MOHITO, a novel mouse cytokine-dependent T-cell line, enables studies of oncogenic signaling in the T-cell context

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ABSTRACT

The mouse pro-B cell line Ba/F3 has gained major interest as a model system to investigate oncogenic tyrosine kinases and to determine the efficacy of kinase inhibitors. While Ba/F3 cells are suitable to study oncogenic kinases derived from various cell types, the signaling networks in Ba/F3 cells are B-cell specific. We have established a mouse CD4+CD8+ double positive T-cell line (named MOHITO, for MOuse Hematopoietic Interleukin-dependent cell line of T-cell Origin) that has many features of human T-cell acute lymphoblastic leukemia (*Notch1* and *Jak1* mutation, TCR rearrangement) and is dependent on interleukin-7. The MOHITO cell line can be transformed to cytokine independent proliferation by BCR-ABL1 or mutant JAK1. This mouse T-cell line is a novel model system to investigate protein signaling and inhibition in a T-cell specific context and is a valuable tool to study and verify oncogenic capacity of mutations in the kinome and phosphatome in T-cell malignancies.

Key words: cell line, leukemia, thymocyte, interleukin, mouse.

Citation: Kleppe M, Mentens N, Tousseyn T, Wlodarska I, and Cools J. MOHITO, a novel mouse cytokine-dependent T-cell line, enables studies of oncogenic signaling in the T-cell context. Haematologica 2011;96(3):779-783. doi:10.3324/haematol.2010.035931

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of the thymocytes that is caused by a stepwise accumulation of genomic lesions. Specific subgroups of T-ALL have been defined based on the expression of transcription factors such as TAL1, TLX1, TLX3, HOXA, CALM-AF10 or LYL1. In addition to those aberrations, deletions or mutations have been identified that target a variety of oncogenes or tumor suppressor genes including NOTCH1, FBXW7, CDKN2A, MYB, WT1, LEF1, PHF6 and others.[1-4] Of these, NOTCH1 is one of the most important oncogenes in T-ALL, since more than half of these leukemias harbor NOTCH1 mutations, and the possibility to target NOTCH1 signaling by gamma-secretase inhibitors has generated wide interest in NOTCH1 as a target for therapy.[1,5,6] In addition, several kinases are known to be activated in T-ALL through overexpression (LCK), gene fusion (NUP214-ABL1), or mutation (JAK1 and AKT1). Deletion and mutation of the phosphatases PTEN and PTPN2 are alternative mechanisms that lead to activation of kinases and additional signaling pathways, and may also influence the sensitivity of the T-ALL cells to specific inhibitors.[6,7]

Many human leukemia cell lines have been derived from various leukemias, which show many of the mutations and chromosomal rearrangements that are observed in human leukemias. Cell lines have been a valuable tool for the analysis of oncogenic properties of oncogenes and oncogenic pathways, for drug and RNAi screens and for pre-clinical drug testing. Unfortunately, each of these cell lines has a set of very specific genomic alterations, and not all mutations that are found in primary T-ALL samples are present in the available cell lines. For example, JAK1 mutations are present in 10% of the T-ALLs, but no T-ALL cell line is available with a JAK1 mutation.[8]

The interleukin-3 (IL-3) dependent murine pro-B cell line Ba/F3 has taken up an important role as a model system to evaluate the oncogenic potential of novel cancer-associated mutations in tyrosine kinases. Ba/F3 cells are IL-3 dependent for their proliferation and survival, and can easily be engineered to become dependent on an oncogenic tyrosine kinase, such as BCR-ABL1, instead of IL-3.[9,10] In this way, the Ba/F3 cell line can be used to study the oncogenic potential of newly identified kinase mutations such as those identified through high-throughput sequence studies.[11] In addition, Ba/F3 cells can be used to perform retroviral insertion mutagenesis screens[12] for the study of kinase inhibitors and the generation of comprehensive resistance profiles,[13,14] up to the point of evaluation of small molecule inhibitors *in vivo*.[15,16] Although Ba/F3 cells can also be used to study T-cell specific oncogenes such as NUP214-ABL117, downstream signaling is likely to be different between the B-cell and the T-cell context. Here we report a novel mouse cytokine dependent leukemic T-cell line and demonstrate its applicability to study oncogenic signaling of T-cell malignancies.

