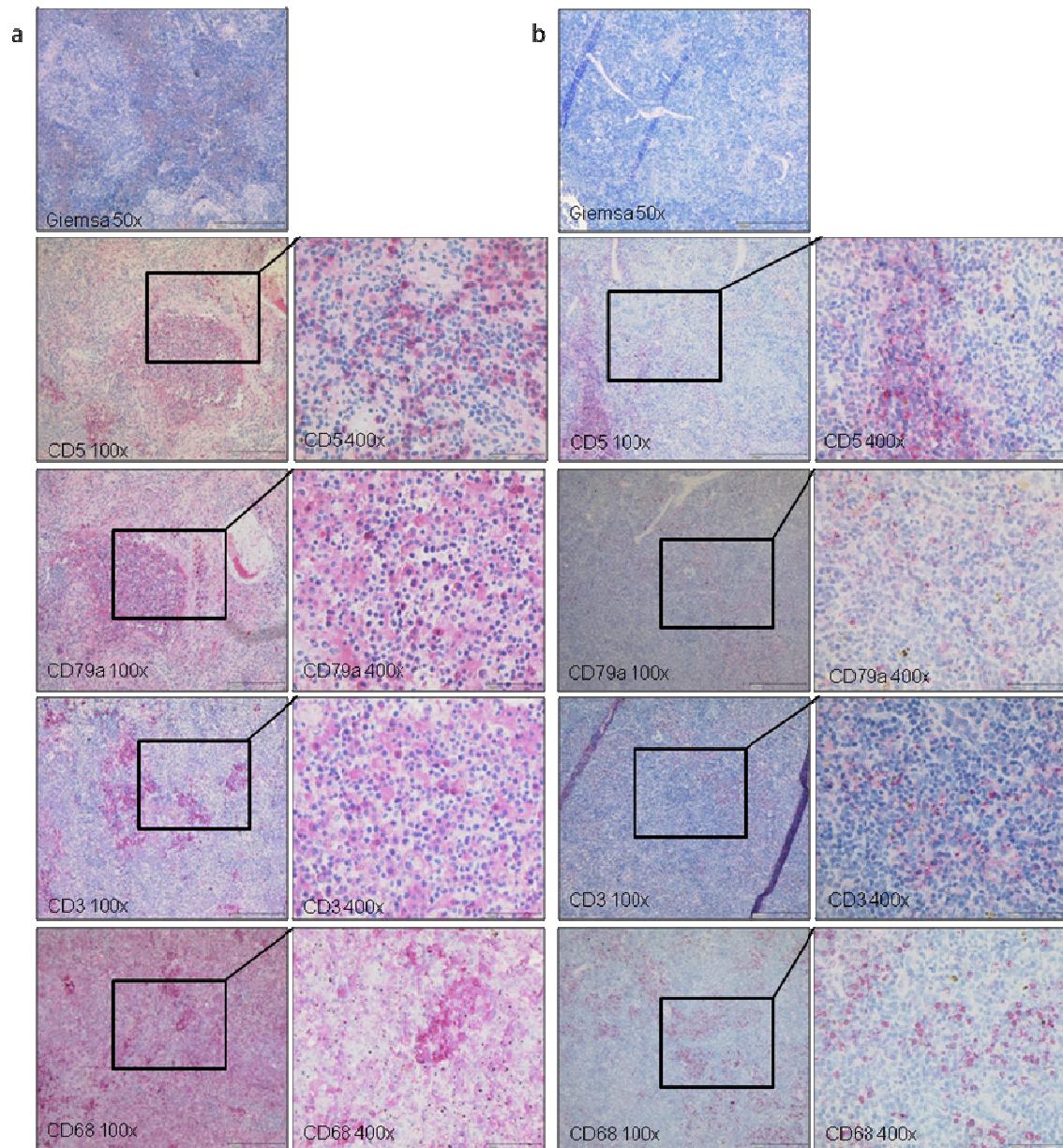


Figure 24: Representative immunohistochemical staining of spleen from a $TCL1^{+/wt}$ $MIF^{+/+}$ mouse (a) and a $TCL1^{+/wt}$ $MIF^{-/-}$ mouse (b). Staining was done for CD5, CD79a, CD3 and CD68. Giemsa stain was performed in order to get an overview. The normal structure of red and white pulp does no longer exist in these spleens due to extensive infiltration by lymphocytes.

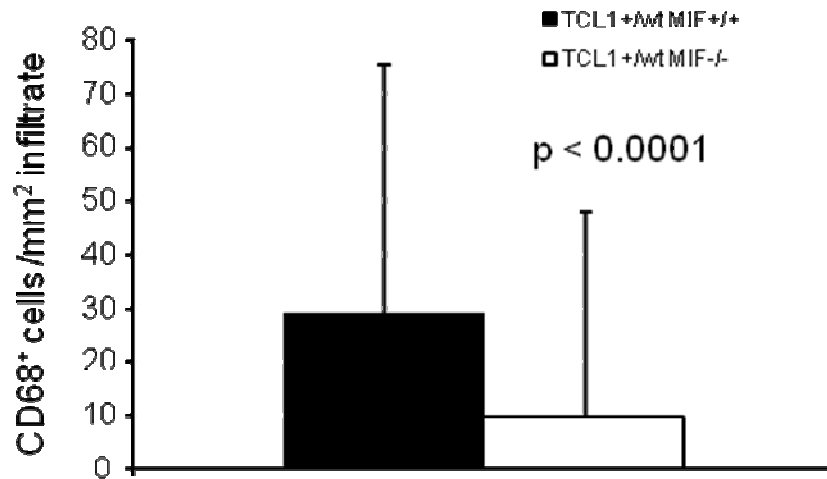


Figure 25: Higher number of macrophage infiltration in $TCL1^{+/wt}$ $MIF^{+/+}$ mice. CD68⁺ cells were counted in the infiltrated regions of the leukemic spleens of $TCL1^{+/wt}$ $MIF^{+/+}$ mice (n = 18) and $TCL1^{+/wt}$ $MIF^{-/-}$ mice (n = 14). $TCL1^{+/wt}$ $MIF^{+/+}$ mice have a significantly higher number of macrophages in the lymphocytic infiltrate compared to the $TCL1^{+/wt}$ $MIF^{-/-}$ mice ($p < 0.0001$).

The bone marrow was stained in a similar fashion as spleens and livers. The Giemsa stain of bone marrow of the $TCL1^{+/wt}$ $MIF^{+/+}$ mice and the $TCL1^{+/wt}$ $MIF^{-/-}$ mice also showed infiltration by small lymphocytes which uniformly stained positive for CD79a and CD5, again confirming the diagnosis of CLL. To further analyse the expression of other cell types like T cells and macrophages, staining for CD3 and CD68 were done (Fig. 26).

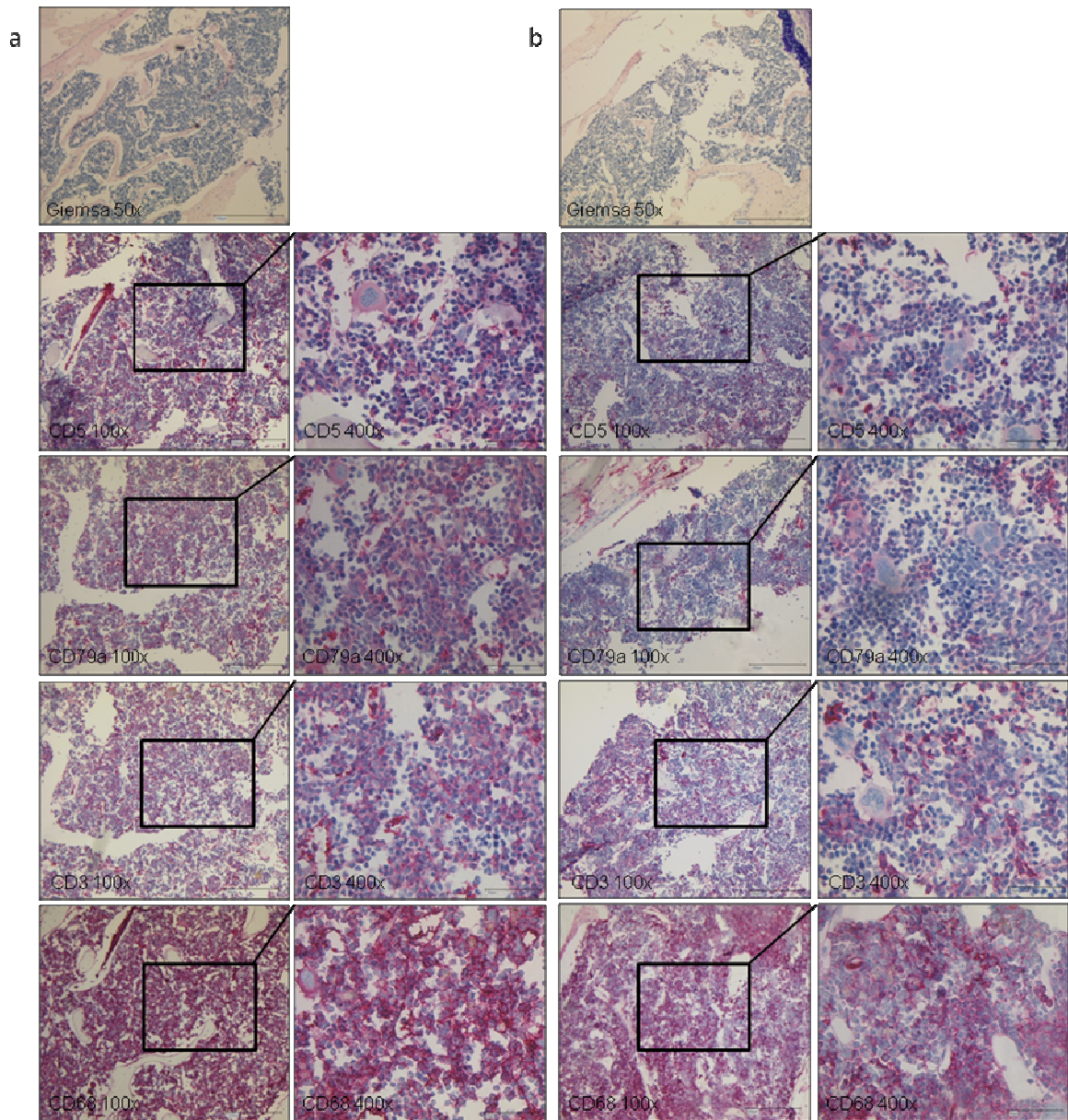


Figure 26: Representative immunohistochemical staining of bone marrow from a $TCL1^{+/wt}$ $MIF^{+/+}$ mouse (a) and a $TCL1^{+/wt}$ $MIF^{-/-}$ mouse (b). Staining was done for CD5, CD79a, CD3 and CD68. Giemsa stain was performed in order to get an overview.

4.8 MIF affects overall survival in murine CLL

Both experimental groups as well as control mice ($TCL1^{+/+}$, $MIF^{-/-}$, $MIF^{+/+}$) were also analysed for overall survival. To this end, animals were observed from birth until death, or, if the performance status of the mouse was too severely affected (e.g. weight loss > 10 %, apathy, swollen abdomen), to the time point at which the animal had to be euthanized. For the group of $TCL1^{+/wt}$ $MIF^{+/+}$ and for the group of $TCL1^{+/wt}$ $MIF^{-/-}$, we analysed a total of

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n = 40 mice for each genotype. These mice were also screened for the presence of leukemia by a 3-monthly blood examination as described before.

Mice were analysed for up to 750 days. The median survival for $TCL1^{+/wt} MIF^{+/+}$ mice is 380 days, whereas the animals with the genotype $TCL1^{+/wt} MIF^{-/-}$ have a median survival of 460 days. The difference of 80 days is significant ($p = 0.04$) (Fig. 27).

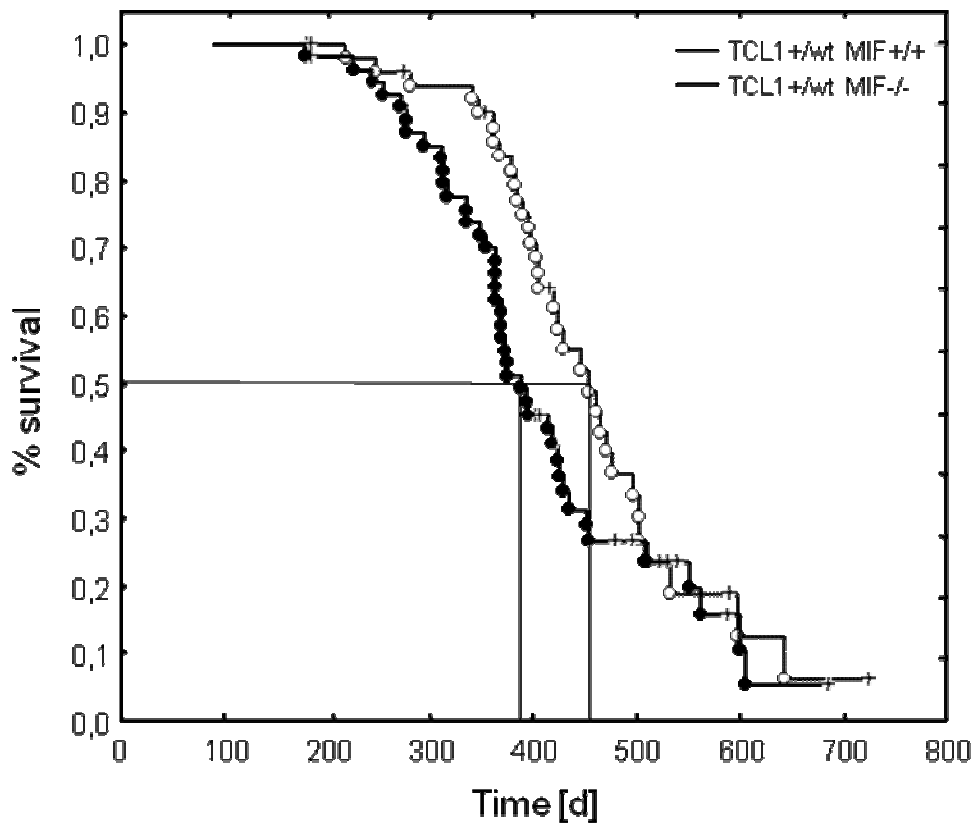


Figure 27: Kaplan Meier curve of cumulative survival of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ animals. $TCL1^{+/wt} MIF^{+/+}$ (filled circles) and $TCL1^{+/wt} MIF^{-/-}$ (empty circles) are observed from birth to sudden death or time of euthanization. The median survival of $TCL1^{+/wt} MIF^{+/+}$ is 380 days, whereas the $TCL1^{+/wt} MIF^{-/-}$ animals have a median survival of 460 days. The difference in overall survival of 80 days is statistically significant with a p-value of 0.04 by the logrank test.

