Antagonization of the maturation block mediated by the leukemia-specific fusion oncoprotein AML1/ETO

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1. Introduction

1.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia are a heterogeneous group of diseases characterized by infiltration of bone marrow and other tissues by neoplastic cells of the hematopoietic system. The incidence of acute myeloid leukemia (AML) is ~3.6 per 100,000 people per year, and the age-adjusted incidence is higher in men than in women (4.4 versus 3.0). The AML incidence increases with age; it is 1.7 in individuals younger than 65 years old and 16.2 in those older than 65 years old (Anthony S. Fauci 2008).

Acute Myeloid Leukemia (AML) Classification System

French-American-British (FAB) Classification
M0: Minimally differentiated leukemia
M1: Myeloblastic leukemia without maturation
M2: Myeloblastic leukemia with maturation
M3: Hypergranular promyelocytic leukemia
M4: Myelomonocytic leukemia
M4Eo: Variant: Increase in abnormal marrow eosinophils
M5: Monocytic leukemia
M6: Erythroleukemia (DiGuglielmo's disease)
M7: Megakaryoblastic leukemia

World Health Organization Classification
I. AML with recurrent genetic abnormalities
   AML with t(8;21)(q22;q22);AML1(CBFα)/ETO
   AML with abnormal bone marrow eosinophils [inv(16)(p13q22) or t(16;16)(p13;q22);CBFβ/MYH11]
   Acute promyelocytic leukemia [AML with t(15;17)(q22;q12) (PML/RARα and variants]
   AML with 11q23 (MLL) abnormalities
II. AML with multilineage dysplasia
   Following a myelodysplastic syndrome or myelodysplastic syndrome/myeloproliferative disorder
   Without antecedent myelodysplastic syndrome
III. AML and myelodysplastic syndromes, therapy-related
   Alkylating agent–related
   Topoisomerase type II inhibitor–related
   Other types
IV. AML not otherwise categorized
   AML minimally differentiated
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma


Transformation events in cancer have been defined as initiation events (contributing to the early stages of neoplastic transition) or progression events (referring to subsequent transformative processes). The product of oncogenes can be classified into six broad groups: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators (Croce 2008).

1.1.1. AML1/ETO positive acute myeloid leukemia

AML of FAB subgroup M2 is a morphologically differentiated form of AML that frequently contains Auer rods and stains positively for myeloperoxidase. The t(8; 21) which is usually found in the M2 FAB subtype of AML generates an invariant AML1/ETO transcript and protein which consists of 177 aa of AML fused to 575 aa of ETO. The t(8;21) and the inv(16)(p13;q22)/t(16;16)(p13;22) chromosomal rearrangements, are grouped into the Core Binding Factor Leukemias (CBF). These are defined by their chromosomal rearrangements that result in the disruption of CBF-α (AML1) and CBF-β genes encoding subunits, respectively (Renneville et al. 2008).
1.1.1.1. The translocation (8;21)

The majority of chromosomal rearrangements result in the formation of a chimeric gene (Fröhling and Döhner 2008).

AML1 (RUNX1) is a member of the Runt family of transcription factors that heterodimerize with CBFβ to form a stable DNA binding complex (Licht 2001). AML1 is also the major gene involved in chromosomal translocations in leukemia. Specifically, the AML1/ETO-associated translocation is observed in approximately 40% of cases of acute myeloid leukemia of the FAB M2 classification, while present in approximately 12% of all AMLs. The chimeric fusion protein contains the N-terminal portion of AML1 up to its Runt homology DNA-binding domain, fused to most of the ETO (MTG8) protein (Peterson and Zhang 2004). The ETO gene is one of the family members of ETO/MTG repressor proteins that have homology to the Drosophila Nervy repressor proteins (Davis et al. 2003), (Wolford and Prochazka 1998). The zinc-finger motifs of ETO are involved in protein-protein interaction, but not protein-DNA interaction.

AML1/ETO negatively regulates AML1 target genes (Biggs et al. 2006). This suppression occurs mostly through interaction of the ETO moiety with the nuclear receptor corepressor N-CoR-mSin3-HDAC1 complex that recruits histone deacetylase activity; This interaction results in a lower level of histone acetylation, less accessible chromatin, and thereby repression of transactivation activity of wild-type AML1 (Müller et al. 2008), (Gelmetti et al. 1998).

The disruption of normal cell proliferation, differentiation and survival contribute to the development of leukemia. In various stably transfected AML1/ETO-expression hematopoietic cell lines, AML1/ETO is shown to block myeloid and erythroid differentiation (Burel et al. 2001), (Puccetti et al. 2002), (Amann et al. 2001). In U937 cells the block in differentiation is accompanied by a decrease in CAAT-enhancer binding protein-α transcription (C/EBPα), (Burel et al. 2001), (Pabst et al. 2001). Also by blocking C/EBPα activity, AML1/ETO impairs granulocytic differentiation (Pabst et al. 2001). Furthermore, AML1/ETO showed a negative effect on cell cycle and survival, including a G1 arrest associated with decreased CDK4 and c-myc expression (Burel et al. 2001), (Amann et al. 2001). AML-associated translocation products block Vitamin D3 induced differentiation of U937 cells by sequestering the Vitamin D3 receptor (Puccetti et al. 2002). AML1/ETO inhibits AML1/CAAT-enhancer binding protein-α mediated activation of the CD11c promoter and represses CD11c expression in HL60 cells (Heidenreich et al. 2003).
1.1.1.2. LAT2 is a novel AML1/ETO target gene

As mentioned before, AML1/ETO regulates several genes critical for hematopoietic differentiation. LAT2 is one of the genes down-regulated by this fusion protein, also called WBSCR5, NTAL or LAB (Fliegauf et al. 2004).

LAT2 comprises 11 exons and encodes a 243aa protein. The gene is located on chromosome 7 within segment q11.23, a region that is deleted in the human autosomal dominant disorder Williams-Beuren syndrome (WBS, OMIM 194050). WBS is characterized by a number of symptoms including vascular, neurological, behavioral and skeletal abnormalities, including dental malformation (Grimm and Wesselhoeft 1980); (Francke 1999). LAT2 was found to be expressed in spleen and hematopoietic cells such as B cells, NK cells, monocytes and masts cells, and represents a 25-30 kDa phosphoprotein, with a 6aa N-terminal extracellular peptide, a single putative hydrophobic trans-membrane domain and a cytoplasmic signaling domain composed of nine tyrosine residues (Brdicka et al. 2002); (Janssen et al. 2003). LAT2 is expressed at high levels in peripheral blood monocytes but it is undetectable in granulocytes (Duque, Solari et al., to be submitted; (Duque 2008).

LAT2 possesses no inherent catalytic activity; it acts as an adaptor molecule, which recruits and fastens critical signaling molecules into the signalosome (Iwaki et al. 2007). This property depends on the phosphorylation of specific tyrosine residues contained within its cytosolic tail. Both Lyn and Syk are required for LAT2 phosphorylation in mast cells following antigen-dependent FεRI aggregation (Tkaczyk et al. 2004). The c-Kit receptor can also directly phospholylate LAT2 (Tkaczyk et al. 2004). In LAT2 knock-down cells, it was observed that the phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 was delayed but enhanced (Tessarz et al. 2007). However, the precise role that LAT2 could play in the function of hematopoietic cells remains unclear.

LAT2 knock-out mice mast cells are hyperresponsive via FcεRI I and c-Kit receptor. The T cells are hyperactivated and are responsible for the autoimmune state of these mice (Zhu et al. 2006). Although LAT2 is highly expressed in B cells, no defect was found in B cell development or function (Wang et al. 2005). LAT2 -/- mice have increased β glucuronidase after FcRI mediated degranulation. Tyrosine phosphorylation of LAT is also
enhanced. Moreover, LAT2 negatively regulates the PI3K activity and enhances PLCγ and Calcium response in LAT2−/− mice (Volna et al. 2004).

### 1.1.2. c-Kit and other receptor tyrosine kinases

The expression of AML1/ETO is not sufficient to induce leukemia (Nimer and Moore 2004). The t(8;21) results in haploinsufficiency of AML1 and ETO, and it generates the AML1/ETO fusion (Nimer and Moore 2004).

FLT3 activating mutations (most commonly an internal tandem repeat of a juxtamembrane portion of the receptor) are found in 20-30% of human AML samples (Nimer and Moore 2004). Such mutations occur most commonly in AML with t(15;17), but they are also found in ~9% of t(8;21) positive AMLs (Kottaridis et al. 2001).

Activating point mutations in the c-Kit tyrosine kinase receptor have also been found in t(8;21) and in inv(16) AML (Care et al. 2003); (Paschka et al. 2006).

It appears as though AML1/ETO positive cells need additional proliferative signals. Such signals come from mutated and activated tyrosine kinase, like c-Kit or FLT3, but these are not independent. It is now clear that cooperative effects are necessary to generate leukemia (Nimer and Moore 2004).

