C/EBPβ expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway

Natasa Anastasov,[1,2] Irina Bonzheim,[3,4] Martina Rudelius,[5] Margit Klier,[3,4] Therese Dau,[1] Daniela Angermeier,[1,5] Justus Duyster,[6] Stefania Pittaluga,[7] Falko Fend,[3,4] Mark Raffeld,[7] and Leticia Quintanilla-Martinez[1,3,4]

[1]Institute of Pathology, Helmholtz Center Munich, German Research Center for Environmental Health, Germany; [2]Institute of Radiation Biology, Helmholtz Center Munich, German Research Center for Environmental Health, Germany; [3]Institute of Pathology, Eberhard-Karls-University, Tübingen, Germany; [4]University Hospital and Comprehensive Cancer Center, Tübingen, Germany; [5]Institute of Pathology, Technical University Munich, Germany; [6]Internal Medicine III, Technical University Munich, Germany, and [7]Hematopathology Section, Laboratory of Pathology, NCI, NIH, Bethesda, MD, USA

ABSTRACT

Background
Anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma is characterized by the t(2;5) chromosomal translocation, resulting in the expression of a fusion protein formed of nucleophosmin (NPM) and ALK. Recently, we reported the abnormal expression of the transcription factor CCAAT/enhancer binding protein-beta (C/EBPβ) in ALK-positive anaplastic large cell lymphomas, and demonstrated its dependence on NPM-ALK activity.

Design and Methods
In this study, the role of C/EBPβ in proliferation and survival of ALK-positive anaplastic large cell lymphomas was investigated, as well as the mechanism of its expression and activity. Highly effective short hairpin RNA sequences and/or pharmacological inhibitors were used to abrogate the expression or activity of C/EBPβ, signal transducer and activator of transcription 3 (STAT3), AKT, extracellular signal-related kinase 1/2 (ERK1/2) and mammalian target of rapamycin (mTOR).

Results
Interference with C/EBPβ expression resulted in a dramatic decrease in cell proliferation in ALK-positive anaplastic large cell lymphomas, with a mild induction of apoptosis after 6 days. Down-regulation of STAT3 resulted in a marked decrease in C/EBPβ mRNA and protein levels with impairment in cell proliferation and viability, underscoring the important role of these two proteins in ALK-mediated oncogenesis. Additionally, we demonstrated that reduction of ERK1/2 activity led to C/EBPβ Thr^{235} dephosphorylation and moderate growth retardation. The AKT/mTOR signaling pathway did not have any influence on C/EBPβ expression or C/EBPβ phosphorylation.

Conclusions
These findings reveal the convergence of STAT3 and ERK1/2 signaling pathways activated by NPM-ALK in mediating the regulation of C/EBPβ expression, a transcription factor central to NPM-ALK transformation.

Key words: anaplastic large cell lymphoma (ALCL), C/EBPβ, STAT3, RNA interference, cell proliferation.

Citation: Anastasov N, Bonzheim I, Rudelius M, Klier M, Dau T, Angermeier D, Duyster J, Pittaluga S, Fend F, Raffeld M, and Quintanilla-Martinez L. C/EBPβ expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway. Haematologica 2010;95:760-767. doi:10.3324/haematol.2009.014050

MR and LQ-M contributed equally to this work

Funding: supported in part by a grant from the Deutsche Forschungsgemeinschaft (SFB-685) to LQ-M and FF

Acknowledgments: we are grateful to Dr. David Baltimore and to Dr. Abel Sánchez-Aguilera for donating the pFUGW vector and packaging plasmids for lentivirus production. The authors thank Dr. Ludger Hengst for donating pSuper vector and Theresa Davies-Hill and Ulrike Reich for excellent technical assistance. The authors also thank Dr. Tim Schröder and Mrs. Andrea Hermann for assistance with the FACS analysis.

Manuscript received on July 13, 2009. Revised version arrived on September 14, 2009. Manuscript accepted on October 28, 2009.

Correspondence: Leticia Quintanilla-Martinez, M.D., Institute of Pathology, University Hospital Tübingen Liebermeisterstr. 8 72076 Tübingen Germany. E-mail: leticia.quintanilla-fend@med.uni-tuebingen.de

The online version of this paper has a Supplementary Appendix.