The online version of this article has a Supplementary Appendix.

Funding: this work was supported by grants from the K.U.Leuven (concerted action grant to JC, IW), the FWO-Vlaanderen (G.0287.07, J.C.), the Foundation against Cancer (SCIE2006-34, JC), and an ERC-starting grant (JC).
Manuscript received on October 20, 2010. Revised version arrived on December 6, 2010. Manuscript accepted on December 22, 2010.
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Design and Methods

Cell lines

Human 293T cells and mouse pro-B cell line Ba/F3 were purchased from DSMZ and cultured in either DMEM or RPMI-1640 medium both supplemented with 10% fetal calf serum (FCS). Ba/F3 cells were cultured in the presence of IL-3 (1 ng/mL) (Peprotech). MOHITO cells were cultured in RPMI-1640 containing 20% FCS supplemented with IL-7 (10 ng/mL) and IL-2 (5 ng/ml) (Peprotech). MOHITO cells were frozen in RPMI-1640 media containing 20% FBS and 5% DMSO. Cells were thawed in presence of 50 μg/mL DNase (Sigma Aldrich). For analysis of cytokine dependency, cytokines were removed through two rounds of washing with PBS and cell proliferation was monitored in 24-well plates. The MOHITO cell line will be available to scientists upon request.

Mouse experiments

Six week old female Balb/c mice were obtained from Harlan and maintained in a specific pathogen free facility. All procedures were approved by the Animal Ethic Committee (K.U.Leuven, Leuven, Belgium). For secondary transplants, 106 spleen cells were injected into the tail vain of sublethally irradiated female recipient mice. Mice were euthanized by first signs of disease development.

Virus production and retroviral transduction of cells

Viral production and transduction were performed as described previously.[17] In case of MOHITO cells, 6-well plates were coated with RetroNectin solution overnight (final concentration 5 μg/cm^2, Takara Bio Inc.) and blocked with 2% FBS in PBS for 30 min before use. Viral supernatant was pre-loaded onto RetroNectin coated plates by centrifugation (2000 g, 30 min, 30°C). After centrifugation, viral supernatant was discarded, plates were washed with PBS and cells were added at a density of 0.5×10^6 cells/mL. Retroviral transduction was achieved using standard spin-infection procedure (2000 g, 90 min, 30°C). Cells were placed in an incubator for 72 h to recover before determining transduction efficiency and performance of subsequent experiments.

Statistical analysis

Student's t-test was applied to determine significant differences between the mean of two groups. Normality tests were used to test the assumption of a normal distribution. All graphs represent mean values ± s.e.m. Additional methods are described in the *Online Supplementary Appendix*.

Results and Discussion

Establishment and characterization of a novel cytokine-dependent mouse T-ALL cell line

A sublethally irradiated female Balb/c mouse developed a hematologic malignancy with enlargement of the spleen, thymus and lymph nodes six months after irradiation. Histopathological analysis from tissue sections and immunophenotyping of single cell suspensions obtained from spleen, bone marrow and thymus confirmed the presence of a CD4$^+$CD8$^+$CD3$^-$ T-ALL (Figure 1A). The leukemia cells were transplantable to secondary and tertiary recipient mice (Figure 1B), and showed a characteristic immunophenotype and a single T-cell receptor (TCR) rearrangement (Figure 1C).

We were able to culture leukemia cells derived from the spleen of the primary diseased mouse *ex vivo* in the presence of IL-2 and IL-7 for a period of more than five months without indications of exhaustion. The cultured cells have round morphology, grow as single cells in suspension with a doubling time of 16-20 h and can cause leukemia in Balb/c mice with a latency of 3-4 weeks (Figure 1B). These cells have all characteristics of an immortalized cell line that we have named MOHITO (MOuse Hematopoietic Interleukin-dependent cell line of T-cell Origin).