The c-Kit receptor is a member of the type III receptor tyrosine kinase family and plays a crucial role in normal hematopoiesis and acute myeloid leukemia (Ashman 1999). Protein tyrosine kinases are enzymes that catalyze the transfer of phosphate from ATP to tyrosine residues in polypeptides (Krause and Van Etten 2005). Receptor tyrosine kinases are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain (Krause and Van Etten 2005). These become activated when the ligand binds to the extracellular domain, resulting in receptor oligomerization, disruption of the autoinhibitory juxtamembrane interaction, and autophosphorylation of a regulatory tyrosine within the activation loop of the kinase (Krause and Van Etten 2005). Exon 8 deletion plus insertion mutations of c-Kit have been described in patients with core-binding factor (CBF)-AMLs (Gari et al. 1999). c-Kit exon 8 mutations induce hyperactivation of C-Kit receptor in response to its natural ligand stem cell factor (SCF), a c-Kit exon 8 mutant, D816V showed spontaneous dimerization and phosphorylation of mitogen-activated protein kinase (MAPK) (Kohl et al. 2005). In CBF leukemias, c-Kit mutations accumulate within
exons 8 and 17. These exons are uncommon targets in other neoplasias, as mutations typically involve exons 11 or 9. Exon 17 translates into the activating loop of the second TK domain and corresponds to the D835 location of FLT3. Exon 8 encodes the fifth immunoglobulin-like unit and comprises and evolutionarily highly conserved region in the extracellular domain (Müller et al. 2008).

1.1.3. Tyrosine Kinase inhibitors

The landscape changed radically by the success of imatinib mesylate (Gleevec). Imatinib, a 2-phenylaminopyrimidine compound that is a specific inhibitor of several tyrosine kinases, ABL, ABL-related gene product, c-Kit, and PDGF receptor induces hematologic and cytogenetic remissions in most patients with CML (O'Brien et al. 2003) but it is less effective in the accelerated and blast-crisis phases of the disease (Druker et al. 2001).
Dasatinib is a small molecule, ATP-competitive inhibitor of SRC and ABL tyrosine kinases. It inhibits the kinase activity of both wild type (WT) and mutant c-Kit receptor proteins (Schittenhelm et al. 2006). Dasatinib is a much more potent inhibitor of c-Kit activation loop mutants, like mutation D816, than imatinib (Schittenhelm et al. 2006).

1.2. Epigenetics

Arthur Riggs and colleagues defined epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences” (Bird 2007). There is growing evidence that epigenetic mechanisms are responsible for tissue-specific gene expression during differentiation and that these mechanisms underlie the processes of developmental plasticity (Gluckman et al. 2008). Epigenetic mechanisms include coordinated changes in the methylation of cytidine-guanosine (CpG) nucleotides in the promoter regions of specific genes, changes in chromatin structure through histone acetylation and methylation, and post-transcriptional control by microRNA (Goldberg et al. 2007).

1.2.1. DNA methylation

DNA methylation is the addition of a methyl group to the carbon-5 position of cytosine residues. It is the only common covalent modification of human DNA and occurs almost exclusively at cytosines that are followed immediately by a guanine (CpG dinucleotides). DNA methylation results from the activity of a family of DNA methyltransferase (DNMT) enzymes that catalyze the addition of a methyl group to the cytosine residues at CpG dinucleotides (Bird 1996). Cytosine methylation can interfere with transcription factor binding (Klose and Bird 2006).

1.2.2. Epigenetic therapy

Genes inappropriately silenced by structural chromatin changes that involve DNA methylation can be reactivated by demethylating agents that can reverse these changes and,
therefore, restore principal cellular pathways. Demethylating agents and histone deacetylase inhibitors are currently under intense preclinical and clinical investigation for the treatment of human leukemias (Koschmieder et al. 2007).

Cytosine analogues such as 5-Aza-2'-deoxycytidine (decitabine, DAC) have shown to reactivate the expression of a variety of genes including tumor suppressors in malignant cells in vivo and in vitro by inhibiting DNMTs (Schmelz et al. 2005). These agents are incorporated into the nucleic acids of dividing cells, where they act as mechanism-based inhibitors of DNA methyltransferases. They inactivate DNA cytosine C5- methyltransferases through the formation of stable complexes between the 5-aza-2'-deoxycytidine residues in DNA and the enzyme, thereby mimicking a stable transition state intermediate when bound to the methyltransferase enzyme (Sheikhnejad et al. 1999), (Crystallization and preliminary crystallographic analysis of the (cytosine-5)-DNA methyltransferase NlaX from Neisseria lactamica, Acta crystallographica, 1999).

The DNA-incorporated decitabine not only binds and inactivates DNMTs but in addition acts as an inhibitor of the methylation of histones, both modifications leading to gene reactivation (Schmelz et al. 2005). These powerful inhibitors of DNA methylation can restore gene function to treated cells in culture, which has indicated that they may have potential in treating patients with malignant disease (Lübbert 2000), (Baylin 2002).

1.3. Cell Differentiation

“The process by which cells or tissues undergo a change toward a more specialized form or function, especially during embryonic development.”

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The use of myeloid cell lines provides appropriate models to study differentiation, since they are constituted by homogeneous populations “blocked” at restricted stages of differentiation. Many of these cell lines can be induced to differentiate to more mature stages (Lübbert and Koeffler 1988).
1.3.1. Monocytic Differentiation

The molecular and genetic basis of monocyte differentiation is still poorly understood. The process of monocyte differentiation involves gain and loss of enzymatic activities and appearance or loss of cell surface markers. All these changes are accompanied by a complex pattern of gene regulation and expression, mediated in part by cytokines. A block of normal hematopoietic cell differentiation to monocytic cells is found in monocytic leukemia. Certain reagents, such as phorbol ester, Vitamin D3 and retinoic acid can induce myeloblastic or monoblastic leukemia cells to further differentiate toward the monocytic lineage. Although these reagents may function by different mechanisms, changes in differentiation include growth arrest in the G0/G1 phase of the cell cycle, the appearance of typical monocytic/macrophage morphology, and in an increase in the expression of a variety of monocytic genes.

12-o-tetradecanoylphorbol 13-acetate (PMA), the most potent phorbol ester, is a protein kinase C activator that promotes cell division in various cells. Due to its long fatty acid side chain, PMA readily partitions into membranes to give high, non-saturable binding (Schimmel et al. 1980). Protein kinase C is present both in membranes and cytosol. The cytosolic form exists as an apo-enzyme, with an absolute requirement for CaZ+ and phospholipid, preferably phosphatidylserine, for activity. In the presence of a limiting concentration of Ca*, protein kinase C can be stimulated by diacylglycerols, e.g. diolein, which appear to function by shifting the Ca+-dose-response curve for activation of the enzyme to lower Ca2+ concentrations (Kishimoto et al. 1981). The phorbol esters lead to abnormal, chronic stimulation of protein kinase C (PKC). They cause an abnormal distribution of activated kinase and in turn may result in an altered pattern of phosphorylation (Blumberg et al. 1984). PKC activates ERK pathway, and activates the transcription of different genes. PMA induction of CD11b gene transcription involves the activation of cis-acting elements including NF-KB (Shelley and Arnaout 1991). PMA has inhibitory effects on the growth of leukemia cell lines and it induces growth arrest and differentiation into monocytes/macrophages (Horiguchi-Yamada et al. 1994).

Vitamin D3 is a steroid hormone family member. Normal human myeloid stem cells can differentiate into macrophages after exposure to Vitamin D3. It increases macrophage colony formation in human bone marrow cultures in the presence of granulocyte-
macrophage-CSF. Vitamin D3 forms a complex with the Vitamin D3 receptor (VDR) in the nucleus. This activated receptor binds to VDR response elements in the promoter or enhancer of Vitamin D3 inducible genes and increases or decreases gene transcription (Zhang et al. 1994).

1.3.2. Granulocytic Differentiation

Retinoids regulate growth and differentiation of normal and malignant cells. In the hematopoietic system, all-trans retinoic acid (ATRA) has been shown to inhibit growth, induce differentiation of myelomonocytic progenitor cells and to enhance self-renewal of more immature multipotent stem cells (de The et al. 1990); (Lawson and Berliner 1999).

Human granulocyte-macrophage colony stimulating factor (GM-CSF) belongs to a family of peptide hormones regulating proliferation, differentiation, and functional activity of hematopoietic cells (Metcalf 1985). GM-CSF is a 22 Kd glycoprotein produced by activated T cells that stimulates in vitro formation of colonies containing neutrophils, eosinophils, and monocytes. In addition to its effects on myeloid progenitor cells, GM-CSF exerts multiple influences on mature effector cells of this lineage, including inhibition of migration, enhancement of chemotaxis and superoxide generation in response to chemotactic agents: increased phagocytosis of bacteria, and augmentation of antibody-dependent cell-mediated cytotoxicity (ADCC) (Vadas et al. 1983).