Introduction

Anaplastic lymphoma kinase (ALK)-positive (ALK+) anaplastic large cell lymphoma (ALCL) is a distinct type of non-Hodgkin's lymphoma with unique morphological features and expression of the CD30 antigen.[1] Most ALK+ ALCL have a characteristic cytogenetic abnormality, the t(2;5) chromosomal translocation, which juxtaposes the *ALK* gene to the nucleophosmin (*NPM*) gene, resulting in the expression of a NPM-ALK fusion protein. However, studies have shown that about one fifth of ALK+ ALCL contain variant translocations in which the *ALK* gene is fused to other partner genes.[2,3] ALK-fusion proteins interact with many adaptor proteins and activate several key signaling pathways involved in cell proliferation, transformation and survival.[3-5] While many of the proximal effects of ALK-mediated lymphomagenesis are now well understood, much less is known about how these activated signaling pathways converge to promote transformation. A promising candidate target gene in ALK-mediated oncogenesis is the transcription factor CCAAT/enhancer binding protein beta (C/EBPβ), which we recently reported to be over-expressed in ALK+ ALCL, as opposed to other lymphoma subtypes.[6] The expression of C/EBPβ in ALK+ ALCL and its dependence on NPM-ALK was corroborated in two recent studies, underscoring the importance of this transcription factor.[7,8] The C/EBP are a family of leucine zipper transcription factors that are involved in the regulation of various aspects of cellular growth and differentiation in a variety of cell types. Several members of this family have been implicated in tumorigenesis, most notably C/EBPα in acute myeloid leukemia.[9-11] Like most other members of the C/EBP family, C/EBPβ is an intronless gene. In rodents, it is transcribed as a single mRNA that can produce at least three isoforms: a 39-kDa liver-enriched activating protein (LAP*), a 36-kDa protein (LAP), and a 20-kDa liver-enriched inhibitory protein (LIP), with the LAP and LIP isoforms constituting the major polypeptides in cells.[12] LIP is an N-terminal truncated form of C/EBPβ that lacks most of the transactivation domain, and, although it is able to dimerize with other C/EBP family members and bind to DNA, its ability to activate transcription is greatly attenuated; it, therefore, appears to act as a repressor of C/EBP-mediated transcription.[12] In our previous study, we demonstrated that C/EBPβ expression was dependent upon NPM-ALK activity;[6] however, the biological significance and the signal transduction pathways potentially responsible for its expression were not investigated. The aim of the current study was, therefore, to investigate both the importance of C/EBPβ expression in ALK+ ALCL survival and proliferation, and to identify which of the NPM-ALK induced signaling pathways might be responsible for its induction and activation.

Design and Methods

Plasmid constructs

Oligonucleotides containing short hairpin RNA (shRNA) sequences for the target genes of interest were used: C/EBPβ-C1 sense – 5'-GAAGACCGTGGACAAGCAC-3',[13] STAT3-Gh1 sense – 5'-GCAGCAGCTGA ACAACATGT-3',[14] mammalian target of rapamycin (mTOR) sense – 5'-GGAGTCTACTCGCTTC-TAT-3'; and AKT sense – 5'-GGGCACTTTCGGCAAGG TG-3'.[15] Oligonucleotides were cloned into the H1 promoter driven vector pSuper (Oligoengine, Seattle, WA, USA) as described previously.[16] A non-targeting shRNA with the sense sequence: 5'-GCCGCTTTGTAGGATAGAG-3' was used for construction of the corresponding shRNA-control transfer vector. The measurement of shRNA knockdown efficiency was performed as recently described.[17,18]

Cell cultures

The ALK+ ALCL (SUDHL-1, Ki-JK, Karpas 299 and SR786) were cultured in RPMI 1640 (Gibco BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany), 2 mM glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The murine pro–B-lymphoid cell line Ba/F3,[5] was maintained in RPMI 1640 and was supplemented with 10% fetal calf serum and 1 ng/mL murine recombinant interleukin-3 (IL-3; R&D Systems, DPC Bierman GmbH, Wiesbaden, Germany). Ba/F3-NPM-ALK-ATP-Abl transformed cells contain a construct in which the ATP-binding site of ALK is replaced by the corresponding domain of the ABL kinase. This substitution renders NPM-ALK protein responsive to imatinib.[6] Ba/F3-NPM-ALK-ATP-Abl cells were treated with 5 μM imatinib (Novartis Pharma, Basel, Switzerland) for 24 h as described elsewhere.[6]

Virus production and viral infection of suspensions of lymphoma cell lines

The virus production and viral infection of lymphoma cells were performed as recently described.[17-19] Cells were analyzed 3 days after infection, on the assumption that this is the normal time it takes for efficient viral genome integration, shRNA expression and corresponding shRNA knockdown.

Cytofluorimetric analysis of infected cells

Gene transduction efficiency was determined by cytofluorimetric analysis using the BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with BD CellQuest Pro™ software. Infected cells were detected on the basis of the fluorescence of green fluorescent protein (GFP). Propidium iodide (1 μg/mL) was used to determine the viability of infected cells and annexin V (Invitrogen, San Carlsbad, CA, USA) was used to determine the percentage of apoptotic cells.

Cell proliferation and viability assay

Triplicate seeding of 2×10^5 cells per well was used for growth experiments from day 1 to 6 after infection. Cell viability and growth retardation was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay (AQueous CellTiter96®, Promega, Mannheim, Germany).

Kinase inhibitors

U0126 and PD98059 (MEK inhibitors) and rapamycin were purchased from Calbiochem (San Diego, CA, USA). The final concentrations used were as follows: 8 μM for U0126, 50 μM for PD98059 and 20 nM for rapamycin.

Western blot analysis

Cells were washed in phosphate-buffered saline, pelleted and lysed, as described elsewhere.[6,17] For immunoblotting the following antibodies were used: C/EBPβ (Santa Cruz Biotechnology), ALK (Zymed), STAT3 (Transduction Laboratories), phospho-eIF2α (Calbiochem), phospho-C/EBPβ (Thr235), phospho-STAT3 (Tyr705), p44/42 MAPK, phospho-p44/p42 MAPK (Thr202/Tyr204), AKT, phospho-AKT (Ser473), mTOR, phospho-

mTOR (Ser2448), phospho-tuberin/TSC2 (Ser1254), phospho-rpS6 (Ser235/236), phospho-4E-BP1 (Thr70), rabbit IgG horseradish peroxidase (HRP)-linked from Cell Signaling (Beverly, MA, USa). α-tubulin antibody (Sigma, St. Louis, MO, USA) was used as a loading control. All experiments were repeated several times and representative data are shown.