We also performed a comparison of the experimental groups with their non- $TCL1$ -transgenic counterparts, i.e. $TCL1^{wt/wt} MIF^{+/+}$ and $TCL1^{wt/wt} MIF^{-/-}$ (n = 20 per group). As expected, $TCL1$ -transgenic animals have significantly shortened survival in comparison to their non-transgenic controls (Fig. 28 upper and lower panel). Gene dosage of $TCL1$ does not seem to affect the overall survival since we did not observe a significant difference between $TCL1^{+/+}$ and $TCL1^{+/wt} MIF^{+/+}$ mice (Fig. 28 upper panel).

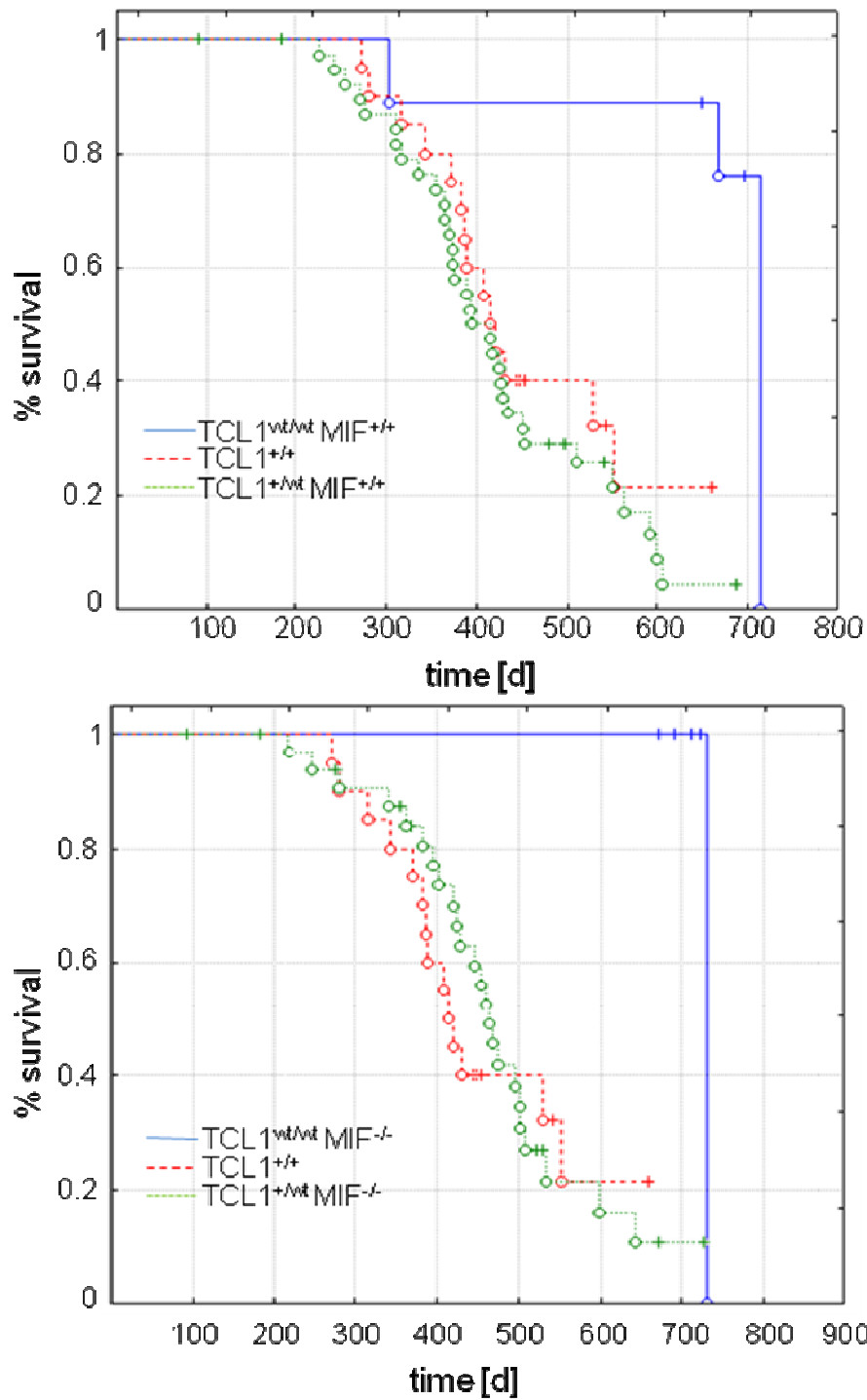


Figure 28: Kaplan-Meier curves of survival for each genotype in comparison to non-transgenic controls and homozygous $TCL1^{+/+}$ mice. Upper panel: Overall survival of $TCL1^{MIF^{+/+}}$ mice. Non-transgenic $MIF^{+/+}$ mice do not develop leukemia and the gene dosage of $TCL1$ shows only a little impact in the presence of MIF . Lower panel: Overall survival of $TCL1^{MIF^{-/-}}$ mice. Non-transgenic $MIF^{-/-}$ mice do not develop leukemia.

The difference in survival between transgenic and non-transgenic animals is very clear and highly significant (TCL1^{wt/wt} MIF^{+/+} vs. TCL1^{+/-wt} MIF^{+/+} with $p = 0.0079$ and TCL1^{wt/wt} MIF^{-/-} vs. TCL1^{+/-wt} MIF^{-/-} with $p = 0.00016$) (Fig. 28). We did not observe any leukemia in those control mice. Occasional deaths were probably due to old age, since pathological analysis did not reveal any organ abnormalities (data not shown).

The gene dosage of TCL1, i.e. a heterozygous or homozygous status of the transgene which we could control by the breeding scheme used, did not seem to have a significant impact on overall survival in MIF^{+/+} mice.

These results confirm that MIF is a relevant regulator of TCL1-driven lymphomagenesis and we therefore attempted to elucidate more of the mechanism by which MIF might promote murine CLL.

4.9 MIF deficiency lowers the apoptotic threshold

MIF is known to affect the rate of apoptosis in splenocytes of the E μ -myc mouse model via a p53-dependent mechanism (Talos et al., 2005). We therefore analysed spleens from animals of the age of 12 months for spontaneous apoptosis *in vivo* and tested their splenocytes *ex vivo* for drug-induced apoptosis.

Paraffin-embedded spleen sections from TCL1^{+/-wt} MIF^{+/+} and TCL1^{+/-wt} MIF^{-/-} mice were stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) in order to detect the *in vivo* rate of apoptosis within the spleen. Propidium iodide (PI) which is used for the counterstain, colours the permeable membrane of dying or dead cells in red.

Fig. 29 shows a representative example out of $n = 5$ independent experiments per group for the differences in TUNEL-positive apoptotic cells in TCL1^{+/-wt} MIF^{+/+} and TCL1^{+/-wt} MIF^{-/-} mice (Fig. 29). A counting of the TUNEL positive cells per visual field resulted in a significantly higher number of positive cells for the TCL1^{+/-wt} MIF^{-/-} spleens compared to the TCL1^{+/-wt} MIF^{+/+} spleens (123 ± 28.1 for MIF knockout vs. 51 ± 16.6 for MIF wildtype, $p < 0.0001$) (Fig. 30). The spleens compared here were all taken from leukemic mice.

We then wanted to further analyse this finding by an *ex vivo* analysis of splenocytes from both leukemic as well as pre-leukemic mice.

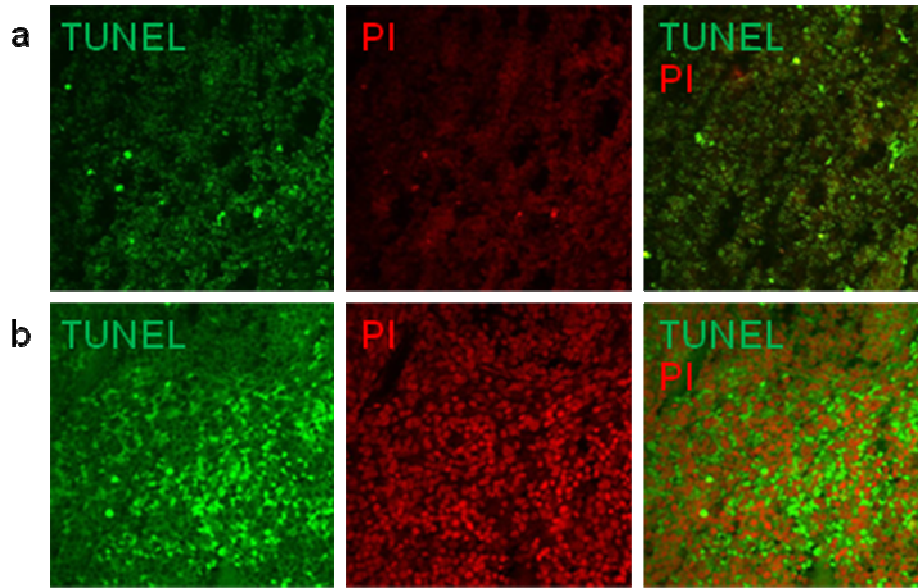


Figure 29: MIF-deficiency increases spontaneous apoptosis in TCL1-trangenic splenocytes in vivo. Representative TUNEL staining of a TCL1^{+wt} MIF^{+/+} spleen (a) and a TCL1^{+wt} MIF^{-/-} spleen (b) from n = 5 independent experiments per group. Counterstain was done with propidium iodide (PI). Pictures were taken on a confocal microscope. In TCL1^{+wt} MIF^{+/+} splenocytes, the rate of TUNEL positive apoptosis is greatly enhanced compared to TCL1^{+wt} MIF^{-/-} splenocytes. The pictures are taken at 600-fold magnification.

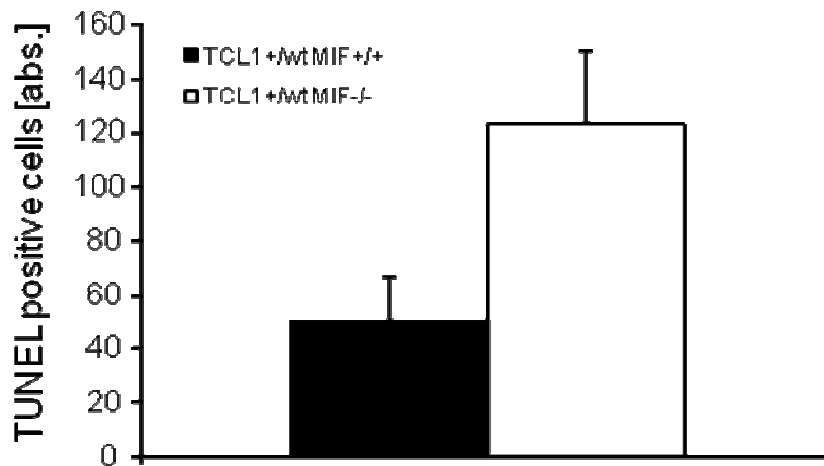


Figure 30: A higher rate of TUNEL positive cells in the TCL1^{+wt} MIF^{-/-} spleens. Counting of the TUNEL positive cells per visual field at a 600-fold magnification. In the TCL1^{+wt} MIF^{-/-} spleens a significantly higher number of apoptotic cells is found in comparison to the TCL1^{+wt} MIF^{+/+} spleens (p < 0.0001).

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For *in vitro* analysis of apoptosis, splenocytes from pre-leukemic (month 3) and leukemic mice were treated with cytostatic drugs and with the corticosteroid prednisolone. We used chemotherapeutic drugs which are in common use for the therapy of patients with CLL nowadays. Fludarabine, a purine analog, inhibits DNA synthesis by interfering with the DNA polymerase in the cells and can be regarded as the standard drug of CLL therapy at the moment. It acts against resting and dividing cells. Vincristine, an inhibitor of the spindle apparatus is used in cancer chemotherapies to disrupt mitosis during the phase of chromosome separation and division of the cell into two identical daughter cells. It therefore only affects dividing cells. As a third drug, we used prednisolone, a glucocorticoid, which is in use for the treatment of lymphatic leukemias and for the treatment of inflammatory and auto-immune diseases. To find out whether the apoptotic response of MIF-expressing and MIF-deficient splenocytes as altered in our model, we tested those three substances on splenocytes of leukemic and pre-leukemic mice from $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ animals *ex vivo*.