The granulocyte colony-stimulating factor (G-CSF) belongs to a family of hemopoietic growth factors regulating the production of granulocytes and macrophages. G-CSF has an outstanding capacity to induce terminal differentiation and suppression of self-renewal in myeloid leukemic cells (Nicola NA, Int J Cell Cloning, 1987; Souza et al., Science, 1986)
1.3.3. Determination of myeloid Differentiation by flow cytometry

**CD11b/CD18**

The adhesion molecule CD11b/CD18 (also termed Mol or Mac-1) is a member of a family of three heterodimeric cell surface glycoproteins and thus part of the broad gene family of integrins involved in cell-cell interactions during host defense and immune response. CD11/CD18 surface molecules share a common P-subunit (CD18, Mac-1 P, Mol P) of 95-Kd molecular weight that is non-covalently linked to a unique α-subunit of higher molecular weight. The α-subunits have been designated CD11a (LFA-1, a L), CD11b (Mol a, Mac-1 a, a M), and CD11c (~1.50,a X), respectively (Lübbert et al. 1991).

CD11b/CD18 is an important marker of terminal myeloid differentiation because of its high specificity for myeloid cells. The CD11b/CD18 surface antigen is first expressed during the myelocytic and monoblastic stages of maturation and is up-regulated during granulocytic and monocytic differentiation. Functions of this molecule include binding of a complement component (C3b fragment) as well as promotion of homotypic granulocyte adhesion and adhesion of granulocytes and monocytes to endothelial cells. In addition, CD11b/CD18 molecule is associated with granulocytic oxidative burst, phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC). The gene for CD11b resides on chromosome 16p11, clustered with CD11a and CD11c, and the CD18 gene is located on
chromosome 21q22. Expression of CD11b mRNA is sharply up-regulated during induction of granulocytic or monocytic differentiation of HL-60. This up-regulation of CD11b transcripts is paralleled by an increase in both the number of CD11b molecules per cell as well as the number of CD11b-positive cells (Lübbert et al. 1991).

The structures of the genes encoding CD11b, CD11c, and CD11a are likely to be similar since they probably arose from a common ancestor by gene duplication (Shelley and Arnaout 1991).

**1.3.3.2. CD11c/CD18**

As mentioned before, CD11c is up-regulated during differentiation and its promoter contains binding sites for AML1 and C/EBPα (Drescher et al. 2003). The structure of the CD11c gene has been determined and, although the first exon and 5' flanking sequence were not isolated, the first intron was shown to interrupt the 5' noncoding region and the second to interrupt the codon specifying the 13th amino acid of the leader polypeptide. Therefore, the second intron of the CD11c gene appears analogous to the first intron of the CD11b gene, which may have lost an intron in its 5' noncoding region (Shelley and Arnaout 1991).

**1.3.3.3. CD14**

CD14, a 55-kDa glycoprotein expressed in a tissue-specific manner on the surface of mature monocytes and macrophages, attaches to the cell membrane through a glycosylphosphatidylinositol anchor. Its expression is undetectable on the surface of myeloid precursor cells and increases dramatically during the process of monocytic differentiation. Therefore, it has been used as a monocyte differentiation marker and is an excellent model to study the mechanism of Vitamin D3-induced differentiation of myeloid cells (Zhang et al. 1994).

Functionally, CD14 is an important molecule involved in the immune response. It has been reported to be a receptor for the complex LPS and LPS-binding protein. The binding of LPS to CD14 triggers a series of monocytic immune responses, including the synthesis of TNF-α, IL-6, and IL-8. Other studies with anti-CD14 antibodies indicate that CD14 is
involved in the monocyte-mediated activation of T cell proliferation and monocyte adhesion to cytokine-activated endothelial cells. Human CD14 is encoded by a single gene located on the long arm of chromosome 5 that encodes several myeloid-specific growth factors and growth-factors receptors, including IL-3, granulocyte-macrophage-CSF, macrophage-CSF, macrophage-SCF receptor and the receptor for platelet-derived growth factor (Zhang et al. 1994).
2. Materials and Methods

2.1 Materials

2.1.1 Cell lines

- **Kasumi-1**
  Established from the peripheral blood of a 7-year-old Japanese boy with acute myeloid leukemia (AML FAB M2) (in 2nd relapse after bone marrow transplantation) in 1989; cells carry the t(8;21) AML1-ETO fusion gene.

- **U937**
  Established from the pleural effusion of a 37-year-old man with generalized diffuse histiocytic lymphoma in 1974; cells were described to express markers and properties of monocytes.

- **HL60**
  The HL-60 (Human promyelocytic leukemia cells) cell line was derived from a 36-year-old woman with acute promyelocytic leukemia at the National Cancer Institute. HL-60 cells are predominantly a neutrophilic promyelocyte (precursor).

- **Jurkat**
  Established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL) at first relapse in 1976.

- **MEC 1**
  MEC1 grew spontaneously on two subsequent occasions from the peripheral blood (PB) of a patient with B-chronic lymphocytic leukemia (B-CLL) in prolymphocytoid transformation.

- **9/14/18 and 9/10/7**
The 9/14/18 cell line was developed from the U937 cell line, it has an Ecdyson inducible expression vector pVgRXR and the AML1/ETO construct in pIND-vector (Invitrogen) (Fliegauf et al. 2004). This cell line was developed by Dr. Manfred Fliegauf PhD (Fliegauf et al. 2004), and is one of the 3 AML1/ETO inducible systems (Alcalay et al. 2003), (Burel et al. 2001). 9/10/7 cells are a LacZ clone and were used as established control.

- **U937 TD-N4/N5 ShRNA** (Obtained from Dr. rer. nat. Cerwenka’s group at the German Cancer Research Center)

Stable U937-derived cells were obtained by retroviral infection, the cell line U937-TD was transduced with pMX-pie-DAP12 and pMX-neo-CD8L-FLAG-TREM-1. U937-TD were transduced also with the construct indicated, TD-N4(RVH1-N4), TD-N5 (RVH1-N5) containing oligonucleotides that encode shRNAs directed against LAT2 (Tessarz et al. 2007).

### 2.1.2. Materials for tissue culture (see 6.0 for abbreviations)

<table>
<thead>
<tr>
<th>Material for cell culture</th>
<th>Company</th>
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<tbody>
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<td>5-Aza-2'-deoxycytidin-DAC (decitabine)</td>
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<td>ATRA (<em>All Trans</em> Retinoic Acid)</td>
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### 2.1.3. Reagents

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## Gels

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## Antibodies for Western Blot

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<td>Ab 5506-100</td>
<td>Rabbit polyclonal anti-c kit Abcam</td>
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<td>Ab 5616-50 Lot: 201636</td>
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<td>PE anti-human CD11b</td>
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<td>PE anti-human CD11c</td>
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<td>PE anti-human CD14</td>
<td>BD Biosciences 555398</td>
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<tr>
<td>PE mouse IgG Isotype Control</td>
<td>BD Biosciences 555749</td>
<td>3.5 µl/100µl</td>
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</table>

### 2.1.4. Solutions

#### 10 x TBS +1% Tween 20 (TBS-T):

24g Trisma Base, 80g NaCl, deionized water up to 800ml, pH=7.6, 10ml 100% Tween 20. Deionized water up to 1l.

#### 1x TBS+ 0.1% Tween 20:

100ml 10 x TBS +1% Tween 20, 900ml deionized water

**Western Blot blocking solution:**

7g milkpowder, 100 ml 1x TBST.

**Western Blot Running Buffer:** 1xNuPaGE MOPS SDS Running Buffer

50ml 20x NuPaGE MOPS SDS Running Buffer, 950ml deionized water, 500ml Antioxidant (if needed).

**Western Blot Transfer buffer:**
50 ml 20x NuPaGE Transfer Buffer Buffer, 100ml Methanol (one gel), 200ml Methanol (for two gels), 1 ml Antioxidant (if needed).

### 2.1.5. Kits

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### 2.1.6. Other Materials

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### 2.1.7. Equipment

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## Materials and Methods

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<td>Fridge -80°C</td>
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<td>Microscope Zeiss axioskop 2 MOT and Axiocam</td>
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<td>Incubator shaker series 25</td>
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<td>Centrifuge Biofuge 15</td>
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### 2.1.7. Software

Summit v 4.2 (www.dakousa.com).

Dakocytomation, California, USA.

FlowJo v.6.2 (www.flowjo.com).

Tree Star Inc, Oregon, USA.
2.2. Methods

2.2.1. Cell Culture
The cells were cultured at 37°C, 5% CO₂, and 100% humidity, in an incubator. Medium was changed three times a week (or less according DSMZ recommendations), cells were centrifugated for 5 min at 1200 rpm, supernatant was discarded and the pellet was re-suspended in the new medium. Work was always performed under sterile conditions.
Medium was RPMI-1640 with 5ml penicillin-streptomycin (100U/ml) and 10% Fetal Calf Serum.
Cell cultures were seeded at 2.5 x 10^5 cells/ml, and cell concentration was always kept under 1x10^6 cells/ml.

2.2.2. Freezing cells
5x10^6 cells were centrifugated 5 min at 1200 rpm and supernatant thrown away. Then the pellet was re-suspended in 1ml freezing solution (20%FCS, 10% DMSO, and 70% medium) and stored in a freezing ampoule. Then, stored in an isopropanol carousel for 24hrs at -80°C and, finally, stored in Liquid Nitrogen.