Real-time quantitative reverse transcriptase polymerase chain reaction analysis

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Gene expression assays from Applied Biosystems were used to quantify mRNA concentration (C/EBPβ, Hs00270923_s1; NPM-ALK, Hs00608289-m1; STAT3, Hs01047572_m1; mTOR, Hs00234508-m1). The TATA box-binding protein (TBP) was used as a control gene, as previously described.[20] Target gene expression was normalized to TBP and the target gene expression was analyzed by the $2^{-\Delta\Delta CT}$ formula. All reactions were performed at least twice in duplicate.

Results

C/EBPβ is necessary for the proliferation and survival of ALK-positive anaplastic large cell lymphoma cells

In a previous study, we showed that ALK+ ALCL expressed high levels of the C/EBPβ and that this expression was dependent on active signaling through the oncogenic protein NPM-ALK.[6] In the current study, to investigate the biological relevance of over-expressed C/EBPβ in the survival and proliferation of ALK+ ALCL, we performed gene silencing experiments in human ALK+ ALCL

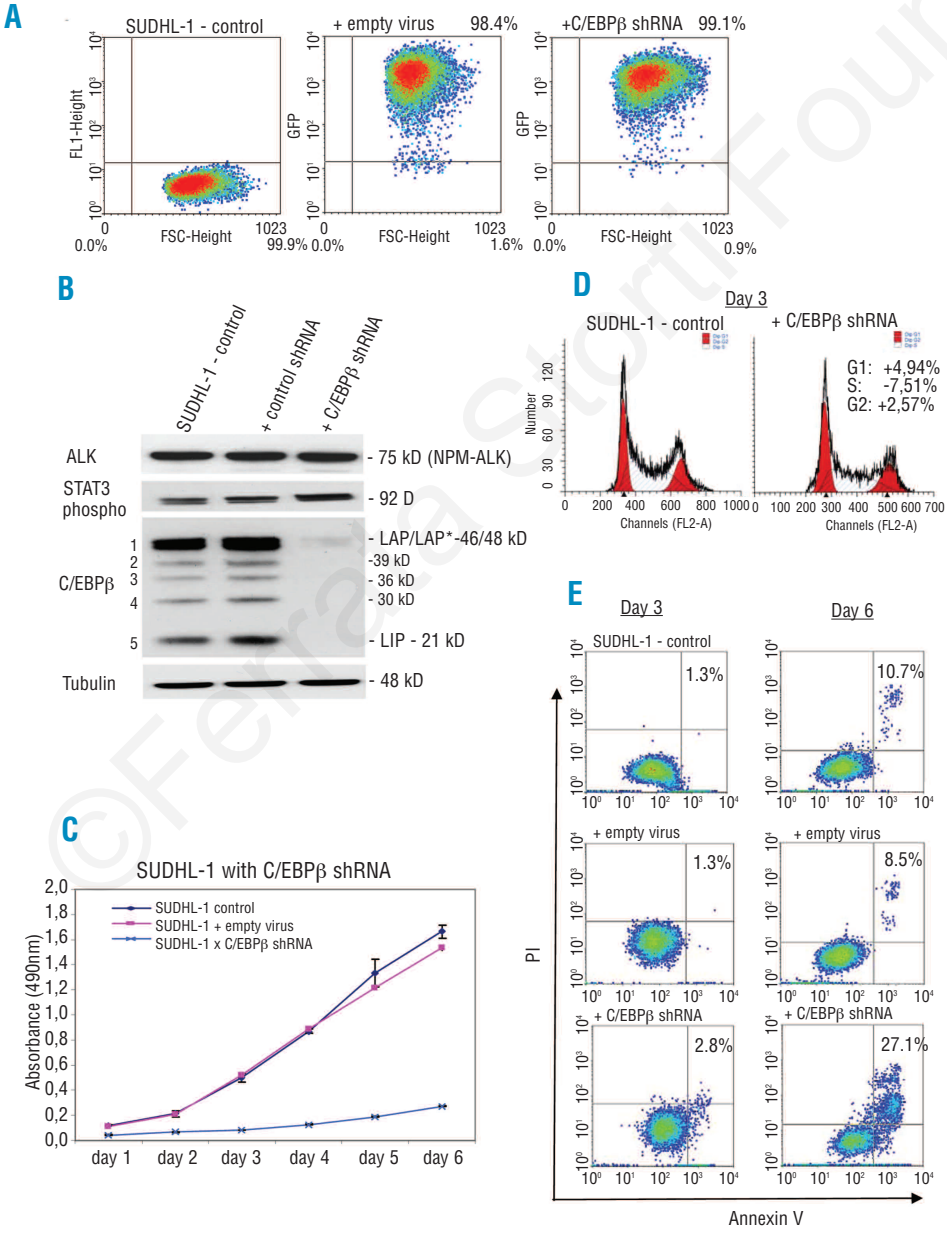

Figure 1. Influence of C/EBPβ shRNA-mediated gene silencing on the expression of the different C/EBPβ isoforms and on cell growth in ALK+ALCL cells. (A) FACS analysis of transduced SUDHL-1 cells with C/EBPβ shRNA and controls 3 days after infection. The percentage of GFP-positive cells represents the infected cells. (B) Western blot analysis of C/EBPβ knockdown three days after infection. The five specific C/EBPβ bands detected are labeled with numbers. The higher molecular weight band corresponds to LAP (46 kDa) and LAP* (48 kDa), and the lower to LIP (21 kDa). Three minor bands were observed at 39 kDa, 36 kDa and 30 kDa, possibly proteolytic breakdown products of the major LAP isoform. Each lane contained 30 μg protein extract. Tubulin was used as a loading control. (C) Proliferation curves of the controls and the C/EBPβ shRNA infected SUDHL-1 cells are depicted up to 6 days after infection. Error bars indicate SD (n=3). (D) Cell cycle distribution of the SUDHL-1 infected cells 3 days after infection with C/EBPβ-shRNA. The differences between the control and the C/EBPβ-shRNA-infected cells are given in percentages on the right side. (E) Annexin V staining of the controls and the C/EBPβ shRNA-infected SUDHL-1 cells 3 and 6 days after infection.

cell lines using C/EBPβ–shRNA (Figure 1A-E). To evaluate the effect of C/EBPβ down-regulation on cell proliferation, growth curves were generated from treated cells with almost 100% infection rate (Figure 1B) for up to 6 days of infection. A comparison between control and C/EBPβ-shRNA-infected cells revealed a dramatic decrease in cell proliferation after 3 days of infection (Figure 1C), even in the presence of ALK and phosphorylated STAT3 (Figure 1B). Analysis of the cell cycle revealed that C/EBPβ-shRNA-mediated down-regulation induced a shift of approximately 8% from the S phase to the G1 and G2 phases in the cell cycle distribution after 3 days of infection (Figure 1D). FACS analysis with annexin V staining showed that C/EBPβ down-regulation caused only mild cell apoptosis, as a late effect, 6 days after initial infection when compared to apoptosis of control cells (27% *versus* 10%, respectively) (Figure 1E). These results demonstrate the key role of C/EBPβ in promoting proliferation and, to a lesser extent, survival in ALK$^+$ ALCL.