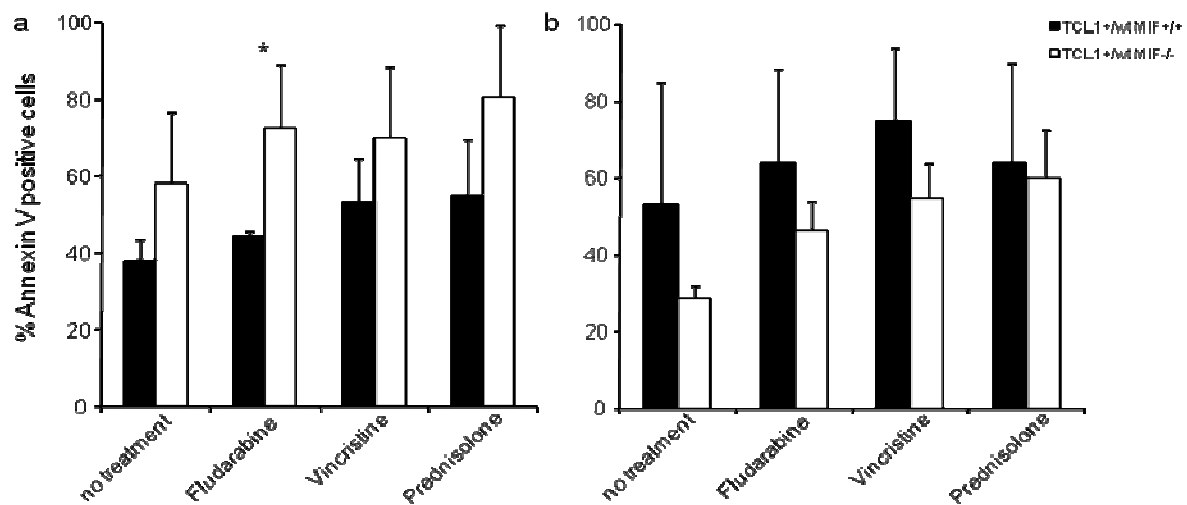


Figure 31: Drug induced apoptosis *ex vivo*. Splenocytes of pre-leukemic (a) and leukemic (b) mice were harvested from spleens of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice and treated with cytostatic drugs (50 μ M fludarabine, 50 ng/ml vincristine, 1 μ M prednisolone) for 24 h *ex vivo*. For each group and genotype n = 5 animals were analysed. * statistical significance with p = 0.04.

Splenocytes of pre-leukemic (n = 5) and leukemic (n = 5) animals of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice were treated with fludarabine (50 μ M), vincristine (50 ng/ml) and prednisolone (1 μ M) for 24 h and then analysed for apoptosis.

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Splenocytes from the pre-leukemic $TCL1^{+/wt} MIF^{-/-}$ mice are more sensitive to the induction of apoptosis than the cells from the pre-leukemic $TCL1^{+/wt} MIF^{+/+}$ mice. Also the rate of spontaneous apoptosis (no treatment) is higher in the MIF-deficient cells. In the example of the purine analog fludarabine, the apoptotic response is significantly pronounced and 72.7 % (± 16.5) of $TCL1^{+/wt} MIF^{-/-}$ cells undergo apoptosis in comparison to 44. % (± 1.3) of the cells from the $TCL1^{+/wt} MIF^{+/+}$ mice ($p = 0.04$) (Fig. 31 a). Prednisolone shows the best effects in the pre-leukemic cells, more than 80 % of the cells are dead after 24 h. At an earlier time point (8 h), differences between $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ were not apparent (data not shown).

Interestingly, for the splenocytes of the leukemic $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice, the response to DNA damage or glucocorticoid stimulation was quite opposite. Here cells undergo spontaneous apoptosis of about 64.2 % (± 24.1) in the MIF wildtype cells, whereas only 46.5 % (± 7.3) of the MIF knock out cells die after 24 h (Fig. 31 b). This observation extends also to unstimulated cells which exhibited reduced spontaneous apoptosis in the absence of MIF. Since previous studies had shown that MIF acts as an essential regulator of the DNA damage pathway (Nemajerova et al., 2007), we tested two different pathway inhibitors. On the one hand, we used NSC-663284, a complete CDC25 inhibitor. CDC25 phosphatases activate Cyclin-dependent kinases (CDKs) and thereby regulate the cell cycle. In mammals there are three CDC25 family members (CDC25A, B and C), which collaborate in coordinating timely activation of multiple cyclin/CDKs at different phases of the cell (Sadhu et al., 1990; Galaktionov and Beach 1991; Nagata et al., 1991; Boutros et al., 2007). CDC25A regulates the G1-S transition as well as the transition of G2-M (Hoffmann et al., 1994; Busino et al., 2004) making it an essential component for all phases of the cell cycle. CDC25B and C are essential regulators of the G2-M transition (Lammer et al., 1998; Perdiguero and Nebreda 2004). On the other hand we used Pifithrin- α , a reversible p53 inhibitor (Komarov et al., 1999) to find out whether the difference in the rate of apoptosis was dependent on the p53-pathway. Those two inhibitors were used in combination with fludarabine. As a third component we used the therapeutic proteasomal inhibitor bortezomib, currently in use for the treatment of multiple myeloma and mantle cell lymphoma. Bortezomib binds the 26S proteasome with high affinity and inhibits its function by regulating protein expression (e.g. NF- κ B) and degradation of ubiquitylated proteins. Since intracellular MIF inhibits the metalloprotease c-jun activating and binding protein-1 (Jab-1), it co-regulates the activity of the skip-cullin-F-box protein

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complex (SCF complex) and might affect proteasomal control of intracellular protein degradation (Fingerle-Rowson and Petrenko 2007; Nemajerova et al., 2007).

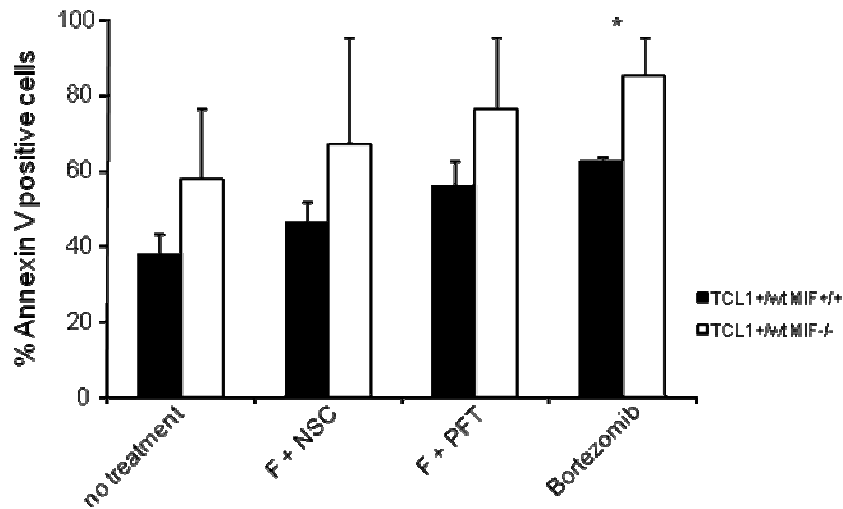


Figure 32: Apoptosis induction in pre-leukemic mice. Testing whether the apoptotic effect is dependent on either p53 or CDC25 with the inhibitors NSC-663284 (10nM) and PFT- α (10 μ M) in combination with 50 μ M fludarabine. Bortezomib (5 ng/ml), a proteasomal inhibitor shows significance after 24h of treatment ($p = 0.02$).

Treatment of the splenocytes in combination with fludarabine and the CDC25 inhibitor NSC-663284 as well as a combination of fludarabine and the p53 inhibitor PFT- α was not able to abrogate the difference between the two genotypes. Cells undergoing spontaneous or fludarabine-induced apoptosis still show the same differences in the apoptotic rates as without these inhibitors. These results indicate that those two pathways tested do not play the key role in apoptosis-induction for the MIF^{+/+} and MIF^{-/-} TCL1^{+/wt} mice. Also the proteasomal inhibitor bortezomib did not abrogate the difference in the two genotypes and therefore we conclude that MIF's intracellular activity of regulating the SCF complex is not responsible for the observed effects either (Fig. 32).

Taken together, while the *in vivo* TUNEL staining yielded clear results, the *ex vivo* experiments were somewhat contradictory within themselves and did not solve the question of which pathway might be involved in the increased apoptosis sensitivity of TCL1^{+/wt} MIF^{-/-} splenocytes *in vivo*.

4.10 Analysis of cellular proliferation

Differences in the incidence of leukemia may also reflect differences in the proliferative capacity of malignant cell. In order to determine the spontaneous rate of DNA synthesis as a surrogate marker for cell cycle entry $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ ($n = 10$ per genotype) were injected with bromo-desoxy-uridine (BrdU) *in vivo*. BrdU, a thymidin analog, intercalates with the DNA during the S-phase of the cell cycle and can be detected by anti-BrdU-specific antibodies. It was injected into the peritoneal cavity (i.p.) over 4 days at a concentration of 50 $\mu\text{g}/\text{kg}$ bodyweight. On day five, the animals were killed, splenocytes were taken out and stained for BrdU and further analysed by FACS. We measured the percentage of BrdU incorporation into the malignant clone ($CD5^+IgM^+$ cells).

In the pre-leukemic (aged 4 months, $n = 4$ for each genotype) splenocytes, no differences in the *in vivo* BrdU incorporation could be measured. Splenocytes of leukemic $TCL1^{+/wt} MIF^{-/-}$ mice ($n = 6$ for each genotype), however, incorporated more BrdU into the DNA of the $CD5^+IgM^+$ cells than their wildtype counterparts (13.9 % \pm 5.4 for $MIF^{+/+}$ vs. 37.4 % \pm 19.7 for $MIF^{-/-}$) (Fig. 33).

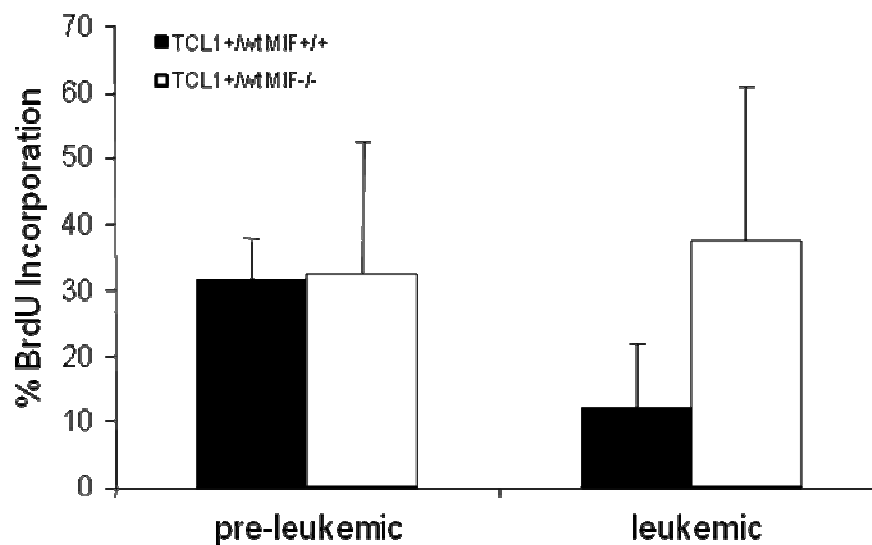


Figure 33: BrdU incorporation in splenocytes of pre-leukemic (month 4) and leukemic $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice. Although there was a trend towards increased BrdU uptake in MIF-deficient splenocytes, this difference was not significant.

To control this result of the *in vivo*-proliferation, we tested pre-leukemic splenocytes for *ex vivo* proliferation by incorporation of EdU. EdU is a similar component to BrdU, it also intercalates the DNA during the S phase and can be measured by flow cytometry. The cells

were stimulated with 1 $\mu\text{g/ml}$ of lipopolysaccharid for 24h, which stimulates B cell proliferation. After 24 hours, the splenocytes were pulsed with EdU and further incubated for another 24 hours. Cells were then stained according to the protocol from Invitrogen (Karlsruhe, Germany) and the analysis was done on the FACS Canto.

In this system, the $\text{TCL1}^{+/wt} \text{MIF}^{+/+}$ cells seemed to proliferate more vigorously than the $\text{TCL1}^{+/wt} \text{MIF}^{-/-}$ cells (336.3 ± 188.7 for $\text{TCL1}^{+/wt} \text{MIF}^{+/+}$ vs. 71.5 ± 9.2 for $\text{TCL1}^{+/wt} \text{MIF}^{-/-}$, $p = 0.13$) (Fig. 34).

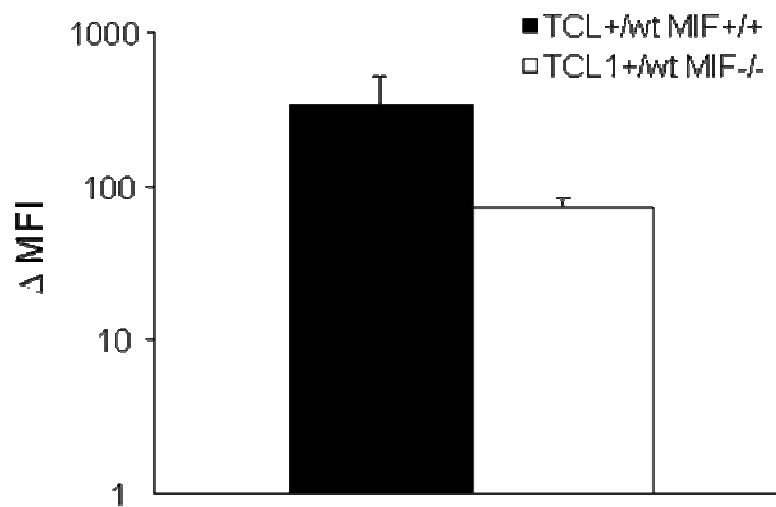


Figure 34: EdU incorporation into splenocytes *ex vivo*. EdU incorporation in splenocytes from pre-leukemic $\text{TCL1}^{+/wt} \text{MIF}^{+/+}$ ($n = 3$) and pre-leukemic $\text{TCL1}^{+/wt} \text{MIF}^{-/-}$ ($n = 3$) animals. Cells were stimulated with 1 $\mu\text{g/ml}$ LPS for 24 h, followed by a 24 h pulse of 7.5 $\mu\text{g/ml}$ EdU. Cells were then fixed, stained and analyzed by FACS. The difference between the two genotypes is not significant ($p = 0.13$).