2.2.3. Thawing cells
Ampoule was thawed in a water-bad (37°C). Cells were transferred to a 15 ml Falcon with 9 ml pre-warmed medium and mixed gently. Then centrifugated 5min at 1200rpm and supernatant discarded. Pellet was suspended in 10 ml medium, and a small sample was taken to determine cell number and viability. Sample was again centrifugated and cells were re-suspended again in fresh medium adjusting concentration to cell line requirements. In some cases cells were re-suspended in 20% FCS medium for a few days. Also size of the bottle was adjusted to cell concentration and volume.
Medium was changed again always after 24hrs, and culture was adjusted to cell growth.

2.2.4. Cell counting and viability
Cell number and viability were assessed using the trypan blue dye. Cells were solved 1:1 with trypan blue and counted using a Neubauer Chamber.
Number of cells/ml = total counted cells/number of counted quadrants x $2 \times 10^4$

% of Viable cells = living cells/ (living + dead cells) x 100

2.2.5. Morphology: Cytospins and staining.

Cytospins

1 x $10^5$ cells were centrifugated and washed with 1ml PBS 5min at 5000rpm in an eppendorf centrifuge. Supernatant was discarded and pellet re-suspended in 200µl PBS. Slides (previously washed with ethanol 70%) and filters were placed into appropriate slots in the cytospin with the cardboard filters facing the center of the cytospin. Then 100µl suspension of each sample was aliquoted into the appropriate wells of the cytospin. Cytospins were spun at 800rpm for 3 min.

May-Grünwald Giemsa staining

Slides were stained in May-Grünwald solution for 3 minutes, and then placed in distilled water for 1min. Slides were then stained with Giemsa solution (1:20 solved in PBS) for 15 min. Finally slides were washed with distilled water. Slides were air dried.

2.2.6. Cell treatments

2.2.6.1. Induction of AML1/ETO protein in 9/14/18 cells.

9/14/18 cell line was cultured always in the presence of 600µg/ml Geneticin and 150µg/ml Zeocin. Conditional expression of AML1/ETO was induced by adding PonA 5µM (PonA was solved in ethanol) to the cell culture.

2.2.6.2 Design for differentiation experiments

For all experiments cells were seeded at $2.5 \times 10^5$ cells/ml and cultured at a volume of 10-40ml. Viability was always over 90%.
Cells treated with PMA became adherent and were detached from the bottle using, 5 min incubation with trypsin at 37°C and inactivation with 2 ml medium and scraping the bottle with a cell scraper.

- **Experiments with 9/14/18 and 9/10/7 cells**

  Ponasterone A (PonA) 5µM was added to 9/14/18 (or 9/10/7) cells at time 0, and then added on at 24hrs. At 8hrs Vitamin D3 250ng/ml (600nM) or PMA 10nM were added. Flow Cytometry was performed at 0, 24, 48 hours. Pellets were obtained at all time points.

- **1,25-(OH)-2 Vitamin D3 induced differentiation of U937 cells**

  U937 cells were differentiated with 60 nM Vitamin D3 (1,25-(OH)2-VitD3; VITAMIN D3) dissolved in ethanol and treated for 72hours.

- **LAT2-knock-down system by short hairpin RNA (shRNA) in U937 cells**

  To induce monocytic differentiation, cells were treated with Vitamin D3 (60 nM for 48hrs), Vitamin D3 + TGF-β (1ng/ml for 96hrs), PMA (10nM for 48hrs), IL-4 (50ng/ml for 144hrs), or GMCSF (50 and 100ng/ml for 144hrs) according to previous publications.
  
  To induce granulocytic differentiation, GCSF (50ng/ml for 96hrs), or ATRA (0.5 µM for 72hrs) were used. Flow Cytometry was performed at the last day of each experiment. Cell growth and viability was also determined every day.

- **Treatment of Kasumi-1 cells with decitabine and other inducing agents**

  Decitabine was added in three pulses every 24hrs (changing medium every day). Concentrations used were 50 nM, 100 nM and 200 nM. At 48hrs ATRA 1µM or Vitamin D3 250 ng/ml were added. Medium was changed every day; cell growth and viability were also assessed every day. At 96hrs flow cytometry and cytopspins were performed, and cell pellets were frozen.

- **Dasatinib cell growth and differentiation experiments.** Dasatinib was added in two pulses, at 0 and 48 hours (after changing medium), at 0.1 nM, 1.5 nM, 10 nM and 100 nM. Viability
and cell growth were assessed at 24, 48 and 96 hours, and flow cytometry was performed at 96 hours.

**Inhibiting phosphorylation with dasatinib.** Cells were treated for 120 min with 0 or 100 nM dasatinib +/- 100ng/ml SCF, pellets were collected for protein isolation.

### 2.2.7. Protein isolation

Cells from culture were centrifugated and washed with PBS. Cell pellets were stored at -80°C, or immediately used. Pellets were solved in 100µl of lysis buffer, then vortexed for 10'', and mixed in an orbital shaker at 4°C for 15'. Finally cells were centrifugated for 15' at 13000rpm at 4°C, supernatant was transferred to a fresh eppendorf tube and stored at -80°C.

### 2.2.8. Protein measurement

On a flat bottom 96 well-plate, add 200µl PBS per well and 1µl protein lysate. 50µl of Protein Biorad assay solution (colorimetric Bradford Protein Assay) were added in each well. Using the spectrometer the samples were measured, the total protein concentration of the sample is determined taking BSA concentration as standard curve.

### 2.2.9. Western Blot

Western Blotting (WB) allows us to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. The samples are prepared from tissues or cells. The proteins are extracted with a lysis buffer that protects the protein of interest from degradation. The sample is then separated using SDS-PAGE and then transferred to a membrane for detection. The membrane is incubated with a generic protein (such as milk protein) to bind to any remaining sticky places. A primary antibody is then added to the solution which is able to bind to its specific protein. Finally, a secondary antibody-enzyme conjugate, which recognizes the primary antibody, is added.

**Preparing the samples**

Protein: 20-50µg

LDS Buffer 4x: 4µl
Fill with deionized water up to final volume: 15μl

Samples are heated, for denaturation of proteins, at the heat-block at 70°C for 10 min. Then shortly centrifugated at max speed. For WB under reducing conditions 1.5 μl Reducing Agent was added.

**Protein electrophoresis**

It is a method used to separate proteins according to their size. Since different proteins with similar molecular weights may migrate differently due to their differences in secondary, tertiary or quaternary structure, SDS, an anionic detergent, is used in SDS-PAGE to reduce proteins to their primary (linearized) structure and coat them with uniform negative charges.

The NuPAGE Electrophoresis system (Invitrogen) was used. A Gel 4-12% Bis-Tris was inserted on the X Cell Sure Lock Mini-cell. Then, the inner chamber was filled with 200ml 1x NuPAGE SDS MOPS Running Buffer. For reduced samples, the running buffer contained 500μl antioxidant. Samples were loaded on the gel together with Protein marker See Plus Blue 2 (Invitrogen). Outer chamber was filled with 600ml 1x NuPAGE SDS MOPS Running Buffer. The running was then made for about 1hr, depending on the size of the protein of interest, at 155V.

**Blotting**

It is the electrotransfer to a suitable membrane. The X Cell II Blot Module was used.

First, blotting pads and filter paper were soaked in Transfer Buffer. Second, the membrane was activated for 1 min in methanol and briefly immersed in deionized water, and finally it was immersed at least 5 min in Transfer Buffer.

Two pre-soaked blotting pads were placed into the blotting module core, carefully with out leaving any bubble between the pads; we place one filter paper, also without leaving bubbles, then carefully the gel (picked up with the gel knife), the other filter paper, and finally three pre-soaked blotting pads. Add enough blotting pads into the core, to rise 0.5 cm over rim.

Holding the blotting module firmly it was placed into the chamber. The module was filled with Transfer Buffer until the assembly was covered and outer chamber was filled with 650 ml deionized water.
The blotting was performed for 1 hour at 30V.

**Blocking**
After transfer, the membrane was placed in blocking solution, over a shaker. It was performed overnight at 4°C or at least one and half hour at room temperature.

Note for phosphorylated proteins: block the membrane with 5%w/v BSA in TBST.

**1st and 2nd antibody incubation**
Membrane was placed with the 1st antibody solution (concentration depending on the antibody, always solved in TBST + Milk 7%) overnight at 4°C or at least one hour at room temperature (depending on the antibody). Membrane was washed in TBST, 3 times shortly, then 15 min shaking, then 3 times 5 min each time. Second antibody was incubated for one hour at room temperature, shaking. The membrane was washed idem as before.

**Detecting System**
ECL Plus Western Blotting Detection System was used. Solutions A+B were mixed 40:1 and the membrane was covered with the mixed solution for 5 min, membrane was packed in plastic film and placed in the cassette for developing. Membranes were exposed to Film (Hyperfilm Amersham) as needed and then placed into the developing machine (Developer x-Omat M35 Kodak) in a dark room.