Cytogenetic analysis using karyotyping, m-FISH and array-CGH on both primary cells and established cell cultures at several time points revealed a stable karyotype (43,XX,t(5;12),+der(5)(5;12),+10,+14,+15) with a limited number of additional duplications or deletions, including deletion of Bcl11b and Dicer1 (Figure 1D and *Online Supplementary Appendix*). Cells harvested from secondary transplants did not harbor additional cytogenetic aberrations as determined by complementary m-FISH and array CGH analysis, indicating that these cells have a stable genome (*data not shown*).

Additional gene expression profiling did not reveal ectopic expression of specific transcription factors (*Online Supplementary Appendix*), but similar to human T-ALL, sequence analysis revealed mutations within Notch1 and Jak1 (Figure 1E and F).

Deregulated activation of NOTCH signaling is frequently detected in human T-ALL and is associated with point mutations in the heterodimerization domain (HD) or insertion/deletion mutations in the PEST domain of the NOTCH1 gene.[18] Sequence analysis of Notch1 in MOHITO cells identified a point mutation in the HD domain and a frameshift mutation in the PEST domain (Figure 1E). Western blot analysis confirmed the presence of cleaved Notch1 protein, and treatment with a gamma-secretase inhibitor dose-dependently reduced activated Notch1 protein levels and proliferation of the cells (*Online Supplementary Appendix*).

Sequence analysis of Jak1 identified a novel S1042I point mutation in the kinase domain (Figure 1F). Strikingly, MOHITO cells were homozygous for the T to G Jak1 mutation suggesting either the presence of a uniparental disomy or complete loss of the wild-type allele. Experiments in Ba/F3 cells confirmed that the Jak1(S1042I) mutant is a constitutively active kinase that transforms Ba/F3 cells (*Online Supplementary Appendix*), but seems not to be able to transform MOHITO cells to cytokine independency at endogenous expression levels.

Additional genetic, immunophenotypic and functional characteristics of the leukemia cells are described in the *Online Supplementary Appendix*.

MOHITO cells are dependent on exogenous IL-7

Proliferation and apoptosis assays demonstrated a high dependency of MOHITO cells to exogenous cytokines. Depletion of both IL-2 and IL-7 resulted in complete inhibition of proliferation and substantial cell death within 72 h as determined by Annexin V staining (Figure 2A and B). Interestingly, removal of IL-2 caused only mild effects on the proliferation of MOHITO cell when IL-7 was present, identifying IL-7 as the major proliferation and survival stimulus (Figure 2C). Both cytokines activate the JAK/STAT signaling pathway through binding to their respective surface receptors.[19] In order to test if Jak1 represent the crucial driving force of proliferation, we performed knockdown experiments using a mouse Jak1 specific small interfering RNA (siRNA). Reduction of Jak1