NPM-ALK induced signaling pathways in ALK-positive anaplastic large cell lymphoma

The status of signaling pathway activity was analyzed in four ALK$^+$ ALCL cell lines (Ki-JK, SR-786, Karpas 299, and SUDHL-1); the results are presented in Figure 2A. All four cell lines showed significant phosphorylation of STAT3, mTOR, ERK1/2 and AKT, confirming that these pathways are active in ALK$^+$ALCL. C/EBPβ was also expressed in all cell lines in different amounts.

To establish the dependence of these signaling pathways on active NPM-ALK, we utilized the model system reported previously.[6] To avoid the background effects of growth factors on the different signaling pathways, the Ba/F3 parental cell line was investigated with and without IL-3 (Figure 2B). In the absence of IL-3, the Ba/F3 parental cell line, in addition to lacking C/EBPβ, showed no phosphorylation of STAT3, ERK1/2 and AKT, whereas there were low basal levels of phosphorylation of mTOR. Addition of IL-3 resulted in the induction of ERK1/2 phosphorylation, a slight increase in mTOR phosphorylation, and no change in STAT3 or AKT phosphorylation status. In contrast, NPM-ALK-ATP-Abl-transformed Ba/F3 cells, which are IL-3-independent, showed high levels of C/EBPβ (murine LAP and LIP are 36 and 21 kDa, respectively) and phosphorylated STAT3, AKT, and ERK1/2 (Figure 2B), indicating that their activation is dependent on NPM-ALK. Imatinib treatment of Ba/F3 NPM-ALK-ATP-Abl-transformed cells resulted in the inhibition of C/EBPβ expression, loss of STAT3 and AKT phosphorylation, and a decrease in ERK1/2 phosphorylation, without influence on mTOR phosphorylation. Taken together, these experiments show that the induction of C/EBPβ coincides with activation of STAT3, ERK1/2 and AKT signaling, suggesting that any of these pathways might be responsible for the direct induction of C/EBPβ.

Effective gene silencing of STAT3 reduces C/EBPβ expression at the mRNA and protein levels

To determine the influence of specific signaling pathways on the expression of C/EBPβ in ALK$^+$ ALCL, the STAT3 signaling pathway was targeted with a specific STAT3 shRNA construct (Figure 3A). STAT3 protein levels analyzed by western blot were significantly reduced 3 days after infection. The reduction in STAT3 protein correlated with a decrease in C/EBPβ protein (Figure 3B). The percentage of viable cells after STAT3 shRNA infection was approximately 80% 3 days after infection (*Online Supplementary Figure S1*) showing that the effect of STAT3 on C/EBPβ expression is not secondary to cell death. These results were corroborated by quantitative RT-PCR showing coordinated reduction of STAT3 and C/EBPβ mRNA levels (Figure 3C), suggesting that the reduction of C/EBPβ mRNA level is a consequence of the STAT3 knockdown with an important effect on cell proliferation (Figure 3D).