**2.2.10. Flow cytometry**
The flow cytometry is used to study the properties of cell subsets identified using monoclonal antibodies to cell-surface proteins. Individual cells within a mixed population are first tagged with specific monoclonal antibodies labeled with fluorescent dyes, or by specific antibodies followed by labeled anti-immunoglobulin antibodies. The mixture of labeled cells is then forced with a much larger volume of saline through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the labeled monoclonal antibodies and hence on the expression of cell-surface proteins by each cell (Charles A. Janeway 2001).
**Preparing the Samples**

2 x $10^5$ cells were placed on a flow cytometry tube with PBS, centrifuged for 5 min at 1200rpm, supernatant was discarded. Afterwards, cells were dyed with the antibody, and left in the dark at 4°C for 20 min. Cells were again washed with PBS, and supernatant discarded. Before the measurement cells were suspended again in PBS.

**Measurement of surface antigens**

Measurement was performed in a Dakocytomation flow cytometry machine. Analysis of the data was performed with Summit v 4.2 and Flow Jo softwares.

**Gating Strategy**

~1 x $10^4$ cells were gated.

Threshold for positivity was considered after 1% positivity with the Isotype Control for each antibody.
3. Results

3.1. Monocytic differentiation of U937 cells is partially blocked by AML1/ETO

Since in another model of conditional AML1/ETO expression a block of myeloid differentiation has been observed (Burel et al. 2001), the Ponsterone A (PonA) inducible cell line U937-9/14/18 was interrogated for cellular, immunocytologic and morphologic effects of AML1/ETO expression on myelo-monocytic differentiation. Upon PonA-induced AML1/ETO expression (confirmed by Western blot, see for example Figure 2.-), a statistically significant decrease of CD11b expression was noted, accompanied by a modest decrease in CD11c expression (Fig. 1). The AML1/ETO-mediated repression of CD11b was not observed in the negative control, the AML1/ETO-negative subclone 9/10/7-U937 containing the Lac-Z gene (Fig 1.- lower panel). Treatment of 9/14/18-U937 and 9/10/7-U937 with phorbol ester (PMA) alone resulted in the expected striking induction of CD11c, with little effect on CD11b which in these subclones of U937 is already highly expressed. The combined treatment of 9/14/18-U937 with PonA and PMA did not overcome the AML1/ETO-induced repression of CD11b, and resulted in a somewhat blunted increase in CD11c, compatible with a partial block of monocytic differentiation imposed by AML1/ETO. Repression of CD11b by AML1/ETO in 9/14/18-U937 was only partially overcome by treatment with Vitamin D3 (250 ng/ml, data not shown). Cytomorphologic signs of monocytic differentiation (adherent growth, wider cytoplasm vacuolization) were less marked in the presence of AML1/ETO in these experiments (data not shown).
Figure 1. - Conditional AML1/ETO expression in U937 cells results in a block of monocytic differentiation.

Upper panel: Flow cytometry analysis (mean of fluorescence intensity) for CD11b-PE and CD11c-PE of 9/14/18-U937 cells treated for 24 hours with Ponasterone A (PonA) to express AML1/ETO and/or treated for 24 hours with PMA 10 nM to induce monocytic differentiation. Lower panel: similar flow cytometry analysis in 9/10/7-U937 cells, a Lac-Z clone. Graphics express the mean of at least three
3.2. **Possible role of LAT2 in myelopoiesis.**

**LAT2 is repressed by AML1/ETO in U937 cells.**

The expression of the AML1/ETO protein was checked by Western Blot in the same samples showed in Figure 1, observing its up-regulation 24hrs after the treatment with PonA (Fig.-2). In agreement with previous results (Fliegauf et al., 2004) (Fliegauf et al. 2004), the adaptor molecule LAT2 (NTAL/LAB/WBSCR5), a novel *in vivo* AML1/ETO target gene, was strikingly repressed after conditional AML1/ETO expression in 9/14/18-U937 cells (Fliegauf et al. 2004). After 48hrs AML1/ETO induction with PonA, LAT2 was almost completely repressed. (Fig 2.-)

![Fig 2.-LAT2 is repressed by AML1/ETO in U937 cells.](image)

Western Blot of 9/14/18-U937 cells. AML1/ETO was expressed after 24hrs. LAT2 was down-regulated 48hrs after AML1/ETO expression. The cells were also treated with PMA. β-actin was used as loading control.

3.2.1. **LAT2 is up-regulated during monocytic differentiation.**

U937 and HL60 cells were treated with Vitamin D3 to induce monocytic differentiation and expression levels of LAT2 protein were detected by Western blotting at different time
points. This showed a transient up-regulation of LAT2 at 12 hours decreasing again at 48hrs in U937 cells (Fig. 3 –A). Vitamin D3 treatment in HL60 cells induced monocytic differentiation and up-regulation of LAT2 protein at 6 hours (Fig. 3 – B). As shown before in Fig. 2., LAT2 induction by vitamin D3 or PMA is partially blocked by AML1/ETO. Taking these results together, we hypothesized that LAT2 could play a role during monocytic differentiation.

Fig 3. - LAT2 is induced during Vitamin D3-induced monocytic differentiation.

A.- Western Blot showing the up-regulation of LAT2 protein in U937 cell line after 12 hours treatment with Vitamin D3, after 48 hours there was a down-regulation of LAT2 protein. B.- Western Blot showing up-regulation of LAT2 protein in HL60 cell line after 12 hours treatment with Vitamin D3. β-actin was used as loading control.

3.2.2. Induced monocytic differentiation of U937 in the presence or absence of LAT2

To assess whether LAT2 plays a role in monocytic differentiation, we used a LAT2-shRNA knock-down model in U937 cells developed in Heidelberg by Dr. Adelheid Cerwenka's group (Tessarz et al. 2007) (Fig. 4.-A). Treatment of the mock U937 cells with PMA showed a strong up-regulation of LAT2 protein after 24 hours (Fig. 4- A). Differentiation of LAT2 knock-down cells with PMA showed that, even though the knock-down model is very effective LAT2 protein is moderately up-regulated (Fig. 4-A).
I hypothesized that induced monocytic differentiation could be partially blocked by the down-regulation of LAT2 in U937 cells, shown by a decrease in the expression of surface molecules markers CD11b, CD11c, CD14. All the nine different treatments used to induce differentiation are shown in Table 2.

A significant difference between the mock transfected cells and the LAT2 knock-down was found on CD11b expression after treatment with the phorbol ester PMA for 48 hours; but not with other compounds (Table 2).

Expression of CD11b after 48 hours treatment with PMA was statistically significantly different ($p=0.002$; $n=9$, 5 independent experiments) in the presence (38.4% +/- 6.0) and absence (29.3% +/- 4.0) of LAT2 (Fig.4); no significant difference was found in CD11c or CD14 expression, with Vitamin D3 treatment (data not shown). Also at 24 hrs or at 72 hrs there was no statistical significance.
Figure 4.- Differentiation of U937 cells in a LAT-2 knock-down model.

A.- Western Blot showing the U937-TD (empty vector), U937-TD N4 and N5 (LAT 2 knock down cell lines). LAT2 was strongly induced after treatment with PMA in the mock and modestly induced in the knock-down. β – actin was used as loading control.

B.- CD11b expression in U937 after monocytic differentiation in the presence or absence of LAT2. Flow cytometry results comparing CD11b expression in U937-TD mock and LAT2 knock down after inducing differentiation with several agents. Treatment with PMA for 48hrs, induced a statistically significant difference in CD11b induction between mock and knock down (p=0.002, n=9, 5 independent experiments). CD11c and CD14 showed no difference.
<table>
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<th>Treatment</th>
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<th>U937-TD CD11b (%)</th>
<th>p-value</th>
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<tr>
<td>Untreated</td>
<td>4.4 +/- 1.8</td>
<td>4.8 +/- 2.4</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Vitamin D3 24 hrs 60 nM</td>
<td>13.7 +/- 5.5</td>
<td>17.3 +/- 2.5</td>
<td>0.96</td>
<td>(Koschmieder et al. 2007, Puccetti et al. 2002)</td>
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<td>Vitamin D3 48 hrs 60 nM</td>
<td>33.35 +/- 5.03</td>
<td>30.72 +/- 5.7</td>
<td>0.77</td>
<td>(Koschmieder et al. 2007, Puccetti et al. 2002)</td>
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<td>Vit D3 + TGFβ 96hrs 250ng/ml + 1ng/ml</td>
<td>59.0 +/- 1.4</td>
<td>59.0 +/- 5.6</td>
<td>1</td>
<td>(Testa et al., 1993)</td>
</tr>
<tr>
<td>PMA 24 hrs 10 nM</td>
<td>13.6 +/- 4.3</td>
<td>10.9 +/- 5.0</td>
<td>0.8</td>
<td>(Yamato et al., 1990)</td>
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<td>PMA 48 hrs 10 nM</td>
<td>38.45 +/- 6.02</td>
<td>29.32 +/- 4.02</td>
<td>0.002</td>
<td>(Yamato et al., 1990)</td>
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<td>ATRA 5.10 ^10^ M, 72hrs</td>
<td>8 +/- 1.0</td>
<td>7.8 +/- 1.9</td>
<td>0.81</td>
<td>(Grignani et al. 1993)</td>
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<td>ATRA + TNF 5.10 ^6^ M, 10ng/ml</td>
<td>4.6 +/- 0.7</td>
<td>5.8 +/- 2.2</td>
<td>0.54</td>
<td>(Grignani et al. 1993; Glasgow et al., 2005)</td>
</tr>
<tr>
<td>ATRA + G/GMCSF 72hrs 5.10 ^6^ M, 50ng/ml, 0,5ng/ml</td>
<td>14.7 +/- 0.4</td>
<td>14.2 +/- 1.1</td>
<td>0.72</td>
<td>(Glasgow et al., 2005)</td>
</tr>
<tr>
<td>IL4 + GMCSF 96hrs 50ng/ml 100ng/ml</td>
<td>9.4 +/- 2.2</td>
<td>9.6 +/- 2.1</td>
<td>0.93</td>
<td>(Koss et al., 1995)</td>
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**Table 2. – Induced differentiation of U937 in the presence or absence of LAT 2.**