4.11 Analysis of Metaphase-Chromosomes

TCL1 itself is a rather weak oncogene which by itself does not induce leukemia, but instead induces genomic instability and secondary genomic damage subsequently leading to leukemic development. Since MIF has been implicated in the regulation of genome protection (Nemajerova et al., 2007), we performed spectral karyotyping (SKY) of metaphase chromosomes from purified splenocytes of leukemic animals ($\text{TCL1}^{+/wt} \text{MIF}^{+/+}$ and $\text{TCL1}^{+/wt} \text{MIF}^{-/-}$) in cooperation with the group of Dr. C. Rudolph and Prof. Dr. B. Schlegelberger from the Institute for Cellular and Molecular Pathology of the Hannover Medical School (MHH).

Results

We were able to analyse $n = 3$ animals for the $TCL1^{+/wt} MIF^{+/+}$ and $n = 4$ animals for the $TCL1^{+/wt} MIF^{-/-}$ group. Overall, we observed trisomy, tetraploidy, deletions and translocations. The most prominent cytogenetic aberration found in both groups is the trisomy 15, manifested in two of three or four mice per genotype (table 2). We did not find significant differences in the number of malignant clones or type of the cytogenetic aberration between both groups.

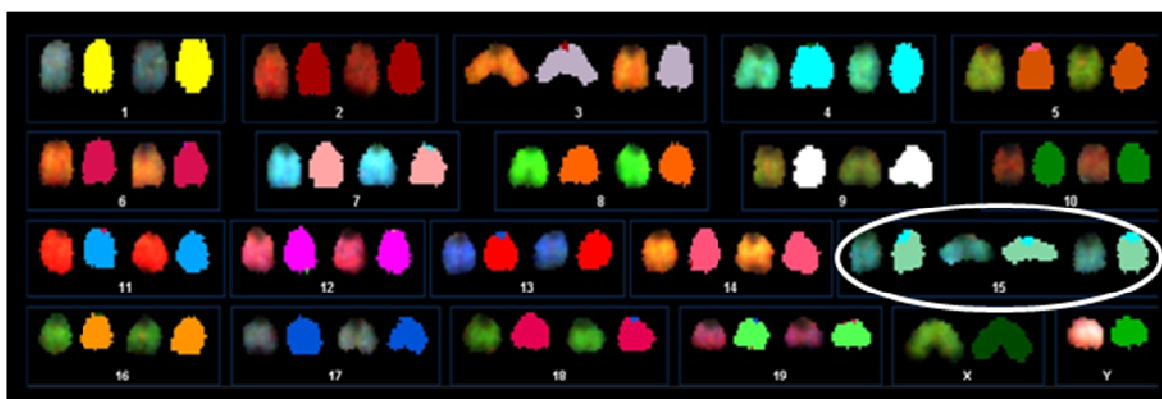


Figure 35: Representative example of a SKY-analysis of a $TCL1^{+/wt} MIF^{+/+}$ mouse. The picture displays the karyogram of the analyzed animal. Trisomy 15 was the most prominent aberration found throughout both groups.

Probe	Genotype	Cytogenetics
FIN 3558	$TCL1^{+/wt} MIF^{+/+}$	41,XY,+15 [6]/ 42-43,XY,+10,+Del(15) [cp5]/ 40,XY [4]
FIN 3587	$TCL1^{+/wt} MIF^{+/+}$	80<4n>,XXYY [8]/ 40,XY [7]
FIN 3568	$TCL1^{+/wt} MIF^{+/+}$	41,XY,+15 [14]
FIN 3275	$TCL1^{+/wt} MIF^{-/-}$	40,X,-X,+15 [2]/ 41,XX,+15 [1]/ 40,XX [8]
FIN 3319	$TCL1^{+/wt} MIF^{-/-}$	71~75,XXY,-Y,Der(1)T(1;?10), Der(4)T(4;?),Der(15)T(7;15),mar [cp9] /40,XY [4]/ 80<4n>,XXYY [2]
FIN 3317	$TCL1^{+/wt} MIF^{-/-}$	40,XY [10]
FIN 3555	$TCL1^{+/wt} MIF^{-/-}$	40,X,-Y,+15 [9]/ 41,XY,+15 [5]

Table 2: SKY analysis of metaphase chromosomes from splenocytes of $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice.

Spectral karyotyping was also done on prepared metaphases of splenocytes from leukemic $TCL1^{+/+}$ mice (table 3). Here we were able to analyse $n = 9$ animals. As seen in the

Results

TCL1^{+wt} MIF^{+/+} and TCL1^{+wt} MIF^{-/-} mice, the trisomy 15 is a recurrent finding of all groups. In the TCL1^{+/+} mice, it is manifested in nearly 80 %, giving it a large impact on the leukemia development. Also the tetraploidy is a frequent finding. The important result of the analysis of the homozygous TCL1 mice is the occurrence of even more and partly pretty severe genetic aberrations. Those findings need to be further analysed as some of the findings have not been described.

Probe	Genotype	Cytogenetics
FIN 1486	TCL1 ^{+/+}	41,XY,+X [2]/ 39~42,X,-Y,-1,+6,?T(8;10),Del(8), Der(14)T(1;14),+15,+15,Ins(16;?) [cp13]
FIN 1913	TCL1 ^{+/+}	40,XX,-12,+15 [12]/ 80,<4n>,XXXX [3]
FIN 2142	TCL1 ^{+/+}	39~42,XY,Der(8)T(8;18),Del(13),Der(18)T(13;18) or T(13;18),+15 [cp10] 41~42,XY,Del(5),+6,+13,Der(16)T(16;5;16) [cp3] 40~41,XY,Der(14)T(13;14) [3] 41~42,XY,Del(5),Der(6)T(6;11;?6;11),-11,+13 [2]
FIN 2681	TCL1 ^{+/+}	41~43,XX,T(2;2),+9,+15,+18,+19 [cp15]
FIN 3094	TCL1 ^{+/+}	41,XY,+15 [6]/80,<4n>,XX,YY [4]/41,XY,+X [1]/40,XY [4]
FIN 3147	TCL1 ^{+/+}	40,XY,Ins(19;16) [15]
FIN 3190	TCL1 ^{+/+}	40-44,X,?Del(X) or Del(5),?Der(2)T(2;5),Der(5)T(5;11), Der(11)T(2;11),+14,+15,+15,Der(17)T(16;17),+19 [cp11]/ 40,X0, T(1;8),+15 [2] /40,XY [1]
FIN 3267	TCL1 ^{+/+}	38-39,XX T(4;9),Del(5),Ins(6;?5),T(6;13),Der(16)T(12;16) [cp11]/ 40,XX [4]
FIN 3836	TCL1 ^{+/+}	41,XY,T(12;12),+15 [cp13]/ 40,XX [1]/ 40,XX,Der(17) [1]

Table 3: SKY analysis of metaphases from leukemic TCL1^{+/+} mice. A high number of chromosomal aberrations is found in these splenocytes. Trisomy 15 and tetraploidy are the most prominent ones.

4.12 Expression analysis of known tumor regulators in leukemic and pre-leukemic mice

On murine chromosome 15, the proto-oncogene *c-myc* is known to be located. *C-myc* is an important transcription factor and regulator of cell cycle control (Obaya et al., 1999). To further evaluate whether *c-myc* protein is upregulated in TCL1-induced leukemia, western blot analysis of leukemic and pre-leukemic protein-lysates from splenocytes were analyzed for *c-myc* expression in the nuclear extracts. We also wanted to know whether other tumor-regulators such as B cell lymphoma-2 (*Bcl-2*), *MDM2* and *p53* were upregulated or altered in the mice.

Cytoplasmic and nuclear extracts of splenocytes from pre-leukemic and leukemic $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice were done. To determine the protein levels of tumor genes and the altered pathways, the protein lysates were separated on a SDS-PAGE and analysed by incubation with specific antibodies. As positive control we used Raji cells, a cell line derived from Burkitt lymphoma, belonging to the aggressive B cell non-Hodgkin lymphomas (lane 1 in the leukemic WB (Fig. 36 a) and the last lane for the pre-leukemic (Fig 35 .b) lysates).

Western analysis of protein lysates from pre-leukemic and leukemic mice showed few differences between $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice. For the leukemic mice (Fig. 36 a) we could show, that the in the $TCL1^{+/wt} MIF^{-/-}$ mice, the anti-apoptotic protein *Bcl-2* is upregulated. More interestingly though is the observation that we found a strong overexpression of the tumor suppressor *p53* in the $TCL1^{+/wt} MIF^{+/+}$ lysates.

With respect to *c-myc* expression, we could not detect any significant differences between the genotypes, but we see significant overexpression of *c-myc* in individual mice which needs to be further correlated to the presence of the trisomy 15 in the animals.

Also in the lysates of the pre-leukemic splenocytes (Fig. 36 b) we see a similar picture emerging. Again, we find more *Bcl-2* in the $TCL1^{+/wt} MIF^{-/-}$ lysates, and *p53* is again upregulated in the *MIF* wildtype mice.

The observed *p53* overexpression is reminiscent of mutated *p53* and experiments are ongoing to determine the mutational status of *p53* in our animals.

Results

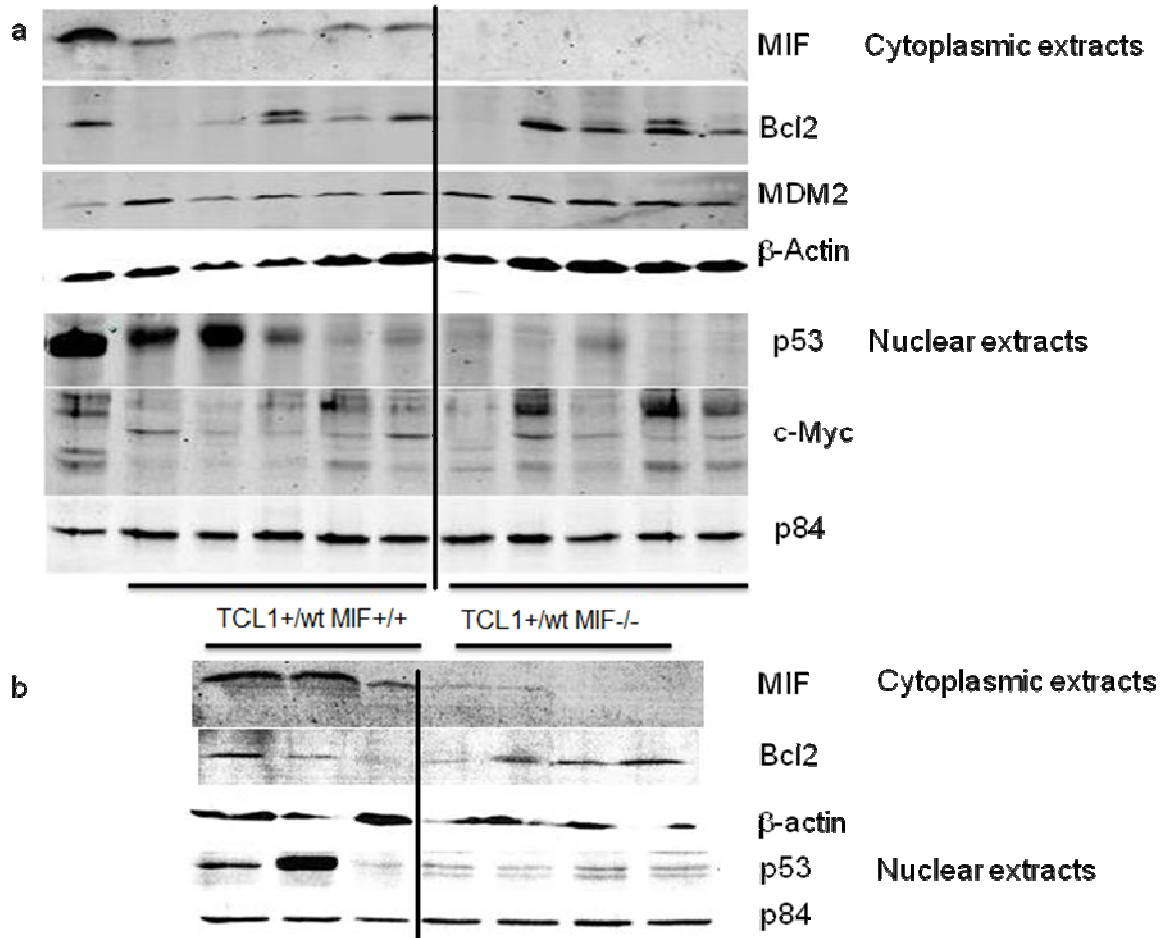


Figure 36: Western blot analysis of cytoplasmic and nuclear cell lysates of leukemic (a) and pre-leukemic (b) $TCL1^{+/wt}$ $MIF^{+/+}$ and $TCL1^{+/wt}$ $MIF^{-/-}$ mice. MIF (1:1000), Bcl-2 (1:1000), MDM2 (1:200) were measured from cytosolic lysates, p53 (1:200), c-myc (1:200) from nuclear lysates. p84 (1:1000) served as loading control for nuclear proteins, β -actin (1:3000) as loading control for cytosolic proteins. Lane 1 (a) and lane 7 (b) positive control (Raji cell line). 60 μ g of protein extracts were loaded onto the gels.