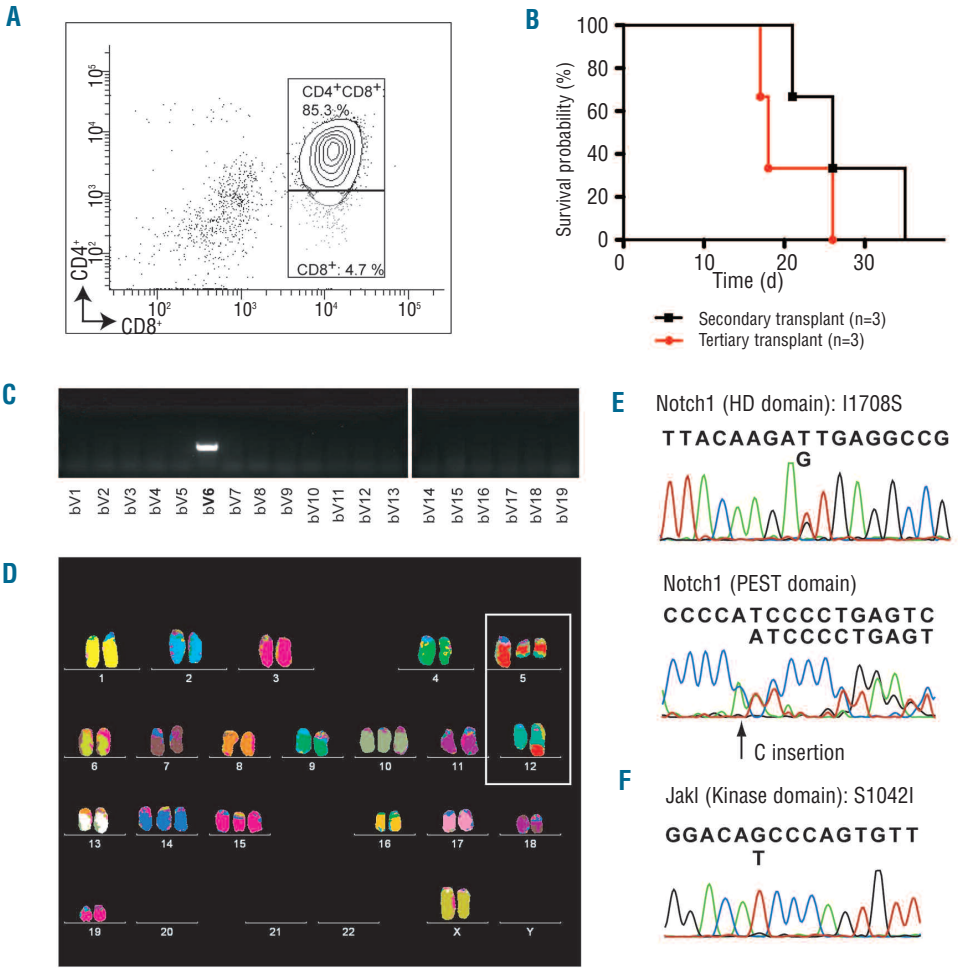

Figure 1. Characterization of MOHITO cells. (A) Flow cytometry analysis defined the majority of isolated leukemic cells as immature with a $CD4^+CD8^+$ phenotype. (B) Spleen cells from the primary mouse were injected (tail vain) into sublethally irradiated syngeneic mice (n=3). Survival curves of a representative secondary (black) and tertiary (red) transplantation experiment are shown. (C) PCR assay results for detection of rearranged TCR, in secondary cells. Clonal TCR, rearrangement between V,6 was almost exclusively present in primary cells. (D) Representative m-FISH analysis of MOHITO cells. Boxed regions highlight the presence of a reciprocal translocation between chr5 and chr12. (E) Sequence analysis of MOHITO cells identified a point mutation (5124T>G) in the heterodimerization domain (HD, upper panel) and an insertion/frameshift (7463-64insC, lower panel) mutation in the PEST domain (exon 34) of Notch1 resulting in a truncated protein (ENSMUST00000028288). (F) MOHITO cells carried a novel point mutation in the kinase domain of Jak1 (exon 22, 3125G>T; ENSMUST00000102781).