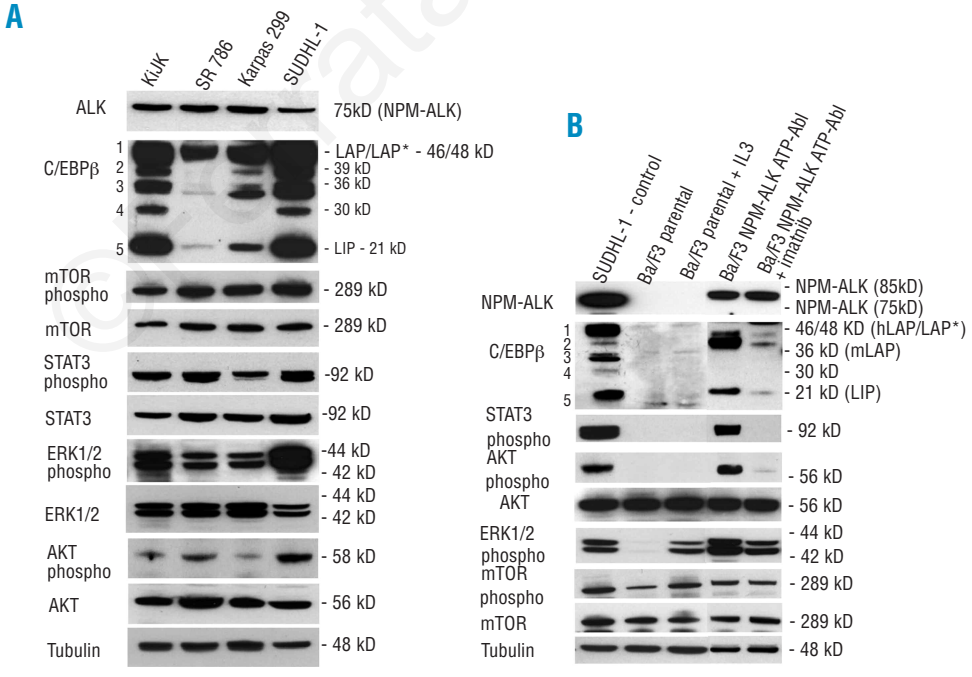

Figure 2. ALK1, C/EBPβ, pmTOR, pSTAT3, pERK1/2 and pAKT expression in ALK$^+$ ALCL and Ba/F3 cells. (A) The cell lines Ki-JK, SR 786, Karpas 299 and SUDHL-1 represent ALCL cell lines with the t(2;5) translocation. The five specific C/EBPβ reactive bands detected are labeled with numbers. (B) Western blot analysis of Ba/F3 cells with and without IL3, and after transfection with the NPM-ALK-ATP-Abl construct untreated or treated with 5 μM imatinib for 24 h. The NPM-ALK-ATP-Abl construct produces a positive protein band of 85 kDa, 5 kDa larger than the protein band detected in the SUDHL-1 cell line used as a control. Each lane contained 30 μg protein extract. Tubulin was used as a loading control.

Inactivation of ERK1/2 signaling reduces C/EBPβ phosphorylation

To determine the consequences of ERK1/2 activity on C/EBPβ expression in ALK+ ALCL, cells were treated with MAPK kinase (MEK) inhibitors (U0126 or PD98059). Both inhibitors showed similar effects and representative data are illustrated in Figure 4A. SUDHL-1 cells treated with MEK inhibitors showed 40% growth retardation (Figure 4B). MEK inhibitors blocked ERK1/2 phosphorylation completely after 2 h and the effect remained for 24 h. Since no effect on total C/EBPβ protein expression was observed, the influence of ERK1/2 on the phosphorylation of C/EBPβ-Thr235 was also investigated (Figure 4A). Inhibition of ERK1/2 resulted in reduced phosphorylation of C/EBPβ after 24 h (Figure 4A) with minimal influence on STAT3 phosphorylation. Collectively, these data imply that ERK1/2 has an important role in the phosphorylation of C/EBPβ.

mTOR protein does not influence C/EBPβ protein level in ALK-positive anaplastic large cell lymphoma

To analyze whether targeting mTOR alone may have any influence on C/EBPβ protein expression, ALK+ ALCL cells were transduced with an mTOR-shRNA construct (Figure 5A-C). Three different mTOR shRNA were analyzed. The most efficient one (mTOR-shRNA1) was used for mTOR silencing (*Online Supplementary Figure S2A*). As a consequence of the reduction of mTOR protein, there were clear decreases in phosphorylation of the downstream targets rpS6 and 4E-BP1 (Figure 5A), whereas no changes were observed in the phosphorylation of p-TSC2 and p-eIF2a, which are either upstream or independent of the mTOR pathway. Specific down-regulation of mTOR protein had no influence on the expression of C/EBPβ protein or on its phosphorylation on Thr-235 (Figure 5A). These results were corroborated with quantitative RT-PCR showing reduction of mTOR at the mRNA level together with no change in C/EBPβ mRNA expression level (Figure 5B). To confirm the data described above, specific inhibition studies were done with rapamycin to inhibit the functional activity of mTOR (*Online Supplementary Figure S2B*). Rapamycin abrogated the phosphorylation of the mTOR downstream target rpS6 in SUDHL-1-treated cells; however, C/EBPβ expression was