CD11b expression after induction of monocytic and granulocytic differentiation in wild type and LAT2 knock-down U937 cells. CD11b expression was studied by flow cytometry in U937 cells with and without knocking-down LAT2 by shRNA after the induction of monocytic and granulocytic differentiation. Treatment with PMA for 48hrs, induced a statistically significant difference in CD11b induction between mock and knock-down (p=0.002, n=9, 5 independent experiments). CD11c and CD14 showed no difference. Statistics were made using the Student t-test.
3.3. Decitabine, a demethylating agent, “priming” for induction of differentiation in AML1/ETO-positive cell line Kasumi-1.

AML1/ETO blocks differentiation in AML1/ETO positive leukaemia (Burel et al. 2001), (Pabst et al. 2001), (Puccetti et al. 2002). In our inducible system, we showed that ectopic induction of AML1/ETO altered the expression of surface markers related to differentiation, CD11b and CD11c (Fig.-1). Moreover, AML1/ETO positive leukaemias do not respond to ATRA; AML1/ETO recruits HDACs and DNMTs to target promoters of different genes to silence the expression of several proteins such as C/EBPα or Pu.1. The effect of decitabine blocks the DNMTs and there is a demethylation of different genes including RARαβ2 gene, which becomes sensitive to ATRA. Following these data, our hypothesis was that pre-treatment with a demethylating agent like decitabine, could increase sensitivity to differentiation agents like ATRA or Vitamin D3 in an AML1/ETO positive cell line, relieving the block in differentiation. In the past, studies were made demonstrating synergy between decitabine and Vitamin D3 in U937 cells (AML1/ETO negative cell line) (Koschmieder et al. 2007), (Niitsu et al. 2001) or decitabine and phenylbutyrate (Wang Xj et al., 2005) (Santini et al., 2005).

Following the work by Yi Jiang, from our group, we analyzed the effect of decitabine in Kasumi-1 cell line, AML1/ETO positive cell line, with and without adding differentiating agents. Decitabine decreased cell growth and viability. 100 nM decitabine inhibited cell growth in 50%; co-treatment with ATRA also reached 50% inhibition in Kasumi-1 cells (Fig.-6.A). 100nM decitabine and co-treatment with Vitamin D3 reached 75% cell growth inhibition in Kasumi-1 cells (Figs.-7&8.Upper panel). ATRA or Vitamin D3 cooperated, in a dose-dependent way with decitabine to decrease viability in Kasumi-1 cells (Figs. 7&8-Lower panel).

Expression of CD11b, marker of differentiation was up-regulated after treatment with decitabine (Figs.9&10.-). Another sign of differentiation was the up-regulation of CD11c (Fig 9.-). Co-treatment with Vitamin D3 showed higher up-regulation when compared with those treated only with decitabine (Fig.9.-). Co-treatment with ATRA showed a very modest up-regulation of CD11b compared to cells treated only with decitabine (Fig. 10.-)
Figure 7. - Modest Increase of ATRA-Induced growth inhibition in combination with decitabine (DAC) treatment in Kasumi-1 cells. DAC was given as three consecutive pulses, one every 24 hrs, simultaneous with the last DAC pulse ATRA was added. Effect of DAC and/or ATRA on cell viability (similar effect was seen on two independent experiments)
Figure 8. - Modest Increase of Vitamin D3-Induced growth inhibition in combination to decitabine (DAC) treatment in Kasumi-1 cells. DAC was given as three consecutive pulses, one every 24 hrs, simultaneous with the last DAC pulse Vitamin D3 was added. Effect of DAC and/or Vitamin D3 on cell viability (similar effect was seen on two independent experiments).
Figure 9. – Assessment of CD11b and CD11c surface markers expression by flow cytometry (% of positive cells) in Kasumi-1 cells. Vitamin D3 was used at 250ng/ml.
Figure 10. – Assessment of CD11b surface marker expression in Kasumi-1 by flow cytometry (% of positive cells). Upper panel: Dose-dependent induction of CD11b after treatment with decitabine. Lower panel: Co-treatment with ATRA. (Concentrations are in nM)
3.4. Dasatinib as inducer of differentiation in AML1/ETO-positive cells

3.4.1 Robust in vivo differentiation of AML1/ETO-positive AML following dasatinib treatment

A 52 year old patient, with relapsed AML t(8;21) after standard treatments (including Allogeneic bone marrow transplantation) was treated with dasatinib (Fig.11-). This treatment was instituted to inhibit the activated tyrosine kinase c-kit (exon 8, exon 17 activating mutations). 48hs later leukemic growth was mitigated and neutrophils were noted in peripheral blood. After 1-2 weeks, blast clearance and strong emergence of granulocytes derived from the abnormal clone was seen in peripheral blood (FISH 99% positive for AML1/ETO). Leukocytes were proved to be functional. (Chevalier et al., manuscript in preparation) (Fig.12.-). This case led us to the study of c-Kit receptor in Kasumi-1 cells.

![Figure 11.-Treatment of a 52 year old patient with multiple relapse of t(8;21) AML with dasatinib. Blast clearance and strong emergency of leukocytes.](image)
3.4.2. Treatment of Kasumi-1 cells with dasatinib induces growth inhibition in the absence of changes in marker expression.

Point mutations of c-Kit have been identified in 12.8-46.8% of AML subtype M2 with t(8:21) (Beghini et al., Blood, 2000; Hematologica, 2004; Care et al., Br.J.Haematology, 2003). In human masts cells, c-Kit phosphorylates directly LAT2 after binding to SCF (Tkaczyk et al. 2004). Kasumi-1 cells have a homozygous mutation of c-Kit receptor and no expression of LAT2. We hypothesized that the inhibition of the c-Kit receptor by dasatinib could influence the viability, differentiation and cell growth of Kasumi-1 cells. Dasatinib is able to inhibit wild type and mutant c-Kit isoforms 45.

To explore this, Kasumi-1 was taken as a model for an AML1/ETO positive cell line and c-Kit receptor mutant (Becker et al. 2008). After 96 hours treatment with 10nM dasatinib, Kasumi-1
cells showed a strong inhibition of cell growth (Fig.13-Upper panel). Dasatinib does not alter the expression of c-Kit, but does inhibit pospho-Kit after 120 minute treatment with dasatinib 100 nM (Fig. 14- A.B). Expression of CD11b of Kasumi-1 cells treated with dasatinib. CD11b was not up-regulated after treatment with dasatinib for 96 hours (Data not shown).

We performed Western blot with the protein lysate of the previously described patient before and after receiving treatment with dasatinib to check for AML1/ETO expression and LAT2 expression (Fig. 15.-). Unfortunately the expression of AML1/ETO could not be properly assessed. However, we could show that there was no up-regulation of LAT2 in the patient before and after treatment with dasatinib (Fig. 15.-). The attempt was made also to assess C/EBPα looking for its up-regulation after treatment with dasatinib, though it was not successful.
Figure 13. – Upper panel.-Cell growth inhibition of Kasumi-1 cells after treatment with different concentrations of dasatinib over 96 hours. Lower panel.- Viability of Kasumi-1 cells after treatment with different concentrations of dasatinib over 96 hours.
Figure 14.- Inhibition of c-Kit in Kasumi-1 cells by dasatinib. A.- Western Blot showing c-Kit receptor protein expression in Kasumi-1 cells treated with dasatinib 0, 100 and 1000 nM for 120 min. Mononuclear cells were used as negative control. β-actin was used as loading control. B.- Western Blot showing p-c-Kit inhibition after adding dasatinib 100nM for 120 min. β-actin was used as loading control.
Figure 15.- LAT 2 is not up-regulated after treatment with dasatinib. Treatment of a patient with AML-1/ETO positive leukemia with dasatinib did not induce LAT-2 expression. β-actin was used as loading control.
4. Discussion

4.1. AML1/ETO mediates a partial block in monocytic differentiation

The initiating steps in the development of cancer are of considerable clinical importance and are a priority in the development of rational cancer treatment (Croce 2008). The fusion gene AML1/ETO is the product of the t(8;21)(q22;q22) chromosomal translocation, and is associated with approximately 40% of FAB subtype M2 subtype of acute myeloid leukemia (Frank et al. 1995; Gelmetti et al. 1998). It blocks cell differentiation by decreasing C/EBPα (Burel et al. 2001) PU.1 or MEF2c via protein-protein interactions (Mao et al. 1999); (Jakubowiak et al. 2000); (Pabst et al. 2001) and by sequestering the Vitamin D3 receptor (Puccetti et al. 2002). Decrease of AML1/ETO by siRNA in a cell line model was followed by a partial differentiation (up-regulation of CD11b) (Heidenreich et al. 2003). β-integrin CD11c was shown to be repressed by AML1/ETO in HL-60 cell line through C/EBPα down-regulation (Drescher et al. 2003).