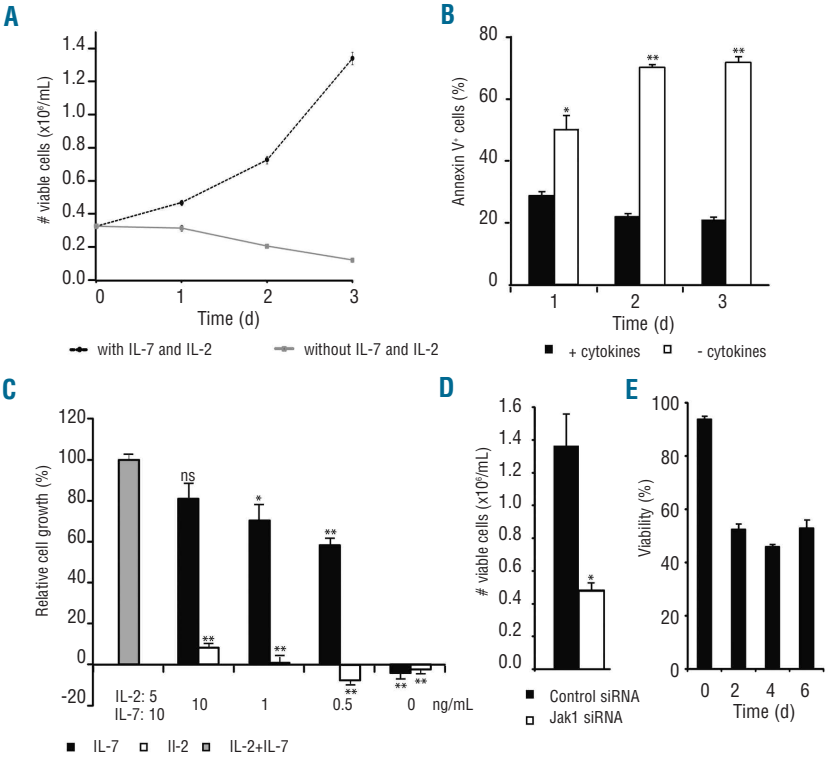

Figure 2. MOHITO cells are dependent on exogenous IL-7 for their growth and survival *ex vivo*. (A) Removal of cytokines resulted in complete inhibition of proliferation of MOHITO cells. Cell numbers were determined in triplicate every 24 h. Averages ± s.e.m. are shown. Dashed black lines: cells cultured in presence of cytokines; solid gray line: cells cultured in absence of cytokines. (B) Cytokine depletion induced profound cell death. Annexin V staining was used to determine percentage of apoptotic cells by flow cytometry. Graph shows annexin V positive cells (%) as function of time (days). Closed bars: with cytokines; open bars: without cytokines. *P< 0.05; **P<0.005. (C Removal of IL-2 in presence of high IL-7 (10 ng/mL) only mildly affected cell growth. MOHITO cells were cultured in variable cytokine combinations and relative cell proliferation was monitored after 72 h. Gray bar: IL-2+IL-7, black bars: IL-7, white bars: IL-2. Bars represent an average of three determinations±s.e.m. *P<0.05; **P<0.005; ns, no statistical significance. (D) Knockdown of endogenous Jak1 (Jak1 siRNA, open bar) significantly blocked proliferation of MOHITO cells. Control cells were transfected with scrambled non-silencing siRNA (control siRNA, closed bar). Absolute cell counts of three determinations at 72 h are given. *P<0.005. (E) MOHITO cells display reduced dependency on IL-2 and IL-7 upon continuous culture for three months. Cell viability (y-axis) was followed for six days after removal of exogenous cytokines.