5 Discussion

Chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world has remained a non-curable malignant disease which significantly reduces the quality of being and the life expectancy of the affected individuals. Thus, the impetus to study CLL pathogenesis and to improve therapeutic options remains high in the field of CLL research.

Until 2002, all evidence generated on CLL pathobiology was based on *ex vivo* analysis of CLL cells and clinical evidence from CLL patients. Molecular targets were validated in the artificial context of *in vitro*-culture or in human studies. With the advent of the E μ -TCL1 transgenic mouse model for CLL in 2002 (Bichi et al., 2002), CLL researchers obtained a new research tool that allows the genetic validation of therapeutic targets before proceeding to human phase I/II trials. The TCL1 mouse has also proven useful for the testing of new therapeutic drugs (Johnson et al., 2006).

We were prompted to study the role of Macrophage Migration Inhibitory Factor (MIF) in the pathogenesis of the murine CLL since we knew that MIF acts as a functional link between inflammation and tumorigenesis and that survival of CLL is strongly dependent on the stimulation by chemokines and chemokine receptors (Burger et al., 2000). Although the nature of MIF remained uncertain for many years and it was discussed to be a hormone, cytokine or enzyme (Swope and Lolis 1999), more and more evidence emerges that MIF functions as an extracellular chemokine-like molecule which binds to the surface receptors CD74/CD44 and CXCR2 (Bernhagen et al., 2007). Both these surface receptors are known to be involved in leukemogenesis/lymphomagenesis, but have not been studied in the context of CLL.

Taken together, our results show that MIF acts as a relevant promoter of CLL development. Specifically, MIF shortens the pre-leukemic phase and increases the incidence of leukemia, promotes organ infiltration by the malignant cells and increases the tumor load. Acceleration of disease by MIF is substantial since it shortens overall survival of mice by 80 days. This amounts to about 8 % of a mouse's life time of approximately 1000 days (ref. Jackson labs for mouse life span). To our knowledge MIF is the first target molecule for which a survival effect has been shown. Given the almost ubiquitous expression of MIF and its many known effects on proliferation, apoptosis, differentiation or malignant transformation, the big question in this project was to elucidate the mechanism by which MIF promoted leukemia development.

5.1 MIF promotes the accumulation of CD68⁺ macrophages

It has been shown that the expansion and survival of CLL cells depends on the presence of “nurse-like” cells (NLCs) that seem to be monocyte-derived components of the marrow stroma (Burger et al., 2000; Tsukada et al., 2002). NLCs express cytoplasmic vimentin and stromal-derived factor 1 (SDF-1) and SDF-1 is known to interact with CXCR4, one of the MIF chemokine receptors (Amara et al., 1999; Mohle et al., 1999). Despite expressing antigens in common with blood monocytes, monocyte-derived dendritic cells, and macrophages, NLCs expressed significantly higher levels of CD68 than some of these other cell types.

In the immunohistochemical staining of infiltrated organs, T- and B-lymphocyte infiltrations were found in both groups of mice, but the number of macrophages is significantly reduced in the infiltrated areas of the spleen and bone marrow in the absence of MIF. Also in the non-infiltrated areas of these organs, the number of macrophages is reduced. This effect is not entirely novel, since it was described before, that MIF acts as a survival factor for macrophages during stress (Mitchell et al., 2002). It is further known that MIF blockade inhibits the accumulation of CD68⁺ cells (Schober et al., 2004; Burger-Kentischer et al., 2006). In addition to that, the production of MIF by bone marrow residing dendritic cells was recently shown to be essential for the presence of mature B-cells in the bone marrow of mice (Sapoznikov et al., 2008).

Macrophages produce a number of cytokines and substances which may promote the survival of B cells. This process is mediated by a number of cytokines, e.g. tumor necrosis factor- α or interleukin-1 and on the other hand mediated by a variety of chemokines and cell adhesion molecules.

The CLL-like disease in our model expresses the MIF receptors CD74/CD44 and CXCR2, all of which are highly relevant for leukemogenesis and survival of malignant cells. The expression of CD44 and CXCR2 was even upregulated in the malignant CD5⁺IgM⁺ cells compared to non-malignant IgM⁺ cells.

CD74 is expressed on B cells and directly involved in the survival of the mature B cell population (Shachar and Flavell 1996; Matza et al., 2002; Matza et al., 2003). CD44, a co-receptor of the CD74 receptor complex, is highly upregulated in the leukemic mice, and CD44 surface expression in human CLL is known to be correlated with a significantly poorer survival (De Rossi et al., 1993; De Rossi et al., 1994). Recently, colleagues of mine could show that CD44 acts as a survival factor for CLL cells in culture by activating CD44

with its ligands hyaluronic acid and chondroitin sulphate (Peer Zada et al., manuscript in preparation).

The chemokine receptor CXCR2, a leukocyte-specific receptor of MIF, is also significantly upregulated in the leukemic mice and is known to play a central role in macrophage accumulation in atherosclerosis and the progression of the atherosclerotic lesions (Boisvert et al., 2006).

CXCR2 has several ligands, a prominent one being interleukin-8 (IL-8) which has been identified as a component of the signaling cascade triggered by activated CD74 which leads to apoptosis resistance in human CLL (Binsky et al., 2007; Gore et al., 2008). Binding of MIF to CXCR2 recruits leukocytes to the inflammatory sites of atherosclerosis and targets monocytes and neutrophils (Bernhagen et al., 2007; Dewor et al., 2007).

In analogy to these effects, it is conceivable that CXCR2 or CD74/CD44 also contribute to the accumulation of nurse-like cells in our model in the presence of MIF. Further genetic studies are ongoing in the TCL1 mouse model which focus on the role of CD74, CD44 and CXCR2 in CLL development and nurse-like cell function.

5.2 MIF inhibits apoptosis *in vivo* and *in vitro*

The increased presence of CD68⁺ macrophages in normal and leukemic organs suggests that the surrounding B cells may receive enhanced pro-survival signals *in vivo*. Although CLL is characterized by a defect in the apoptotic machinery of the cell (Kitada et al., 1998), the CLL cells are nevertheless still prone to apoptosis and require constant stimulation by pro-survival factors in order to overcome spontaneous apoptosis. A higher rate of apoptosis may therefore be due to less anti-apoptotic stimulation.

Our *in vivo*-experiment reveals that the rate of spontaneous apoptosis is increased in MIF-deficient mice. The *ex vivo* experiments with pre-leukemic and leukemic splenocytes were less clear which may be due to MIF present in the FCS of the media used. Nevertheless, the treatment of splenocytes with cytostatic drugs commonly used in the treatment of CLL showed that pre-leukemic splenocytes had a near significant trend to higher apoptosis in the absence of MIF.

A higher threshold for apoptosis in MIF-expressing animals or cells might be the reason for the accelerated accumulation of the malignant clone, leading to increased tumor load and reduced overall survival. We currently do not have an explanation why leukemic MIF-

deficient splenocytes were less responsive to DNA damage. Further experiments are required to clarify the role of MIF in apoptosis regulation of leukemic cells.

Treatment of the splenocytes *ex vivo* with the p53-inhibitor PFT- α and the CDC25-inhibitor NSC during DNA damage induction with cytostatic drugs did not show any differences in the pre-leukemic cells. In both groups, cells behaved the same as with the normal fludarabine-treatment. This indicates that the mechanism of the cells undergoing apoptosis is p53-independent and also independent of CDC25. CDC25 phosphatase regulates the checkpoints of the G1-S and the G2-M transition and is known to be upregulated in different aggressive tumors and tumors with poor prognosis (Kristjansdottir and Rudolph 2004). We have indications that genetic inactivation of p53 may occur in the leukemias since we found cases with strongly overexpressed p53, a typical feature of mutated p53 (de Vries et al., 2002). Nevertheless, formal proof by sequencing is still outstanding. Alternatively, functional inactivation of p53 may also occur via p53 inhibitory molecules such as MIF (Hudson et al., 1999; Fingerle-Rowson et al., 2003; Talos et al., 2005). In this context, it is noteworthy, that p53 overexpression occurred more frequently in MIF-expressing than in MIF-deficient leukemias.

Bortezomib, a well known proteasomal inhibitor was chosen to test whether apoptosis induction was dependent on the known ability of MIF to increase SCF-dependent ubiquitylation and proteasomal degradation. Like the cytostatic drugs, bortezomib also induces apoptosis in CLL cells *in vitro* (Liu et al., 2008), effectively killed the malignant splenocytes but failed to abolish the difference. Still the $TCL1^{+/wt}$ $MIF^{-/-}$ splenocytes show a significantly higher rate of apoptotic cells after 24 h in comparison to the $TCL1^{+/wt}$ $MIF^{+/+}$ cells ($p = 0.02$). Thus, the use of pathway inhibitors did not help to get further insights into the mechanism of MIF action in apoptosis of CLL cells. It has been shown recently that activation of CD74 via MIF leads to NF- κ B activation which may also affect the rate of apoptosis in response to DNA damage. Therefore, additional experiments looking at NF- κ B pathway activation in CLL are required in order to find out whether MIF involves the NF- κ B pathway *in vivo*.

5.3 MIF and *in vitro* and *in vivo* proliferation

Our study of MIF-dependent proliferation in CLL yielded a more complex picture of what might be going on *in vivo*.

We found no differences in the rate of BrdU incorporation in B cells of the pre-leukemic animals of the $TCL1^{+/wt} MIF^{+/+}$ and the $TCL1^{+/wt} MIF^{-/-}$ group. In contrast, in leukemic animals splenocytes from the MIF knockout mice showed more BrdU incorporation than the wildtype cells. This contrast to the effects seen in the apoptosis assay, but MIF is also described to act as a growth promoting molecule (Mitchell et al., 2002; Calandra and Roger 2003; Morand 2005; Swant et al., 2005; Bernhagen et al., 2007). We cannot prove but we speculate that the leukemic cells of the $MIF^{-/-}$ animals have a combined defect in cell cycle regulation, with both an increased rate of proliferation and an increased rate of apoptosis at the same time. Such effects may occur in molecules which regulate the G2-M checkpoint. Indeed, MIF has been found recently to act as an important regulator of the G2-M checkpoint on a p53-deficient background (Nemajerova et al., 2007).

5.4 Spectral karyotyping highlights the importance of trisomy 15 in $TCL1$ -driven leukemogenesis

Typically, a dysregulation of G2-M checkpoint function leads to an accumulation of genomic damage in the following generation of daughter cells, and we therefore tested by SKY analysis whether the $TCL1^{+/wt} MIF^{-/-}$ mice had accumulated more severe chromosomal aberrations. However, our set of SKY analyses in $TCL1^{+/wt} MIF^{-/-}$ and $TCL1^{+/wt} MIF^{+/+}$ spleens could not prove this hypothesis, yet, but this may require a larger number of analyses.

Nevertheless, in $TCL1^{+/+}$ leukemic mice we found an almost 100 % incidence of trisomy +15, whereas all other genomic aberrations were less frequent. This suggests that trisomy +15 may be an important secondary genetic event leading to CLL development. The trisomy +15 aberration is described for many different mouse models, e.g. a model for Down's syndrome (Morice et al., 2008) and also in the pE μ -B29- $TCL1$ mouse model, where $TCL1$ is overexpressed in T and B cells (Shen et al., 2006). In both cases, this trisomy goes ahead with overexpression of c-myc.

It is known that the protooncogene c-Myc is located on murine chromosome +15 (Bain et al., 1997; Linardopoulos et al., 2000; Marinkovic et al., 2004). C-myc plays a pivotal role in the regulation of proliferation, differentiation and apoptosis (Askew et al., 1991; Shi et al., 1992; Evan and Littlewood 1993; Hoffman and Liebermann 1998). Alterations of c-myc on the protein-level are associated with many hematological malignancies, e.g. Burkitt's lymphoma, T-ALL (T-cell acute lymphoblastic leukemia) or multiple myeloma

(Boxer and Dang 2001). Overexpression of c-myc is known to lead to progression in the G1-S phase of the cell cycle (Perez-Roger et al., 1999). In our mice, however, we do not observe a significant upregulation of c-myc in leukemic spleens. Currently, experiments are ongoing which measure c-myc gene expression in TCL1-induced leukemias of MIF wildtype and knockout mice.

5.5 Outlook

The role of MIF in the pathogenesis of murine CLL is partly explained by the results of this work. MIF acts as a tumor promoter and it recruits macrophages as potential nurse-like cells to the infiltrate which in turn protect CLL cells from looming apoptosis. Our work also depicts downstream effects of MIF such as its contribution to apoptosis resistance, proliferation and clonal cytogenetic evolution of the leukemia. Our results bring also the tumor suppressor p53, the protooncogene c-myc as well as the surface receptors CD74, CD44 and CXCR2 into the larger picture of this mouse model.

A lot more, but fascinating questions have been raised by this work. We will continue on this avenue primarily focussing on the role of MIF for nurse cell function in CLL. In addition, we are running experiments which aim at elucidating the contribution of CD74, CD44 and CXCR2 to the development of murine CLL.

Our hope is that this work will provide a novel detail to the widening picture of how CLL develops and evolves in humans. With MIF being an extracellular molecule, we believe that targeting MIF in humans may be technically possible and scientifically reasonable for the treatment of human CLL.

