NPM-ALK dependent expression of the Transcription factor CCAAT/enhancer binding protein (C/EBP) β in ALK-positive Anaplastic Large Cell Lymphomas

Leticia Quintanilla-Martinez¹,², Stefania Pittaluga², Cornelius Miething³, Margit Klier¹, Martina Rudelius², Theresa Davies-Hill², Natasa Anastasov¹, Antonio Martinez², Angelica Vivero², Justus Duyster³, Elaine S Jaffe², Falko Fend⁴, Mark Raffeld².

¹Institute of Pathology, GSF-National Research Center for Health and Environment, Neuherberg, Germany, ²Hematopathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD, and the ³Department of Internal Medicine III and ⁴Institute of Pathology, Technical University Munich, Munich, Germany

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Correspondence Author: Mark Raffeld, Senior Investigator, Laboratory of Pathology, National Cancer Institute, Bldg 10, Rm 2N110, 9000 Rockville Pike, Bethesda, MD 20852
E-mail: mraff@box-m.nih.gov
ABSTRACT:

C/EBPβ is one of a six-member family of CCAAT/enhancer binding proteins (C/EBP). These transcription factors are involved in the regulation of various aspects of cellular growth and differentiation. Although C/EBPβ has important functions in both B and T-cell differentiation, its expression has not been well studied in lymphoid tissues. We, therefore, analyzed its expression by immunohistochemistry and/or Western blot in normal lymphoid tissues and in 248 well characterized lymphomas and lymphoma cell lines. Non-neoplastic lymphoid tissues and the vast majority of B-cell, T-cell, and Hodgkin lymphomas lacked detectable C/EBPβ. In contrast, most cases of ALK-positive ALCL (40/45; 88%) strongly expressed C/EBPβ. Western blot analysis confirmed the C/EBPβ expression in the ALK-positive ALCL cases, and demonstrated elevated levels of the LIP isoform, which has been associated with increased proliferation and aggressiveness in carcinomas. Transfection of Ba/F3 and 32D cells with both NPM-ALK and a kinase-inhibitable modified NPM-ALK resulted in the induction of C/EBPβ, and demonstrated dependence upon the NPM-ALK kinase activity. In conclusion, we report the constitutive expression of C/EBPβ in ALK-positive ALCL cases, and show its relationship to NPM-ALK. We suggest that C/EBPβ is likely to play an important role in the pathogenesis and unique phenotype of this lymphoma.
INTRODUCTION:

The CCAAT/enhancer binding proteins (C/EBPs) 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. Six members of the family have been isolated and characterized to date (C/EBPα, β, γ, δ, ε, ζ) sharing a strong homology in the carboxyl-terminal region, which contains a basic DNA-binding and dimerization domain and a leucine-zipper motif. In contrast, their N-terminal regions are divergent and contain both transcriptional activation and repression domains. These factors play major roles in such diverse functions as the acute phase response and inflammation, but are also important in cellular differentiation programs including adipogenesis, solid organ development, and hematopoiesis.

Several members of this family have been implicated in tumorigenesis, most notably C/EBPα in AML, C/EBPβ in epithelial tumors, and C/EBPζ in myxoid liposarcoma. C/EBPβ has a number of interesting characteristics that have led investigators to suggest that this gene may have a role in oncogenesis. Like most other members of the C/EBP family, C/EBPβ is an intronless gene. It is transcribed as a single mRNA that can produce at least three isoforms, a 38 kDa liver-enriched activating protein* (LAP*), a 35 kDa protein (LAP) and a 21 kDa liver-enriched inhibitory protein (LIP), with the LAP and LIP forms being the major polypeptides produced in cells. LIP is an N-terminally truncated form of C/EBPβ that lacks most of the transactivation domain, and although able to dimerize with other CEBP family members and bind to DNA, its ability to activate transcription is greatly attenuated and, therefore, appears to act as a repressor of C/EBP mediated transcription.

The LIP/LAP ratio appears to be tightly regulated, and changes in this ratio have been shown to affect cell proliferation and differentiation decisions, with increases in the ratio generally favoring proliferation. Consistent with a role in cellular proliferation, aberrant expression of LIP has been reported in breast tumors, in ovarian carcinoma, and in colorectal carcinoma, where LIP expression levels are associated with the more aggressive tumors.

While C/EBPβ has been most intensely studied for its role in the acute phase
response\textsuperscript{3} and in adipogenesis,\textsuperscript{9} it also plays an important role in the activation and terminal differentiation of macrophages and myeloid cells.\textsuperscript{10,12} In addition, although C/EBP\textsubscript{β} is not constitutively expressed in mature lymphocytes, it has been implicated in B-cell lymphopoiesis,\textsuperscript{22,23} and is a major regulator of the TH1/TH2 response in T-cells.\textsuperscript{24,25} Not surprisingly, C/EBP\textsubscript{β} deficient mice display numerous abnormalities in humoral, innate, and cellular immunity\textsuperscript{26} and develop a lymphoproliferative disorder with plasmacytosis and elevated IL-6 levels similar to Castleman’s disease in humans.