Pucetti et al., and Burel et al., have engineered U-937 cells for the conditional expression of AML1/ETO fusion protein. These cells have been employed to demonstrate that the presence of this fusion protein interferes with granulocytic differentiation (Burel et al. 2001); (Puccetti et al. 2002). To analyze the effect of AML1/ETO in monocytic differentiation, an inducible U937 system 9/14/18-U937 (Fliegauf et al. 2004) was employed to conditional express AML1/ETO by adding Ponsterone A (PonA). We studied its effect using flow cytometry to measure the expression of CD11b and CD11c, membrane proteins up-regulated during monocytic differentiation, after 24 hrs treatment with PMA or Vitamin D3. The LacZ clone 9/10/7-U937 was used as a negative control. Cyto-morphological evidence of maturation (data not shown) were increase of cell size, with wider cytoplasm, appearance of cytoplasmic vacuoles and an increase of cell adherence, which was most striking in cells treated with PMA.

After inducing AML1/ETO in 9/14/18-U937 cells we showed a significant down-regulation of CD11b and CD11c by flow cytometry. This confirms the studies of Pucetti et al. and Burel et al. The induction of differentiation markers after treatment with PMA was also significantly decreased.
Phorbol ester is a strong inducer of monocytic differentiation: cells become adherent and increase their size and cytoplasmic vacuoles besides increasing the expression of differentiation markers. Interestingly, after the induction of AML1/ETO we could show a strong down-regulation of surface markers in cells treated with PMA. These effects were not due to the addition of Ponasterone A, given that they were specific for the 9/14/18-U937 clone and not seen in the LacZ-clone used as control for our experiments.

Overcoming the block in differentiation caused by AML1/ETO is one of the main aims of this work. The fusion protein PML/RARα blocks differentiation and this block has been proved to be overcome using ATRA (Grignani et al. 1993), an approach successfully used in the treatment of acute promyelocytic leukemia patients (Tallman et al. 1997). AML1/ETO recruits a nuclear corepressor complex including HDACs mediated by the ETO moiety (Gelmetti et al. 1998). AML1/ETO also recruits DNMTs (Fazi et al. 2007). Previous data showed that decitabine, a DNA-demethylating agent, induces monocytic differentiation in U937 cells; this inhibits Vitamin D3-induced cell proliferation without compromising cell viability (Koschmieder et al. 2007). Niitsu and colleagues showed that it is possible to sensitize MLL cells to ATRA or Vitamin D3 using 5-aza-2’-deocycytidine (Niitsu et al. 2001). In the present study, treatment with decitabine was able, in a dose-dependent way, to inhibit cell growth and decrease viability and, most importantly, to up-regulate the expression of CD11b in Kasumi-1 cells. However, the cooperation between decitabine and Vitamin D3 or ATRA was rather modest.

By blocking C/EBPα activity, AML1/ETO blocks granulocytic differentiation (Burel et al. 2001). Our data strongly indicates that AML1/ETO also alters monocytic differentiation. In our study AML1/ETO partially blocked monocytic differentiation induced by PMA. To go deeper into the mechanism of this observation, we looked again in one of AML1/ETO target genes involved in monocytic differentiation.

4.2. The AML1/ETO target gene LAT2 is involved in monocytic differentiation

Taking into consideration that LAT2 is a target gene of AML1/ETO in the inducible U937 model (Fliegauf et al. 2004) and that LAT2 is expressed in monocytes but not granulocytes, and up-regulated in alveolar macrophages when compared to circulating
monocytes (Duque 2008); we build the hypothesis that LAT2 could play a role in the AML1/ETO-mediated block in differentiation, regarding the monocyte lineage.

We used Vitamin D3 and Phorbol ester to induce monocytic differentiation in U937 and HL60 cells, showing that LAT2 is transiently up regulated.

To functionally study the role of LAT2 in monocytic differentiation we employed a LAT2 knock-down model (Tessarz et al. 2007). It consists of a stable shRNA knock-down. In the present study we could show that the knock-down model is very robust, however, after using PMA there was still an up-regulation, though mild, of LAT2.

LAT2 possesses no inherent catalytic activity; it acts as an adaptor molecule, which recruits and fastens critical signaling molecules into the signalosome (Iwaki et al. 2007). This property depends on the phosphorylation of specific tyrosine residues contained within its cytosolic tail. The complete pathway still remains unclear. Therefore, in our study we used a wide variety of differentiation treatments in the presence and absence of LAT2, attempting to trigger some of those pathways.

To induce monocytic differentiation in our knock-down model, we choose several agents and combinations. Treatment with ATRA and/or G-CSF/GM-CSF, that activates MAP kinase pathways, promotes myelomonocytic differentiation in murine pluripotent myeloid progenitor cells, U937, Kasumi-1, NB-4 and AML M2 patients (Glasow et al. 2005). Using this treatment, we could induce differentiation: CD11b expression increased from 4% to 14%; however, we could not show a difference in the presence or absence of LAT2. The combination between ATRA and TNF synergistically induces differentiation in NB-4 and U937 cell lines without a concomitant increase in apoptosis levels (Witcher et al. 2003), nevertheless, in our study, this combination failed to induce strong differentiation. Many of the studies performed in the past used different surface markers to look for differentiation; we used CD11b in order to be able to perform a wider variety of treatments, this might explain the difference observed in induction of differentiation between our study and others. IL-4 and GM-CSF induced morphological changes consistent with monocytic differentiation (Koss et al. 1996); in our hands, there was a modest increase of one fold in CD11b. Testa et al., already in 1993, showed that the combination of TGF-β and Vitamin D3 markedly potentiates the differentiating action of D3 on three human cell lines, HL-60, U937 and AML193, however, the percentage of CD11b positive cells was similar upon treatment with D3 alone or in combination with TGF-β, whereas the level of antigen density was significantly higher
in the latter group as evaluated by fluorescence intensity (Testa et al. 1993). Our results also show that this combination was capable of inducing CD11b and monocyctic features, but there was no significant difference between the “mock” cells, expressing LAT2 and the shRNA knock-down. Vitamin D3 can indirectly induce the up-regulation of CD14 through Sp1 (Zhang et al. 1994). PMA, Vitamin D3 and retinoic acid can induce myeloblastic leukemia cells to further differentiate toward the monocytic lineage (Lübbert et al. 1991). Vitamin D3 treatment of the mock and the knock-down could induce differentiation but no difference was assessed in the presence or absence of LAT2.

Interestingly, treatment with PMA at a very specific time point, 48 hours, showed a significant down-regulation of CD11b in the absence of LAT2. This effect is clearly agent specific.

Induction of CD11b gene transcription involves the activation of cis-acting elements including NF-KB (Shelley and Arnaout 1991). PMA activates PKC and PKC activates ERK, this pathway ends with the transcriptional activation of multiple genes mediated in part by NF-KB one of these genes is as it was presented before CD11b gene (Shelley and Arnaout, 1991). On the one hand, LAT2 negatively modulates ERK1/2 phosphorylation, in LAT2 knock-down cells there is a delayed but enhanced ERK phosphorylation after 20 minutes stimulation (Tessarz et al. 2007). However, studies showing later time points were not done yet. This means that at a later time point, and considering that Ca++ influx is negatively modulated by LAT2, it could be that LAT2 positively regulates ERK and in absence of LAT2 the transcriptional activation of certain genes is diminished.

On the other hand, transcriptional activity of NF-kB can be regulated by mechanisms other than cytosolic degradation of Inhibitor Kappa B (IκB) such as p65 phosphorylation. Several reports have demonstrated that the DNA binding (Hayashi et al. 1993) and transactivating capacity (Zhong et al. 1998); (Wang and Baldwin 1998) of NF-kB are up-regulated by inducible phosphorylation of p65. In this context, protein kinases, such as IKKs (Sakurai et al. 1999), protein kinase A (PKA) (Zhong et al. 1998), p38 mitogen-activated protein kinase (MAPK) (Vanden Berghe et al., 1998; Jeferies and O'Neill, 2000), extracellular signal-regulated protein kinase (ERK) (Vanden Berghe et al., 1998; Jeferies and O'Neill, 2000), and PKC (Lozano et al. 1994) have been shown to phosphorylate p65, for example at Ser276 by PKA or at Ser536 by IKKs. This p65 phosphorylation in turn increases the transcriptional activity of NF-kB, and represents another mechanism, independent of IκB
degradation, for enhancing NF-kB activation this argues against the hypothesis that LAT2 down-regulation inhibits PMA-induced differentiation by suppressing genes like the CD11b gene.