6 Material

6.1 Instruments

Automatic pipettes	Gilson, Middleton, WI, USA
Balance	Ohaus, Pine Brook, NJ, USA
Cell counting chambers	Neubauer, Germany
Cell freezing containers	Nalgene, Neerijse, Belgium
Centrifuge (benchtop and 4°C)	Eppendorf, Hamburg, Germany
Centrifuge (cell culture)	Eppendorf, Hamburg, Germany
Electrophoresis system DNA	BioRad, Hercules, CA, USA
Electrophoresis system Protein	Invitrogen, Karlsruhe, Germany
Elisa-Reader	µQuant, BioTek, Bad Friedrichshall, Germany
Film-developer	AGFA, Cologne, Germany
Films	Amersham Bioscience, Buckinghamshire, UK
Flow Cytometers	
FACS Calibur	BD Biosciences, Heidelberg, Germany
FACS Canto	BD Biosciences, Heidelberg, Germany
Fridge and Freezer	
4°C & -20°C	AEG, Stockholm, Sweden
-80°C	Sanyo, Wood Dale, IL, USA
Heater/ Magnetic Shaker	Heidolph, Schwabach, Germany
Incubator (mammalian cell culture)	Labotect, Göttingen, Germany
Laminar flow hood	Heraeus, Hanau, Germany
Microscope	Axiophot, Zeiss, Göttingen, Germany
	Leica DM4000B, Leica Camera AG, Solms, Germany
N ₂ -storage tank	Thermo Scientific, Dubuque, IA, USA
PCR machine (Mastercycler gradient EP S)	Eppendorf, Hamburg, Germany
pH-Meter	Mettler-Toledo, Schwerzenbach, Germany
Shaker	Heidolph, Schwabach, Germany
Spectrophotometer	BioRad, Hercules, CA, USA
Thermomixer	Eppendorf, Hamburg, Germany
Vortex	VWR, Darmstadt, Germany
Waterbath	Medingen, Freital, Germany
X-ray film Exposure cassette	AGFA, Cologne, Germany

6.2 Materials

Gel blotting paper	Schleicher & Schuell, Dassel, Germany
Hyperfilm ECL	Amersham Biosciences, Buckinghamshire, UK

Parafilm	American National Can, IL, USA
Photometer cuvettes	Sarstedt, Numbrecht, Germany
Tissue culture material	
Cell strainer	BD Biosciences, Heidelberg, Germany
Cryotube vials	Nunc, Roskilde, Denmark
Cell scraper	TPP, Trasadingen, Switzerland
Flasks	BD Biosciences, Heidelberg, Germany
Pipette tips, tubes, dishes	Sarstedt, Numbrecht, Germany

6.3 Chemicals and Reagents

2-Mercaptoethanol	Sigma, Steinheim, Germany
5'-Bromo-desoxy-uracil (BrdU)	Sigma, Steinheim, Germany
Acetic Acid	Carl Roth GmbH & Co, Karlsruhe, Germany
Acrylamid 30%	Carl Roth GmbH & Co, Karlsruhe, Germany
Agarose	Biozym, Hess. Oldendorf, Germany
Ammonium peroxdisulfate (APS)	Carl Roth GmbH & Co, Karlsruhe, Germany
Bromphenol blue	Carl Roth GmbH & Co, Karlsruhe, Germany
Bovine Serum albumin	Appllichem, Darmstadt, Germany
Calcium chloride	Merck, Darmstadt, Germany
DMEM	Gibco/Invitrogen, Karlsruhe, Germany
DMSO	Sigma, Steinheim, Germany
ECL	Amersham Biosciences, Buckinghamshire, UK
Ethanol	Carl Roth GmbH & Co, Karlsruhe, Germany
Ethidiumbromide	Carl Roth GmbH & Co, Karlsruhe, Germany
Ethylendiamine tetra acetic acid (EDTA)	Carl Roth GmbH & Co, Karlsruhe, Germany
Fetal Calf Serum (FCS)	Gibco/Invitrogen, Karlsruhe, Germany
Glycerol	Carl Roth GmbH & Co, Karlsruhe, Germany
Glycine	Carl Roth GmbH & Co, Karlsruhe, Germany
Hepes	Carl Roth GmbH & Co, Karlsruhe, Germany
Hydrochloric acid	Carl Roth GmbH & Co, Karlsruhe, Germany
Isopropanol	Carl Roth GmbH & Co, Karlsruhe, Germany
Magnesium chloride	Carl Roth GmbH & Co, Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co, Karlsruhe, Germany
Molecular weight marker	MBI-Fermentas, St. Leon-Rot, Germany
MTT	Sigma, Deisenhofen, Germany
Non fat dry milk powder	Carl Roth GmbH & Co, Karlsruhe, Germany
Nonidet P-40 (NP-40)	Sigma, Deisenhofen, Germany
Paraformaldehyde	Sigma, Deisenhofen, Germany
PBS	Gibco/Invitrogen, Karlsruhe, Germany
PCR Primers	Metabion, Martinsried, Germany

Penicillin/Streptomycin	Gibco/Invitrogen, Karlsruhe, Germany
Ponceau S	Carl Roth GmbH & Co, Karlsruhe, Germany
Potassium Chloride	Merck, Darmstadt, Germany
Prestained protein marker	MBI-Fermentas, St. Leon-Rot, Germany
RPMI	Gibco/Invitrogen, Karlsruhe, Germany
Sodium chloride	Carl Roth GmbH & Co, Karlsruhe, Germany
Sodium dodecylsulfate (SDS)	Serva Electrophoresis GmH, Heidelberg, Germany
Sodium pyruvate	Gibco/Invitrogen, Karlsruhe, Germany
TEMED	Carl Roth GmbH & Co, Karlsruhe, Germany
Tris –(hydroxymethyl)-aminomethane	Carl Roth GmbH & Co, Karlsruhe, Germany
Triton X-100	Sigma, Deisenhofen, Germany
Trypan Blue	Gibco/Invitrogen, Karlsruhe, Germany
Trypsin/EDTA	Gibco/Invitrogen, Karlsruhe, Germany

6.4 Antibodies

6.4.1 Antibodies for Western Blotting

Antibody specificity	Species	Supplier
Akt	Rabbit polyclonal	CellSignaling Technology, MA, USA
ATM	Mouse monoclonal	Novus Biologicals, Littleton, CO, USA
Bcl-2	Mouse monoclonal	Santa Cruz Biotechnology, CA, USA
c-myc	Rabbit polyclonal	Santa Cruz Biotechnology, CA, USA
ERK1/2 (p44/42)	Rabbit polyclonal	CellSignaling Technology, MA, USA
GAPDH	Rabbit polyclonal	Santa Cruz Biotechnology, CA, USA
MDM2	Mouse monoclonal	Santa Cruz Biotechnology, CA, USA
MIF	Rabbit polyclonal	Invitrogen, Karlsruhe, Germany
p19ARF	Rabbit polyclonal	Novus Biologicals, Littleton, CO, USA
p53	Rabbit polyclonal	VisionBiosystems Novocastra, Newcastle, UK
Phospho-Akt	Rabbit monoclonal	CellSignaling Technology, MA, USA
Phospho-ERK1/2 (Phospho-p44/42)	Rabbit monoclonal	CellSignaling Technology, MA, USA
β -actin	Mouse monoclonal	Sigma, Deisenhofen, Germany
β -tubulin	Rabbit polyclonal	Santa Cruz Biotechnology, CA, USA
TCL1	Mouse monoclonal	Provided by Dr. M. Herling

Secondary Antibodies

Anti-mouse-IgG	HRP-conjugated, goat	Dako Cytomation, Glostrup, Denmark
Anti-rabbit-IgG	HRP-conjugated, swine	Dako Cytomation, Glostrup, Denmark

Anti-mouse- IgG	Biotinylated	Dako Cytomation, Glostrup, Denmark
Anti-rabbit-IgG	Biotinylated	Dako Cytomation, Glostrup, Denmark
Anti-rat- IgG	Biotinylated	Dako Cytomation, Glostrup, Denmark

6.4.2 Antibodies for FACS-Analysis

Directly Labeled Antibodies

CD19	APC-Cy7-conjugated	BD Pharmingen, Heidelberg, Germany
CD21/CD35	PE	BD Pharmingen, Heidelberg, Germany
CD23	PE-conjugated	BD Pharmingen, Heidelberg, Germany
CD25	APC	BD Pharmingen, Heidelberg, Germany
CD3	PE-Cy7-conjugated	BD Pharmingen, Heidelberg, Germany
CD4	PE-conjugated	BD Pharmingen, Heidelberg, Germany
CD44	PE-conjugated	BD Pharmingen, Heidelberg, Germany
CD45R/B220	APC-conjugated	BD Pharmingen, Heidelberg, Germany
CD45R/B220	PerCP-conjugated	BD Pharmingen, Heidelberg, Germany
CD5	PerCP-conjugated	BD Pharmingen, Heidelberg, Germany
CD74	FITC-conjugated	BD Pharmingen, Heidelberg, Germany
CD8a	FITC	BD Pharmingen, Heidelberg, Germany
CD93 (AA4.1)	FITC-conjugated	BD Pharmingen, Heidelberg, Germany
CXCR2	PE-conjugated	BD Pharmingen, Heidelberg, Germany
IgD	PE-conjugated	BD Pharmingen, Heidelberg, Germany
IgM	FITC-conjugated	BD Pharmingen, Heidelberg, Germany
IgM	APC-conjugated	BD Pharmingen, Heidelberg, Germany
Isotype control Rat IgG _{2b}	FITC-conjugated	BD Pharmingen, Heidelberg, Germany
Isotype control Rat IgG _{2b}	PE-conjugated	BD Pharmingen, Heidelberg, Germany

6.4.3 Antibodies for Immunohistochemistry

CD3	Rabbit	Thermo Fisher Scientific GmbH, Schwerte, Germany
CD5	Rabbit	Thermo Fisher Scientific GmbH, Schwerte, Germany
CD20	Mouse	Thermo Fisher Scientific GmbH, Schwerte, Germany
CD68	Mouse	Thermo Fisher Scientific GmbH, Schwerte, Germany
CD79a	Rabbit	Thermo Fisher Scientific GmbH, Schwerte, Germany
p53	Rabbit	Thermo Fisher Scientific GmbH, Schwerte, Germany

6.5 Mouse strains

MIF ^{-/-} mouse	Homozygous knockout of the MIF gene	Fingerle-Rowson et al., 2003
MIF ^{+/+} mouse	Wildtype control	C57Bl/6, Charles River
TCL1 ^{+/+} mouse	Transgenic overexpression of TCL1	Bichi et al., 2002

6.6 Primers for Genotyping

MIF-A1	5' – AGG TTA GTC ACT CTA CTG GCC – 3'
MIF-B1	5' – TCT CAC TGT TCT GGT GTG AGG – 3'
MIF-C1	5' – GGC TCC TGG TCT CAG TCA GG – 3'
TCL1-reverse	5' – CAT CTG GCA GCA GCT CGA – 3'
TCL1-universal	5' – GCC GAG TGC CCG ACA CTC – 3'

6.7 Special Reagents and Kits

Reagents/Kits	Supplier
Annexin V-PE Apoptosis detection kit	BD Bioscience, Heidelberg, Germany
BrdU Flow kit	BD Bioscience, Heidelberg, Germany
ClickIt EdU detection kit	Invitrogen, Karlsruhe, Germany
ECL Detection Reagents	Amersham Bioscience, Buckinghamshire, UK
Mouse on Mouse Kit	Thermo Fisher Scientific GmbH, Schwerte, Germany
Protease Inhibitor Cocktail	Roche, Mannheim, Germany
DeadEnd Fluorimetric TUNEL assay	Promega, Mannheim, Germany
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Pierce Biotechnology, Rockford, IL, USA
NSC-663284	Tocris Bioscience, Ellisville, MO, USA
PFT- α	Sigma, Deisenhofen, Germany

6.8 Abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
APS	Ammoniumpersulfate
BPB	Bromphenol blue
BrdU	5'-Bromo-desoxy-uracil
CD	Cluster of Differentiation
CDK	Cyclin dependent Kinase
CDKI	Cyclin dependent Kinase inhibitor
CLL	Chronic lymphocytic Leukemia
DDT	D-Dopachrome Tautomerase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Desoxyribonucleic acid

Material

EDTA	Ethylene diamine tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	fetal calf serum
Ig	Immunoglobulin gene
Jab1	c-Jun-activating binding protein-1
LPS	Lipopolysaccharid
MAPK	Mitogen activated protein kinase
MEF	murine embryonal fibroblast
MIF	macrophage migration inhibitory factor
MIF ^{-/-}	MIF-knockout mouse
MIF ^{+/+}	MIF-wildtype mouse
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NSC-663284	6-Chloro-7-[[2-(4-morpholinyl)ethyl]amino]-5,8-quinolin edione
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFT-α	2-(2-Imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-p-tolyethanone hydrobromide
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
RPMI	Roswell Park Memorial Institute Medium
SDS	Sodiumdodecylsulfate
SDS-PAGE	SDS-Polyacrylamide gelelectrophoresis
TBS	Tris buffered saline
TCL1	T-cell leukemia 1
TCL1 ^{+/+}	T-cell leukemia 1 transgenic mouse, homozygous
TCL1 ^{+wt}	T-cell leukemia 1 transgenic mouse, heterozygous
TCR	T-cell receptor
Tris	tris-(hydroxymethyl)-aminomethane
ZAP-70	zeta-chain associated protein-70

6.9 Software

BD FACS DIVA

BD Cellquest Pro

Endnote

KC4

Microsoft Office

Statistica

SPSS

7 Methods

7.1 Breeding

Breeding of MIF^{-/-} animals to TCL1^{+/+} transgenic mice was done in the animal facility of surgery which is run by the Clinic I of Internal Medicine at the University Hospital of Cologne.