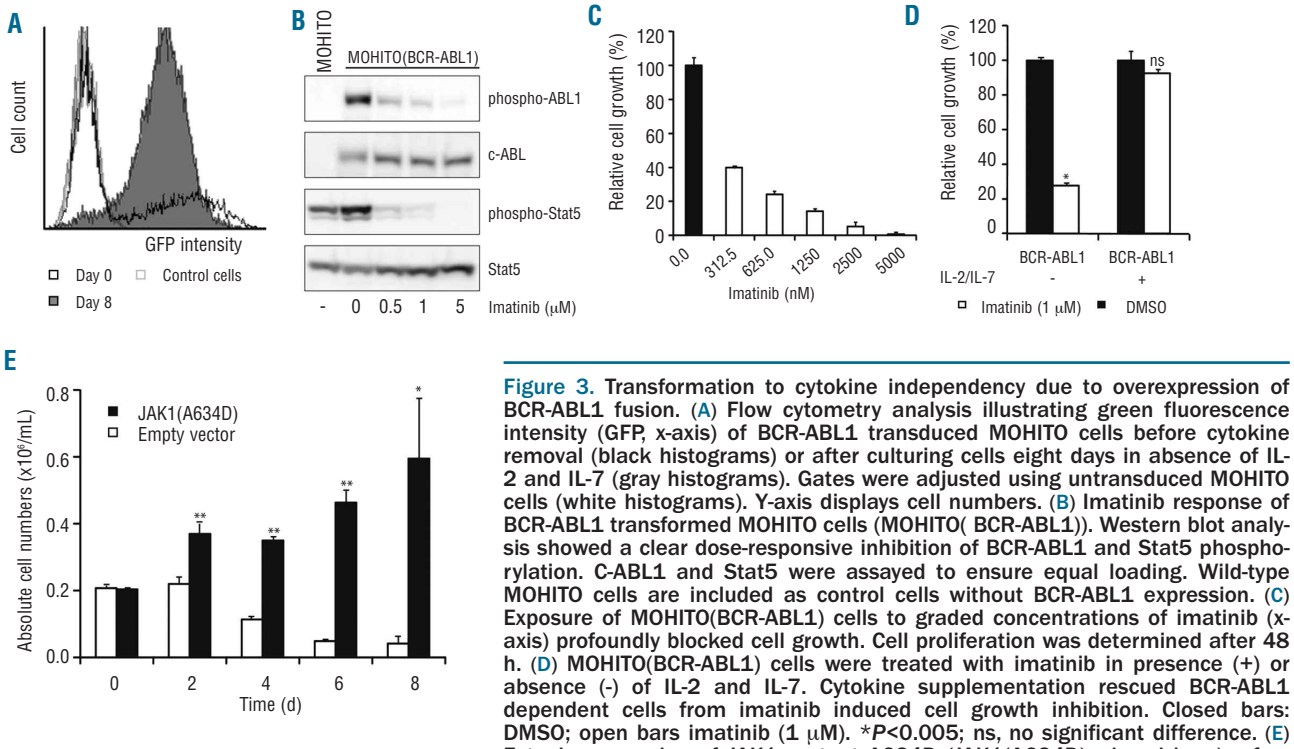

Figure 3. Transformation to cytokine independency due to overexpression of BCR-ABL1 fusion. (A) Flow cytometry analysis illustrating green fluorescence intensity (GFP, x-axis) of BCR-ABL1 transduced MOHITO cells before cytokine removal (black histograms) or after culturing cells eight days in absence of IL-2 and IL-7 (gray histograms). Gates were adjusted using untransduced MOHITO cells (white histograms). Y-axis displays cell numbers. (B) Imatinib response of BCR-ABL1 transformed MOHITO cells (MOHITO(BCR-ABL1)). Western blot analysis showed a clear dose-responsive inhibition of BCR-ABL1 and Stat5 phosphorylation. C-ABL1 and Stat5 were assayed to ensure equal loading. Wild-type MOHITO cells are included as control cells without BCR-ABL1 expression. (C) Exposure of MOHITO(BCR-ABL1) cells to graded concentrations of imatinib (x-axis) profoundly blocked cell growth. Cell proliferation was determined after 48 h. (D) MOHITO(BCR-ABL1) cells were treated with imatinib in presence (+) or absence (-) of IL-2 and IL-7. Cytokine supplementation rescued BCR-ABL1 dependent cells from imatinib induced cell growth inhibition. Closed bars: DMSO; open bars imatinib (1 μM). **P*<0.005; ns, no significant difference. (E) Ectopic expression of JAK1 mutant A634D (JAK1(A634D), closed bars) refers cells to cytokine independent growth. Cells were depleted for IL-2 and IL-7 and cell numbers were recorded in triplicate every two days (x-axis). Y-axis represents absolute cell numbers. **P*<0.05, ***P*<0.005.

expression levels inhibited cell proliferation and reduced viability of MOHITO cells (Figure 2D). In order to ensure retained cytokine dependency during long-term *in vitro* culture, we assayed MOHITO cells for viability and proliferation upon cytokine depletion at various time points. We observed a diminished dependency on exogenous cytokines at three months of continuous culture with prolonged survival of the cells after removal of IL-2 and IL-7 (Figure 2E). Of note, during *ex vivo* culturing, cells altered their surface marker expression to a $CD8^+$ single positive phenotype, while retaining their cytokine dependency. The differentiation process from the immature double positive stage to a mature single $CD8^+$ stage occurred slowly within 4-5 weeks of culture (*data not shown*).[20,21]

BCR-ABL1 and JAK1(A634D) transform MOHITO cells to IL-7 independent proliferation