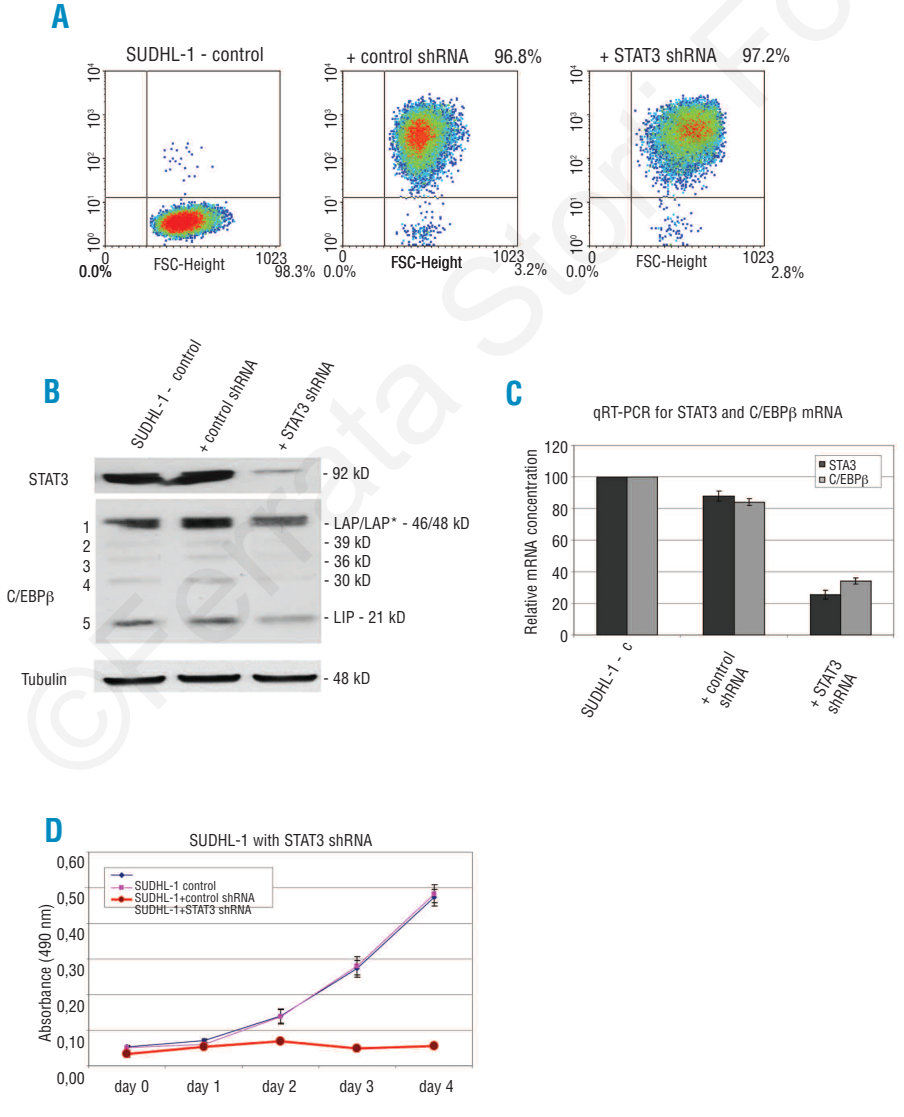

Figure 3. Influence of STAT3 shRNA-mediated gene silencing on the expression of C/EBPβ and on cell growth in ALK+ALCL. (**A**) FACS analysis of transduced SUDHL-1 cells with STAT3 shRNA and controls 3 days after infection. The percentage of GFP-positive cells represents the infected cells. (**B**) Western blot analysis of STAT3 and C/EBPβ in the transduced SUDHL-1 cells 3 days after infection. Each lane contained 30 μg protein extract. Tubulin was used as a loading control. (**C**) Quantitative RT-PCR analysis of STAT3 mRNA (dark boxes) and C/EBPβ mRNA (light gray boxes) in the transduced SUDHL-1 cells 3 days after infection. Values were normalized to TBP and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. Results are depicted as mRNA concentration relative to SUDHL-1 non-infected cells (SUDHL-1-c). Error bars indicate SD (n=2). (**D**) Proliferation curves of the controls and the STAT3 shRNA-infected SUDHL-1 cells are depicted up to 5 days after infection. Error bars indicate SD (n=3).

not changed, confirming the data from the RNA interference experiments. Collectively, these data indicate that the mTOR pathway is not responsible for C/EBPβ expression or its phosphorylation in ALK+ ALCL.

The AKT signaling pathway does not influence total protein level or C/EBPβ phosphorylation

Finally, to determine whether AKT participates in the over-expression of C/EBPβ protein, AKT shRNA was used (Figure 5C). Down-regulation of AKT resulted in a reduction of mTOR phosphorylation, without effects on C/EBPβ protein expression or C/EBPβ Thr-235 phosphorylation. AKT-mediated signal transduction, including the AKT/mTOR overlapping signaling pathway, is not involved in the constitutive expression of C/EBPβ in ALK+ ALCL.