The two strains were bred with each other to obtain TCL1^{+/wt} MIF^{-/-} and TCL1^{+/wt} MIF^{+/+} animals. As the TCL1^{+/+} mouse was bred on a B6C3H background and the MIF^{-/-} mouse was developed on a C57Bl/6 background, the breeding strategy resulted in a mixed genetic background.

The animals were kept at a controlled atmosphere. Semi-annual health checks showed that the mice were virus-free and only carried Helicobacter species as well as Pasteurella pneumonia without clinical signs of disease. The animals were allowed to feed and drink ad libitum and were held on a special breeding diet. They were kept in type II cages at groups of four to five mice.

Breeding was done on a one to one basis and pups were weaned after 21 days.

7.2 Genotyping

Genotyping of the animals is necessary to determine the correct experimental animals from the controls and is done by polymerase-chain reaction (PCR). At the time of weaning, a piece of the tail is cut; DNA is prepared and the region of interest amplified by PCR with gene-specific primers.

7.2.1 DNA-Preparation

DNA from mouse tails was prepared: A piece of tail is put into a tube and lysed over night in 500 µl of lysis buffer plus 10 µl of Proteinase K (1 mg/ml) in a thermomixer at 56 °C and 700 rpm.

The next morning the digested tail is centrifuged at 13000 rpm for 10 minutes. The supernatant is put into a new cup with 500 µl of isopropanol, mixed and again centrifuged for 10 min at 13000 rpm. Discard supernatant and wash pellet twice with 200 µl of 70 % ethanol. Discard ethanol and dry pellet for 30 minutes at room temperature. When the

pellet is dry (no more smell of ethanol) it was resuspended pellet in 100 µl of TE-buffer. Leave the DNA at 4 °C over night to dissolve.

DNA lysis buffer: 100 mM Tris, pH 8.5
 5 mM EDTA
 0.2 % SDS
 200 mM NaCl
 ad 1 l ddH₂O

1 x TE-buffer: 50 mM Tris
 150 mM NaCl
 adjust to pH 7.5
 ad 1 l ddH₂O

7.2.2 Polymerase-Chain-Reaction (PCR)

Amplification of DNA fragments is done by polymerase-chain-reaction (PCR). A piece of DNA is amplified with specific primers binding to the gene of interest. The amplification is done with a Taq (named after the thermophilic bacterium *Thermus aquaticus*) -polymerase by *in vitro* enzymatic replication. The generated DNA is itself used as a template again, so the chain reaction amplifies the DNA exponentially.

Set up the genotyping-PCR by pipetting the mastermix (23 µl per reaction) plus 1 µl of each primer and 1 µl of DNA into a 0.5 ml eppendorf cup. Add 0.4 µl of Taq-polymerase to the reaction and start the program on Eppendorf mastercycler S gradient.

Mastermix: 100 µl 10 x Taq-buffer
 66 µl MgCl₂ (equates to 1.5mM)
 100 µl dNTP's (10 mM each)
 734 µl ddH₂O

TCL1-PCR: Initiation 94 °C 2 min
 Denaturation 94 °C 1 min
 Annealing 62 °C 45 sec
 Elongation 72 °C 2 min 35 cycles (step 2, 3 and 4)
 Final elongation 72 °C 5 min

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<u>MIF-PCR:</u>	Initiation	94 °C	2 min	
	Denaturation	94 °C	1 min	
	Annealing	62 °C	45 sec	
	Elongation	72 °C	45 sec	35 cycles (step 2, 3 and 4)
	Final elongation	72 °C	10 min	

7.2.3 Agarose Gel Electrophoresis

PCR products as well as restriction enzyme digests were analysed by agarose gel electrophoresis to verify the size of the fragments or products.

Therefore 1 x TAE buffer was boiled with the desired amount of agarose, mixed with the intercalating substance ethidium bromide (0.25 µg/ml) and poured into the gel chamber. The comb was directly inserted and taken out when solidification occurred before the chamber was put into the electrophoresis chamber containing 1 x TAE. For large fragments 0.8 % agarose, for smaller ones 2 % was used. 300 to 500 ng of restriction digested DNA or 10 µl of PCR products were mixed with loading dye filled up with H₂O to a final volume of 20 µl and loaded onto the gel. Depending on size of the gel, between 80 and 140 V and 200 mA was chosen for electrophoresis.

50x TAE-buffer: dissolve 242 g of Tris in 500 ml of ddH₂O
 50 mM Na₂EDTA
 57.1 ml glacial acetic acid
 ad 1 l ddH₂O

7.3 Blood-Analysis

7.3.1 Bloodsampling

For the analysis of leukemia, blood of the experimental mice is taken every three months from the tail vein. Mice are warmed up under red-light to improve blood flow. A little cut at the end of the tail is done by a scalpel and the blood is collected in a tube containing 10 µl of 0.5 M EDTA, to prevent the blood from clotting. 100 µl of blood is

taken from each mouse which is then further checked for leukemia in the blood counts and by FACS for the percentage of malignant cells.

7.3.2 Differential Blood Count

To determine the complete blood count, blood is diluted 1:10 with CellPack (BD Biosciences) and measured on a Sysmex XE-2100. Characteristics of the cells are measured by lasers (flow cytometry) where the blood is separated by a semiconductor into a number of channels. The XE-2100 has got five different channels, differentiating the white blood count, the differential leukocyte count as well as reticulocyte count and fluorescence platelet count.

7.3.3 Flow Cytometry

Fluorescence activated cell sorting (FACS) provides a method to study cells based upon the specific light scattering and fluorescent characteristics of each cell. Expression of proteins either surface proteins or intra-cellular proteins can be detected by staining the cells with fluorescently-labelled antibodies, whereas cells expressing endogenous fluorescent proteins can be detected directly.

To determine the grade of leukemia, blood of the mice is labelled with the surface receptor antibodies IgM and CD5. 30 μ l of blood was pipetted into a FACS tube and incubated with 1 μ l of anti-IgM and 1 μ l of anti-CD5 antibody at room temperature in the dark. After 20 min, 2 ml of 1x ACK buffer was added to the cells and incubated on ice for exactly 4 min. Cells were then spun down at 1200 rpm for 5 min followed by a washing step with 3 ml of PBS. Cells are pelleted again and resuspended in 150 μ l of PBS for FACS analysis on the FACS Canto.

10x ACK buffer: 82.9 g ammonium chloride
 10 g potassium bicarbonate
 0.37 g EDTA
 ad 1 l ddH₂O

7.4 Extraction of organs

Organs from the animals were taken out for further examination. If an animal dies or is euthanised because of severe disease, organs like spleen, bone marrow, liver and lymph nodes were taken out and fixed in a 4 % formalin solution and stored in the dark for maximum two weeks. After fixation, the organs were processed in the Thermo Fisher Scientific and embedded into paraffin. Before fixation, parts of the spleen were prepared for further ex vivo analysis by FACS, for detection of the malignant clone or apoptosis and proliferation.

7.5 Eukaryotic cell culture

7.5.1 Culture conditions

All cell lines and primary cells were cultured at 37 °C, a relative humidity of 90 % and 5 % CO₂. DMEM cell culture media was supplemented with 10 % (v/v) FCS, 1 % Na-pyruvate and 1 % Penicillin / Streptomycin. RPMI cell culture medium is supplemented with 10 % (v/v) FCS, 1 % HEPES-buffer and 1 % Penicillin / Streptomycin.

7.5.2 Trypsinization

To detach adherent cells from culture dishes, media was taken off and cells were washed with PBS to remove rests of medium. Cells were then incubated in a small volume of trypsin / EDTA in the incubator until detachment is visible. The reaction was stopped by adding the same volume of media containing 10 % FCS.

7.5.3 Counting

After trypsinization, cells were diluted with trypan blue. Trypan blue is a vital stain which selectively stains dead cells blue. 10 µl of dilution were transferred into a “Neubauer” chamber. Four squares were counted and an average was calculated. The number of cells (n) in one square equals $n \times 10^4$ per ml divided through dilution factor of trypan blue.

7.5.4 Freezing and thawing of Cells

Cells were grown to confluence, trypsinized and pelleted at 1200 g for 5 min. They were then resuspended in freezing medium containing 80 % FCS and 10 % DMSO. Aliquots were pipetted into cryotubes and frozen at -80°C in freezing containers. For long term storage the cryotubes were transferred into the liquid nitrogen tank after one to two days.

For thawing the cells, frozen vials were taken out of the liquid nitrogen tank, carefully thawed at 37°C in the water bath and immediately transferred into a 15 ml tube containing 5 ml of prewarmed culture medium. Pelleting the cells by centrifugation at 1200 g for 5 min removes the toxic DMSO. After resuspension of the cells in fresh culture medium the cells were plated into a 10 cm plate.

7.5.5 Primary splenocytes

Primary splenocytes of $\text{TCL1}^{+/wt} \text{MIF}^{+/+}$ and $\text{TCL1}^{+/wt} \text{MIF}^{-/-}$ were prepared for apoptosis, proliferation tests and chromosomal preparations. The animal was killed by cervical dislocation and the abdominal cavity was opened. First the overall appearance of the animals' organs is checked to see whether there are any abnormalities. Then the spleen is taken out by opening the peritoneum getting it with two tweezers. The organ is then put into a cell strainer (BD, 100 μm diameter) in a 50 ml falcon and a single cell suspension is done by grinding the spleen through the mesh with a syringe. Rinse the mesh with 5 - 10 ml of RPMI media (depending on the size of the spleen) and spin down the cells. Lyse red blood cells with 1x ACK buffer for 5 min on ice, spin again and wash cells with 5ml of PBS. Count cells and plate the appropriate number of cells into well-plates for the required experiments.

7.6 Protein biochemistry

7.6.1 Preparation of cell lysates

To detect the expression pattern of different proteins, they have to be separated on a gel.

A cell pellet of 1×10^7 cells is washed twice in PBS and then lysed in 100 μl of Joe's lysis buffer, sonicated with an ultrasound-sonicator for 20 seconds. The lysed proteins

were spun down in a cooling centrifuge at 400x G for 30 minutes. After centrifugation the clear lysate is transferred into a new tube and then the protein content is determined.

Joe's Lysis buffer: 25 mM Hepes, pH 7.7
 400 mM NaCl
 1.5 mM MgCl₂
 2 mM EDTA
 0.5 % Triton X-100
 ad 1 l ddH₂O

7.6.2 Quantification of Proteins

The protein concentration is determined by a Bradford assay, preparing a standard curve with BSA. Standard proteins and cell lysates are mixed with Roti-Nanoquant (Roth), which was diluted 1:5 in ddH₂O.

For the assay, 2 µl of protein lysate was mixed with 1000 µl of Roti-Nanoquant. To prepare protein standards for calibration of the reader, 2, 4, 6, 8 and 10 µg of bovine serum albumin from a 1 mg/ml stock was diluted in Roti-Nanoquant. 200 µl of this dilution was pipetted into a 96-well plate, also the dilution of the protein samples. The determination of the protein concentration was measured at wavelengths of 450 nm and 590 nm as a reference, using a spectrophotometer. A standard curve of absorbance minus micrograms protein was plotted (OD₅₉₀/OD₄₅₀) and the protein concentration of each sample was determined by the linear regression.

7.6.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated on the basis of their molecular weight.

Two sequential gels were casted, the top gel, so called stacking gel is slightly acidic (pH 6.8) and has a low polyacrylamide concentration, where the proteins are poorly separated but form thin and sharply defined bands. The lower gel, the separating or resolving gel, is more basic (pH 8.8) and has a higher polyacrylamide content, which allows the proteins to be separated according to their molecular sizes, where smaller proteins travel faster than the larger proteins. Sodium-dodecyl-sulfate (SDS), an anionic detergent, denatures the protein and imparts a negative charge, while β-mercaptoethanol, a hybrid of ethylene

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glycol breaks disulfide bonds in the proteins. In an electric field, the negatively charged proteins are attracted towards the anode and they are resolved solely on the basis of their sizes by the pores of the polyacrylamide gel.

Electrophoresis was carried out using a vertical apparatus Mini Protean II (BioRad, Munich, Germany). Protein samples for electrophoresis are prepared by adding 4x sample buffer and boiling the mixture at 95 °C for 5 min. Equal amounts of protein samples and the molecular weight marker were loaded in the slots of the stacking gel. Gels were run at 100 V, until the blue running front has travelled to the bottom of the separating gel.

Solutions for casting SDS-Polyacrylamide gels:

Solutions	Stacking gel	Resolving gel	
		10 %	15 %
ddH ₂ O	2.5 ml	2.075 ml	2.075 ml
1.5 M Tris (pH 6.8)	1.25 ml	-----	
1.5 M Tris (pH 8.8)	----	0.825 ml	2.075 ml
30 % Acrylamide	1.25 ml	0.33 ml	2.075 ml
10 % SDS	50 µl	33 µl	33 µl
10 % APS	13.3 µl	17 µl	33 µl
TEMED	6 µl	3.3 µl	33 µl

Electrophoresis buffer 192 mM Glycin
 25 mM Tris
 0.1 % SDS (v/v)

4x sample buffer 50 ml 1 M Tris pH 6.8
 30 ml ddH₂O
 20 ml Glycerol
 4 g SDS
 200 mg Bromphenolblue
 before use add 200 µl of β-mercaptoethanol to 4.8 ml of stock

7.6.4 Protein Transfer

For the detection of the protein of interest, the resolved protein bands on the gel are transferred on to blotting membranes by the process referred to as blotting. The blotting process was achieved by using Trans-blot cell wet transfer apparatus.

Proteins were transferred onto nitrocellulose membranes. Membranes, filter paper and fibre pads were all pre-soaked in the transfer buffer.