### 4.3. c-Kit inhibition and myeloid differentiation

In mast cells, LAT2 phosphorylation is a pivotal step in the signaling pathways leading to degranulation following FceRI aggregation and c-Kit receptor activation (Tkaczyk et al. 2004). Activating mutations of the activation loop of c-Kit are associated with acute myeloid leukemia. Dasatinib, a small-molecule, inhibits kinase activity of both wild-type and mutant c-Kit (Schittenhelm et al. 2006). One study performed in different cell lines, showed that among many cell lines treated with dasatinib, Kasumi-1 cell line was in the group of greatest response (Kolb et al. 2008). In our studies we took Kasumi-1 cell line as an AML1/ETO positive cell line that also bears a c-kit mutation (Becker et al. 2008), and treated it with different concentrations of dasatinib, showing that it in fact inhibits pospho-kit; however, we could not show any effect in vitro on cell differentiation. Of interest, in one patient with an AML FAB subtype M2, treatment with dasatinib clearly induced differentiation; the molecular mechanism needs to be studied (Chevalier et al., manuscript in process).

Based on our studies, AML1/ETO alters monocytic differentiation in our inducible system. We can also confirm LAT2 repression after induction of this fusion protein. The effect of the absence of LAT2 in differentiation is specific for PMA-induced differentiation, and it was reflected in the down-regulation of CD11b. The relevance of this result is not clear; it is also a matter of discussion if studies in the existent knock-down mice (Volna et al. 2004), (Zhu et al. 2004), should be done to further explore the role of this adaptor molecule in acute myeloid leukemia. The role of C-Kit phosphorylation of LAT2 is also not elucidated yet.
5. Zusammenfassung

## 6. Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABL1</td>
<td>V-abl Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AML1</td>
<td>Acute myeloid leukemia Gene 1</td>
</tr>
<tr>
<td>APL</td>
<td>Akute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATRA</td>
<td>All trans-Retinoic Acid</td>
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<tr>
<td>BCL-2</td>
<td>B cell CLL/lymphoma 2</td>
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<td>BSA</td>
<td>Bovine Serum Albumine</td>
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<td>CBF</td>
<td>Core binding factor</td>
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<td>CD</td>
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<td>C/EBPα</td>
<td>CAATT enhancer binding protein α</td>
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<td>CSF-R</td>
<td>Colony stimulating factor receptor</td>
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<td>c-AMP response element binding</td>
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<td>ERK</td>
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<td>FAB</td>
<td>French-American-British</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>Fetal Bovine Serum</td>
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<td>G-CSF</td>
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<td>Inhibitor of κB</td>
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<td>Inversion</td>
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<tr>
<td>ITD</td>
<td>Internal tandem duplication</td>
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<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
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<td>kDa</td>
<td>Kilo Dalton</td>
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<td>l</td>
<td>Liter</td>
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<tr>
<td>LAB</td>
<td>Linker for activation of B cells</td>
</tr>
<tr>
<td>LAT</td>
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<td>LPS</td>
<td>Lipo-polysaccharides</td>
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MAP: Mitogen-activated protein
MBD: Methyl binding protein
M-CSF: Macrophage colony stimulating factor
MDS: Myelodysplastic Syndrome
Min: Minutes
MTG8: Myeloid translocation gene on 8q22
ml: Milliliter
μl: Microliter
μg: Microgram
μM: Micromolar
mm: Millimeter
mM: Millimolar
mg/mL: Milligram per milliliter
NaCl: Natriumchloride
NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells
nm: Nanometer
ng: Nanogram
n.s: non-significant
NTAL: Non T cell activation linker
PE: Phycoerythrin
PCR: Polymerase-chain-Reaction
PKA: Protein kinase A
PKC: Protein kinase C
PLC-γ1: Phospholipase C gamma 1
PMA: Phorbol 12-Myristat 13-Acetat
pmol: Picomol
PML: Promyelocytic leukemia Gene
<table>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
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<td>Pon A</td>
<td>Ponasteron A</td>
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<tr>
<td>RARβ2</td>
<td>Retinoid acid receptor beta 2</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribo-nucleic-acid</td>
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<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Runt related Transcription-factor 1</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium-dodecyl-sulphate</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
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<tr>
<td>t</td>
<td>Translocation</td>
</tr>
<tr>
<td>tAML</td>
<td>Therapy-related AML</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>U/mL</td>
<td>Units per milliliter</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>WBSCR5</td>
<td>Williams Beuren Syndrome Critical Region 5 Gen</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>μL</td>
<td>Microliter</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μg/μL</td>
<td>Microgram per Micro liter</td>
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<td>WT</td>
<td>Wild type</td>
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7. Bibliography


8. Acknowledgements

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9. Curriculum Vitae

Personal Information

Date of Birth: 10.06.1982
Age: 27
Nationality: Argentine/Italian
E-mail: leticia.solari@uniklinikfreiburg.de
letisolari@gmail.com

9.1 Educational Background

2000-2007 Universidad de Buenos Aires (UBA) – Medical Doctor (Studies).
Class performance: 8.70 out of 10 (8 - 9 being distinguished).

1995-1999 Instituto Libre de Segunda Enseñanza (High School, superintended by the University of Buenos Aires) – graduation date December 1999.
With Honors, Class performance: 8.66 out of 10 (8 - 9 being distinguished).

Research Work

May 2007-Feb.2009 Full time research project for the doctoral thesis at the Hematology and Oncology department of the Albert Ludwig University, Freiburg, Germany

Currently

2009 Resident at the Hematology and Oncology department from the Albert Ludwig University of Freiburg, Freiburg, Germany

Since 2007 Coordinating and Organizing the International Master Program in Biomedical Sciences, IMBS, a joint program from the University of Freiburg and the University of Buenos Aires (UBA)
Experiences Abroad

2006  **Albert Ludwig University**, Freiburg, Germany
      Two months Scholarship (May-June), rotation at the Hematology and Oncology department, as a part of the last medical year clinical rotations.

2005-2006  **Harvard Medical School**
      Visiting student in Harvard Medical School (4 Months, last medical year clinical clerkships) Boston, Ma, USA.
      Beth Israel Deaconess Medical Center: Neonatal Intensive Care Unit
      Brigham and Women Hospital – Children’s Hospital Boston: Clinical Immunology
      Brigham and Women Hospital: Infectious Diseases

1998  School Exchange, **Mc-Gill School**, Mobile, Alabama, USA January

Clinical Experiences

2006  Primary Care Center, City of Buenos Aires, Public Health System
      Clinical Practice in Pediatric Area, October

2006  Ricardo Gutierrez Children Hospital, Buenos Aires
      Pediatrics Intensive Care Unit and primary care facilities
      September-November

2006  Cosme Argerich Hospital, Buenos Aires
      Rotation through the Chirurgic Department
      July-August

2006  British Hospital in Buenos Aires
      Rotation in the Obstetrics and Gynecology department
      March-April

2003- 2007  Emergency Ward, at the University Hospital “Hospital de Clínicas José de San Martin”
      Eight hours shifts weekly
1999 Internship Program, in the Gastroenterological hospital “Bonorino Udaondo”, Organized by the Instituto Libre de Segunda Enseñanza, UBA.

Academic Experiences

2002-2007 Assistant lecturer, from the 3º Academic Unit Histology, Cell Biology, Embryology and Genetics “Professor Dr. Guillermo Jaim Etcheverry”, eight hours weekly.


2004 National Medical Student Contest


Languages

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9.2. Symposium Participation / Congresses/Courses (abbreviated)

-Refresher Course of Internist Oncology-19.06.09-Organized by Sanofi Aventis, Freiburg, Germany

-Basic Life Support-18.06.09-University of Freiburg, Germany

-“Advances in Stem Cell transplantation- 20th anniversary of the first allogeneic hematopoietic cell transplantation in Freiburg” May 07-09 2009 Freiburg, Germany.

-DGHO 10-14 October 2008
Annual Meeting of the German, Austrian and Swiss Societies of Hematology and Oncology

**Poster Presentation:** “Does AML1/ETO-mediated repression of LAT2 play a role in the differentiation block of AML1/ETO positive leukemia?”

- “Project Management Seminar” 05-07.11.2008, Organized by the DAAD, “training and Beratung”, Bonn, Germany.

- 1st Freiburg-Iasi Symposium on Molecular and Clinical Oncology, October 24th-28th 2007, University of Medicine and Pharmacy Gr.Popă, Iasi, Romania

**Presentation:** “AML1/ETO and its target genes: role in differentiation block of AML”


- 13th Annual EuCCU Symposium Clinical and Experimental Oncology, Freiburg, Germany, May 2006.

- Internacional Research Course, Asociación médica Argentina (Argentinean Medical Society) 2005.


- Participant in the 10th International Congress of Intern Medicine from the “José de San Martin” Clinical Hospital (UBA) 2004.