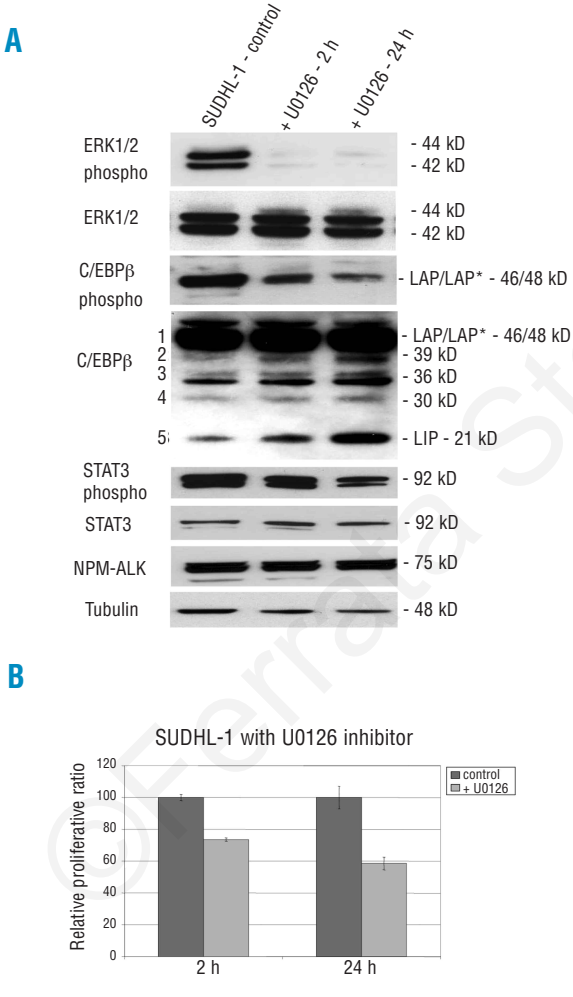

Figure 4. Influence of MEK inactivation on C/EBPβ expression, C/EBPβ phosphorylation, and cell growth. (A) Western blot analysis of the protein extractions from SUDHL-1 cells after treatment with MEK inhibitor (U0126), 2 h and 24 h after inhibition. Each lane contained 30 μg protein extract. Tubulin was used as a loading control. (B) Proliferation assay of the control and the SUDHL-1 cells 2 h and 24 h after treatment with MEK inhibitor (U0126). Error bars indicate SD (n=3).

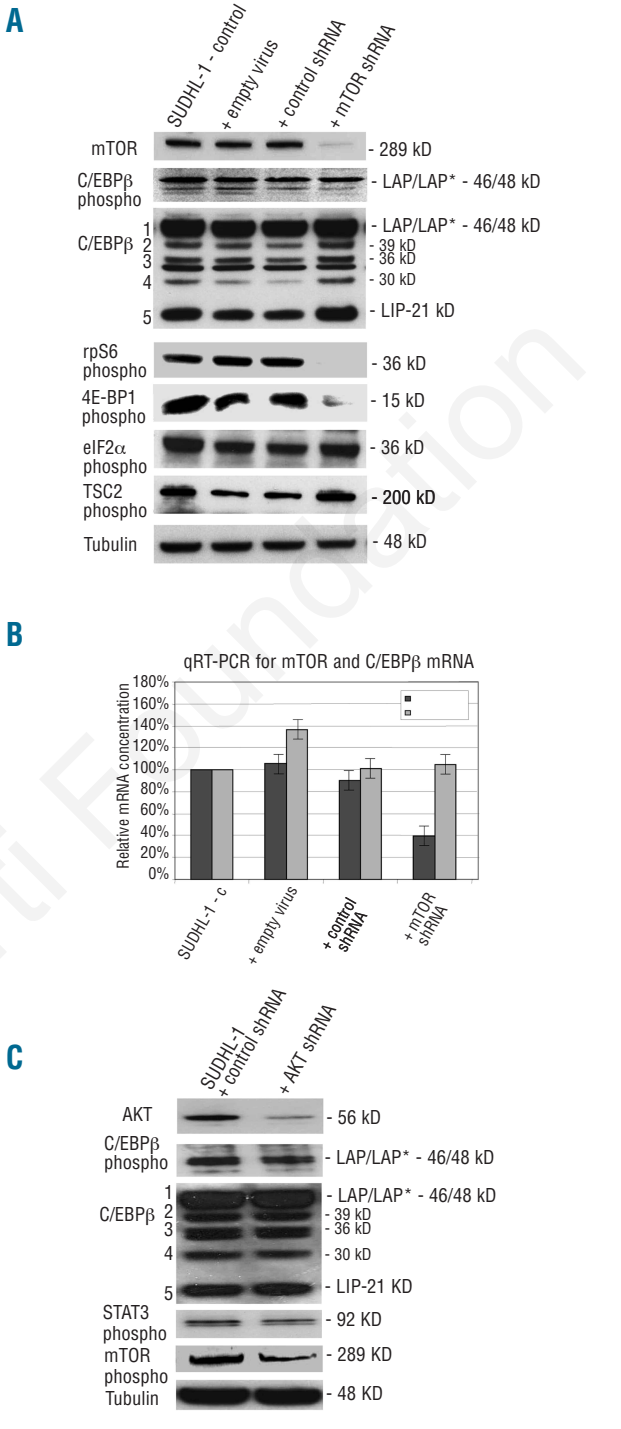

Figure 5. Influence of shRNA-mediated mTOR and AKT inhibition on C/EBPβ expression and C/EBPβ phosphorylation. (A) The level of the knockdown effect for the mTOR protein and downstream targets (p-rpS6, p-4E-BP1) was analyzed by western blot from the SUDHL-1 cell extracts prepared 3 days after infection. Phospho-TSC2 and phospho-eIF2α were used as a control for specificity. (B) Quantitative RT-PCR analysis of mTOR mRNA (dark boxes) and C/EBPβ mRNA (light gray boxes) in the transduced SUDHL-1 cells 3 days after infection. Values were normalized to TBP and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. Results are depicted as mRNA concentration relative to SUDHL-1 non-infected cells (SUDHL-1-c). Error bars indicate SD (n=2). (C) The level of the knockdown effect for the AKT protein and phospho- C/EBPβ was analyzed by western blot from the SUDHL-1 cell extracts prepared 3 days after infection with corresponding controls and AKT shRNA. Phospho-STAT3 and phospho-mTOR were used as a control for specificity.