The following is the order of assembly of the module (sandwich-blotting chamber):

Anode (white)
Fibre pad
Filter paper
Membrane
Gel
Filter paper
Fibre pad
Cathode (black)

Care should be taken to remove all the air bubbles in the assembly. The transfer was carried out at 100 V for 60 min.

Transfer Buffer: 250 mM Tris
 200 mM Glycine
 20 % Methanol

7.6.5 Immunoblotting and Development

Immunoblotting involves the detection of specific proteins on membranes using antibodies. The membranes are first blocked with blocking reagent (5 % non fat dry milk in PBS or 5 % BSA in TBS-T) to prevent non-specific binding of antibodies. Then, primary antibodies that recognize specific proteins are allowed to bind to their targets. Respective primary antibodies are diluted in the blocking reagent and incubate the membrane over night at 4 °C on a shaker. After three washing steps, 10 min each with 1x PBS or TBS-T to remove non-specific binding and excess antibody, secondary antibodies labelled with horseradish peroxidase (HRP), that recognize the primary antibodies are applied to the membrane. The secondary antibody is also diluted in the blocking reagent and incubated with the membrane for 60 min at room temperature. Thereafter the membrane is washed again three times with 1x PBS or TBS-T for 10 min before detection of the protein by chemoluminescent visualization using the enhanced chemoluminescent (ECL) detection system. For the detection 1 ml of freshly prepared ECL working solution is prepared by mixing Solution A and B in a ratio of 1:1 (v/v). The mixture is immediately spread on the membrane and incubated for 1 min. Excess solution is drained off and the membrane was exposed to Hyperfilm in the dark for the appropriate time and developed in an X-ray film processor.

10x PBS buffer: 80 g NaCl
 2 g KCl
 26.8 g Na₂HPO₄-7H₂O
 2.4 g KH₂PO₄
 ad 1 l ddH₂O, adjust pH to 7.4

TBS buffer: 10 mM Tris-HCl, pH 8.0
 150 mM NaCl

TBS-T buffer: 1 % (v/v) Tween-20 in TBS buffer

7.7 Quantification of Apoptosis

7.7.1 Annexin V and 7-AAD staining

The phospholipid phosphatidylserine (PS) is translocated from the inner cytosolic leaflet of the plasma membrane bilayer to the outer cell surface, indicating the early events of the apoptosis machinery. Annexin V is a 35 kDa phospholipid-binding protein with high affinity for PS. PS inversion is also a phenomenon occurring during necrosis, so in order to differentiate apoptosis and necrosis, Annexin V staining is accompanied with a vital dye, 7-amino-actinomycin D (7-AAD), which stains the nucleic acid only when the membrane integrity is lost.

Splenocytes from $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ mice were isolated from the spleen as described in chapter 7.5.5. After preparation and cell count, 1×10^5 cells were plated into a 96-well plate in a volume of 100 μ l. To induce apoptosis, cells were either treated with cytostatic drugs or left untreated until a total volume of 200 μ l was reached. Treated and untreated cells are incubated for 8 and 24 hours in an incubator at 37 °C and 5 % CO₂. After the incubation times, cells were pipetted into FACS tubes and washed once with 3ml of PBS and centrifuged at 1200 rpm for 5 min. After this step, cells were resuspended in 100 μ l of Annexin binding buffer containing 1 μ l of Annexin V and 1 μ l of 7-AAD. The samples were then incubated at room temperature in the dark. After 20 min, the samples are analysed using the FACS-Calibur cytometer. The percentages of treated Annexin V positive cells (% apoptotic) were compared to untreated cells.

7.7.2 TUNEL assay

For detection of apoptosis *in vivo*, paraffin embedded sections of spleen were stained for dT-mediated dUTP-biotin nick end labelling (TUNEL). TUNEL measures nuclear DNA fragmentation, an important biochemical hallmark of apoptosis, in cells.

We used the Dead End Fluorimetric TUNEL System (Promega, Madison, WI, USA), which is a non-radioactive system, measuring apoptotic cells by catalytical incorporation of fluorescein-12-dUTP at the 3'-OH end of the DNA.

Sections of the spleen were deparaffinised in xylene for 5min, followed by a washing step in 100 % ethanol for 5 min. After that, sections were rehydrated by a descending ethanol series (100 %, 95 %, 85 %, 70 %, 50 %) for 3 min each. Another washing step in 0.85 % NaCl follows, then a wash in PBS. Sections have to be fixed by an incubation step in a 4

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% methanol-free formaldehyde solution for 15 min. The samples were washed again two times in PBS for 5 min each. The liquid has to be removed from the tissue and the slides have to be put on a flat surface for the incubation with 100 µl of a 20 µg/ml Proteinase K solution for 10 min. Tip off the Proteinase K and wash the samples twice in PBS for 5 min, followed by another fixation step in 4 % methanol-free formaldehyde solution for 5 min and another wash in PBS for 5 min.

For a positive control, incubate one sample with DNase I to cause DNA fragmentation. Therefore 100 µl of DNase I buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl₂, 10 mM CaCl₂) is added to the sample and incubated for 5 min followed by incubation with DNase I buffer containing 10 units/ml of DNase I for 10 min. Slides need to be washed extensively three to four times in ddH₂O. Positive controls need to be processed in extra Coplin jars now, due to residual DNase I activie which my introduce background staining in experimental slides.

Slides were then pre-incubated with 100 µl of Equilibration buffer (provided) for 5 – 10 min. In the meantime, the reaction mix is prepared sufficient for all samples according to the following table:

Buffer component	Component volume per standard reaction	50 µl	Number of reactions (experimental reactions + positive control)	Component volume
Equilibration buffer	45 µl	x	_____	= _____µl
Nucleotide mix	5 µl	x	_____	= _____µl
rTdT Enzyme	1 µl	x	_____	= _____µl

For the negative control, prepare a control incubation buffer without the enzyme. Most of the equilibration buffer hast to be removed from the slides, then the cells were incubated with 50 µl of prepared rTdT incubation buffer. To protect the samples from drying out, they need to be covered with plastic coverslips, also to ensure even distribution of the reagent. Put the samples in a humidified chamber covered with aluminium foil to protect the samples from light for 60 min at 37 °C.

After that time, the reaction was terminated by putting the slides in 2x SSC for 15 min at room temperature in the dark. Wash the samples three times in PBS for 5 min each to remove the unincorporated fluorescein-12-dUTP. Stain the sample in a freshly prepared propidium iodide (PI) solution (1 $\mu\text{g/ml}$ PI in PBS) for 15 min in the dark to stain the nuclei. Wash the samples again twice in PBS and mount the slides with Mowiol using glass coverslips.

The samples are then analysed under a confocal microscope (Olympus FluoView 1000). A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images.

20x SSC: 87.7 g NaCl
 44.1 g sodium citrate
 adjust pH to 7.2, ad 500 ml ddH₂O

7.8 Quantification of Proliferating Cells

7.8.1 *In vivo*

TCL1^{+wt} MIF^{+/+} and TCL1^{+wt} MIF^{-/-} mice are treated with 5-bromo-2-deoxyuridine (BrdU). BrdU is injected i.p. into the animals at a concentration of 50 $\mu\text{g/g}$ of bodyweight over four days constantly. On the fifth day the animal is killed and spleen, blood, fluid of the peritoneal cavity and bone marrow are checked for the incorporation of BrdU.

10⁶ cells per 50 μl of staining buffer (PBS + 3 % FCS + 0.09 % sodium azide) are incubated with the cell surface markers CD5 and IgM for 15 min in the dark on ice. After the incubation time, cells are washed with 1 ml of staining buffer for 5 min at 300 x g, followed by a fixation and permeabilization step. The cell pellet is resuspended in 100 μl of BD Cytfix/Cytoperm buffer an incubated for 30 min on ice, followed by another washing step. Cells are then resuspended in 100 μl of the BD Cytoperm Plus buffer for 10 min on ice, again followed by a wash and another re-fixation step in 100 μl BD Cytfix/Cytoperm buffer. After the incubation of 5 min at roomtemperature cells are washed and then resuspended in 100 μl of DNase (300 $\mu\text{g/ml}$) and incubated for 1 hour at 37 °C to expose the incorporated BrdU. Resuspension of the cells in 50 μl of BD

wash/Perm buffer containing the fluorescent anti-BrdU antibody follows another washing step. Cells are now incubated for 20 min at room temperature in the dark, washed once more and then analysed on the FACS Canto.

7.8.2 *In vitro*

Splenocytes from mice of the genotype $TCL1^{+/wt} MIF^{+/+}$ and $TCL1^{+/wt} MIF^{-/-}$ are taken and pulsed with 5-ethynyl-2'-deoxyuridine (EdU), an alternative for BrdU. EdU also incorporates into the DNA during DNA synthesis. 10^6 splenocytes are taken and incubated with 1 μ g/ml of lipopolysaccharid (LPS) to stimulate DNA synthesis. After 24 h cells are pulsed with 7.5 μ g/ml EdU to analyse the incorporation after another 24 h respectively 48 h.

Before analysis on the FACS Canto, cells need to be harvested, washed one with PBS + 1 % BSA for 5 min at 500 x g. Cells are then resuspended in 100 μ l of PBS + 1 % BSA and incubated with the cell surface antibodies CD5 and IgM for 15 min at room temperature. After another washing step cells are resuspended in 100 μ l of Click-iT fixative and incubated for 15 min at room temperature protected from light. After another washing step cell are proceeded to cell permeabilization by washing with 3 ml of 1x saponin-based permeabilization buffer, followed by incubation with 500 μ l of the Click-iT reaction cocktail for 30 min at room temperature. For one reaction the Click-iT reaction cocktail contains of:

Reaction component	
1x Click-iT Reaction Buffer	487 μ l
CuSO4	10 μ l
Fluorescent dye azide	2.5 μ l
Reaction Buffer Additive	50 μ l
Total volume	500 μ l

Cells are washed again with 3 ml of 1x saponin-based permeabilization reagent and then analysed on the FACS Canto.

7.9 Chromosomal preparation

Primary splenocytes are isolated from the spleen, counted and then put into a 10 ml cellculture flask. 1×10^5 cells are plated in RPMI-medium supplemented with 10 % FCS, HEPES and sodium-pyruvate. Stimulation is done by treatment of the cells with LPS, ConcanavalinA and β -mercaptoethanol. Cells are stimulated for 48 hours, and then treated with Colcemid, a cell cycle-inhibitor. The cells are incubated for another 24 hours to arrest the cells in G1S-phase.

For the preparation of metaphases, cells are collected and put into a 15 ml centrifugation tube and centrifuged for 10 min at 1500 rpm. Discard the supernatant and leave ~2 ml above the pellet and resuspend on a vortex. Fill tube up to 12 - 13 ml with prewarmed KCl, vortex and incubate at 37 °C for exact 20 min. Add freshly prepared fixative (methanol-acetic acid 3:1) under vortexing and centrifuge for 10 min at 1500 rpm. Discard supernatant, leave again 2 ml above pellet, resuspend pellet and add freshly prepared fixative under vortex. Centrifuge for 10 min at 1500 rpm and repeat this step three to four times. Place one drop of suspended pellet with a glass-pipette onto a pre-cooled glass-slide and check chromosomes under a microscope. If there are too many cell-rests, wash cells again with fixative. Prepared chromosomes can be kept at -20 °C for up to two years.

Preparation and analysis was done in cooperation with the group of Dr. Cornelia Rudolph from the Medizinische Hochschule Hannover.

7.10 Immunohistochemistry

Paraffin-embedded organs are cut on a microtome in the institute of pathology of the University Clinics Cologne. The sections have a diameter of 3 μ m.

For immunohistochemical staining, sections are labelled with the desired antibody. Before staining with the antibody, sections are deparaffinised in xylene for 20 min, then rehydrated in a descending ethanol series (three times 100 % EtOH, three times 70 % EtOH; 2 - 4 min each) and rinsed in deionised H₂O. Depending on the antibody, the slides had to be pretreated with different buffers of different pH (see following table).

Methods

Antibody	Pre-treatment	Incubation -time and - temperature
CD3	Target Retrieval Solution pH 9 (Dako Cytomation #S2367)	15 min, 95 °C
CD5	Target Retrieval Solution pH 9 (Dako Cytomation #S2367)	15 min, 95 °C
CD20	Target Retrieval Solution pH 9 (Dako Cytomation #S1699)	15 min, 95 °C
CD68	Proteolytic Enzyme (Dako Cytomation #S3007)	5 min, room temperature
CD79a	Target Retrieval Solution pH 9 (Dako Cytomation #S2367)	15 min, 95 °C

After pre-treatment, slides are washed in TBS-T for 5 min and then blocked with Thermo Ultra Block (# TA125UB) for 10 min and then incubated with the primary antibody for 45 to 60 min, depending on the antibody. After another washing step with TBS-T, the slides are incubated with LAB Polyvalent Alk Phos Polymer (Thermo #TL-125-AL) for 15 min and then developed with the UltraVision Detection System Liquid Fast Red Substrate System (Thermo #TA-060-AL).

While development with FastRed the slides have to be watched under a microscope that there is no background stain. After the development slides are counterstained with a hemalaun solution for 1 min and embedded with AquaTex.

To get an overview of the infiltration by lymphocytes in the organs, the slides are stained for Giemsa. Erythrocytes in the organs stain pink, platelets show a light pale pink, lymphocyte cytoplasm stains sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. To stain the organs, slides need to be deparaffinised as above and then satined in Giemsa-buffer for 30 min. This stain is followed by a short dip in acetic acid to intensify the red, rinsed shortly with ddH₂O and a dip into 96 % EtOH to remove the excess blue, three washes in 100 % EtOH and two dips into xylene. The slides need to be embedded fast, otherwise the staining will be lost.

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Erklärung

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