The IL-3 dependent murine pro-B cell line Ba/F3 is an extremely convenient model system to study the transforming potential of tyrosine kinase genes, and to test their sensitivity to kinase inhibitors. The Ba/F3 cells are, however, of B-cell origin, and thus not suitable to study T-cell specific signaling. In order to test if the IL-7 dependent MOHITO cell line would serve as a model system to study kinase signaling in the T-cell context, we tested if MOHITO cells could be transformed to IL-7 independent proliferation by BCR-ABL1 or the JAK1(A634D) mutant, two oncogenes known to be implicated in the pathogenesis of T-ALL.[3,22-24]

We transduced MOHITO cells with a bicistronic BCR-ABL1-IRES-GFP construct allowing us to track BCR-ABL1 expressing cells by GFP. With an initial fraction of about 30% GFP positive cells, cytokine withdrawal caused a constant increase of the GFP positive population, which finally completely outcompeted the untransduced cells (Figure 3A). Protein analysis detected high autophosphorylation levels of BCR-ABL1 and phosphorylation of its downstream target Stat5 (Figure 3B). Transformed cells became sensitive to the kinase inhibitor imatinib, which blocked proliferation and induced cell death (Figure 3C). Signal pathway analysis showed a dose-dependent reduction of BCR-ABL1 activation and downstream target Stat5 upon exposure to increasing concentrations of imatinib (Figure 3B). Re-addition of IL-2 and IL-7 rescued cells completely from imatinib induced cell death (Figure 3D).

Similarly, expression of JAK1(A634D) also transformed MOHITO cells to growth factor independency (Figure 3E). Of note, even though cells were highly proliferating in absence of exogenous cytokines, JAK1(A634D) expressing cells rarely exceeded viabilities above 80%. Together with the fact that BCR-ABL1 expressing MOHITO cells displayed high viability (>90%), this finding suggests that the JAK1(A634D) mutant does not activate all survival pathways in T cells as compared to BCR-ABL1.

Signaling downstream of oncogenic kinases in the B- and the T-cell context

Signaling downstream of oncogenic kinases is likely to be different depending on the cell context. Having both BCR-ABL1 or mutant JAK1 transformed leukemic T- and B-cells, we assessed the difference in activation of Src family kinases and MAP kinase pathway activation.

MOHITO cells showed expression of Lck, Fyn, Blk and Lyn, whereas in Ba/F3 cells only Lyn and Fyn expression was detected (*Online Supplementary Appendix Figure S9A*).

Immunoprecipitation of activated Src kinases in cytokine independent MOHITO and Ba/F3 cells determined clear differences in their phosphorylation pattern. Both BCR-ABL1 and JAK1(A634D) dependent MOHITO cells displayed only strong activation of Lck (*Online Supplementary Appendix Figure S9B*). In contrast, transformed Ba/F3 cells revealed activation of Lyn (53 and 56 kD) and Fyn (59 kD), both previously implemented in the signaling cascade initiated by BCR-ABL1 in myeloid cells (*Online Supplementary Appendix Figure S9B*).[25,26] In addition, we also detected major differences in the activation level of the MAP kinase pathway. Both BCR-ABL1 and JAK1(A634D) strongly activated Mek1/2 kinases as well as downstream kinases Erk1/2 in Ba/F3 cells, but only weakly activated this pathway in MOHITO cells (*Online Supplementary Appendix Figure S9C*).

Conclusions

We describe here the establishment of a new cytokine dependent mouse T-cell line. Based on our genomic and functional characterization, we conclude that this cell line has characteristics of human T-ALL and can be used in pre-clinical studies of drugs both *in vitro* and *in vivo*. *In vitro* the cells are largely dependent on IL-7 for their proliferation and survival, but *in vivo* the cells quickly expand and cause a T-ALL like disease when injected in Balb/c mice. Our initial analyses show that the cells are sensitive to gamma-secretase inhibitors, dexamethasone, and JAK inhibitors. Most importantly, the MOHITO cell line can be transformed by oncogenic kinases, which allows for the analysis of the transforming potential of T-cell specific mutations and their consequences on T-cell signaling.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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