Discussion

We previously reported that NPM-ALK induces C/EBPβ expression,[6] but the biological significance of this expression was not explored. In the current study we demonstrated a critical role of C/EBPβ in the proliferation of ALK+ ALCL. Efficient knockdown of C/EBPβ expression resulted in a complete block of ALK+ ALCL cell proliferation, while infection with control shRNA had no effect on cell growth. This was accompanied by an 8% shift from S phase to G1-G2 phase in the cell cycle distribution. Additionally, the apoptosis rate increased to 27%, 6 days after C/EBPβ knockdown. These data indicate a crucial role for C/EBPβ in proliferation and, to a lesser extent, in survival, and complement a recent study,[8] which showed that silencing of C/EBPβ resulted in variable induction of apoptosis. In contrast to the study by Piva *et al.*,[8] our study demonstrates that although the knockdown of C/EBPβ induces mild to moderate apoptosis, the main role of C/EBPβ in NPM-ALK-driven transformation is the induction of proliferation. Our experiments showed that 3 days after effective C/EBPβ knockdown there was a striking decrease in proliferation, whereas apoptosis was minimal (2.8%). Apoptosis became apparent only after 6 days of C/EBPβ knockdown, which strongly suggests that apoptosis is not a direct effect of C/EBPβ knockdown but rather a secondary, late event. These results are in line with those of studies in other human cancers, which have demonstrated that the main effect of C/EBPβ over-expression is induction of uncontrolled proliferation.[13,21-23]

Additionally, the role of several major NPM-ALK activated signaling pathways in C/EBPβ expression and activation was explored. Through the use of gene silencing experiments, we were able to dissect several mechanisms through which NPM-ALK exerts its control on C/EBPβ. Gene silencing of STAT3 resulted in a marked decrease of both C/EBPβ mRNA and protein, indicating that STAT3 activation by NPM-ALK is essential for C/EBPβ expression. Our results confirm a recent study[8] suggesting that STAT3 regulates C/EBPβ in ALK+ ALCL. Of note is that although the C/EBPβ promoter contains no STAT3 DNA binding motif sequences, a novel mechanism indirectly tethering STAT3 to the C/EBPβ promoter has been described.[24-26] This result underscores the important role of STAT3 in orchestrating the transforming capacity of NPM-ALK and support studies suggesting STAT3 as a possible therapeutic target.[27]

C/EBPβ activity is further regulated through specific phosphorylations that determine its transcriptional activation and DNA binding capacity.[24,28,29] The ERK1/2-dependent phosphorylation of human LAP at Thr 235 is a critical "priming" site required for the transcriptional activation of C/EBPβ.[30] In contrast to a previous study,[8] we did not find down-regulation of total C/EBPβ protein upon pharmacological inhibition of ERK1/2 activation. However, we demonstrated that C/EBPβ phosphorylation at Thr 235 is ERK1/2-dependent and unaffected by AKT or mTOR down-regulation. Moreover, inhibition of ERK1/2 led to a 40% growth retardation of ALK+ ALCL cells, indicating that ERK1/2 is important in the regulation of C/EBPβ function. The possible influence of the mTOR and AKT pathways on the expression of C/EBPβ was also investigated. Efficient inactivation of mTOR or AKT did not result in any reduction in C/EBPβ mRNA or protein levels. The results with mTOR inhibition were somewhat unexpected because mTOR activity was shown previously[31,32] to be involved in the control of C/EBPβ isoform expression, through the activation of the translation initiation factor eIF-4E.[33,34] In the current study, although efficient inactivation of mTOR and its downstream targets rpS6 and 4E-BP1 was achieved, no major changes in C/EBPβ protein levels or in the LIP-LAP ratios were observed. One possible explanation might be that the effect on C/EBPβ caused by the mTOR inhibitor everolimus in the earlier study[33] is not the same as that produced by shRNA silencing. Nevertheless, the results obtained with the RNA interference assay were confirmed with mTOR inactivation using rapamycin. It is of note that efficient knockdown of AKT led to similar results in our study. Moreover, these results are in agreement with an earlier study in which specific knockdown of AKT1/AKT2 did not show any influence on the expression of C/EBPβ.[8]

In conclusion, we demonstrated a critical role of the NPM-ALK-induced target gene C/EBPβ in the proliferation and, to a less extent, in the survival of ALK+ ALCL. Furthermore, we explored the role of several major NPM-ALK activated signaling pathways in C/EBPβ expression and activation. Our data indicate that C/EBPβ expression in ALK+ ALCL is controlled primarily by the STAT3 pathway, while its phosphorylation is partially dependent upon the MAPK pathway. Neither mTOR nor the AKT signaling pathway appears to have any influence on C/EBPβ expression or activation in ALK+ ALCL.

Authorship and Disclosures

MR and LQM designed research, analyzed and interpreted data and drafted the manuscript; NA performed research, analyzed and interpreted data and drafted the manuscript; SP and FF designed research and analyzed and interpreted data; JD contributed vital new reagents and material and discussed data; IB, MR, MK, TD and DA performed research.

The authors declare no potential conflicts of interest.

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