Given a role for C/EBP\textsubscript{β} in lymphoid cells, its complex relationship to proliferation and differentiation, and its link to epithelial cancers, we wished to explore whether C/EBP\textsubscript{β} might also contribute to lymphoid neoplasias. For this purpose, we assessed its expression in lymphoma cell lines and in a large series of primary B-, T-cell and Hodgkin lymphomas, and found that C/EBP\textsubscript{β} is consistently overexpressed in ALK-positive anaplastic large cell lymphomas. In addition, we demonstrated that C/EBP\textsubscript{β} expression is transcriptionally induced through the kinase activity of NPM-ALK, opening new insights into the pathogenesis of this lymphoma subgroup.

**MATERIALS AND METHODS:**

**Cell lines**

Twenty-one cell lines were selected for this study, 19 lymphoid cell lines (Table 1) and two epithelial cell lines. The lymphoid cell lines included 4 with expression of Anaplastic Lymphoma Kinase (ALK), as a result of the t(2;5) translocation: KiJK, Karpas 299, SUDHL1, and SR786. The rest of the lymphoid cell lines included 2 mantle cell lymphoma cell lines: Granta 519 and NCEB-1; 3 transformed follicular lymphoma cell lines: SUDHL4, SUDHL6 and SUDHL10; 3 T-cell lymphoblastic lymphoma cell line: Molt4, CEM and Jurkat; 5 multiple myeloma cell lines: KMS12, OPM2, JIM3, JD38 and KMM1; one Burkitt’s lymphoma cell line: ST 486, and 1 HTLV1+ adult T-cell leukemia/lymphoma: Hut102. The epithelial cell lines Hela and MCF7 were used as positive controls for C/EBP\textsubscript{β} expression.

**Tissue samples**
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Formalin-fixed and paraffin embedded biopsies from 229 well-characterized lymphomas, including 107 T-cell non-Hodgkin lymphomas (NHL), 93 B-cell NHL, 9 nodular lymphocyte predominant Hodgkin lymphomas (NLPHD) and 20 classical Hodgkin lymphomas (HL), (Table 2) were selected from the files of the Hematopathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health (Bethesda, MD), and from the files of the Institute of Pathology, Technical University of Munich (Munich, Germany). All cases were classified according to the guidelines of the World Health Organization (WHO) Classification of Neoplastic Diseases of Hematopoietic and Lymphoid Tissues.27 These lymphomas were chosen to represent all major lymphoma subtypes, and included 45 cases of ALK-positive anaplastic large cell lymphoma (ALCL) and 29 cases of ALK-negative ALCL. ALK-negative ALCL resembled their ALK-positive counterparts, strongly expressing CD30, CD4 and/or CD3, but were negative for ALK protein. In addition, different samples of reactive lymphoid tissue including tonsils (5 cases), thymus (5 cases) and lymph nodes (3 cases) were analyzed to determine the distribution of C/EBPβ in normal lymphoid tissue. All diagnoses were confirmed by immunohistochemistry on paraffin-embedded tissue sections, using a panel of antibodies to assess lymphoid phenotype, performed during the diagnostic workup. Anaplastic lymphoma kinase-1 (ALK-1) (DakoCytomation Corp, Carpinteria, CA) immunohistochemistry was performed on all cases of ALCL. Frozen tissue from 1 normal reactive lymph node, 4 primary cases of ALK-positive ALCL, 2 cases of adult T-cell leukemia/lymphoma (ATLL), 1 case of precursor T-cell lymphoblastic leukemia/lymphoma (ALL), and one case of peripheral T-cell lymphoma, unspecified (PTCL) were selected for Western blot analysis.

Immunohistochemistry

All cases were previously studied by paraffin section immunohistochemistry (IHC) with a panel of antibodies to assess lymphoid phenotype, as well as ALK1 in suspected cases of ALCL. The expression of C/EBPβ (clone H7, Santa Cruz Biotechnology, Santa Cruz CA) was investigated on paraffin-embedded sections. Cases were scored as C/EBPβ positive when >20% of the tumor cells showed nuclear positivity.
All immunohistochemical analyses were reviewed by three of the authors (LQ-M, SP and MR).

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ and/or a DakoCytomation autostainer, DakoCytomation, Carpinteria, CA) according to the company’s protocols, with slight modifications. After deparaffinization and rehydration, the slides were placed in a microwave pressure cooker (TenderCooker, Nordicware, Minneapolis, MN) in 0.01 mol/L citrate buffer, pH 6.0, containing 0.1% Tween 20, and heated in a microwave oven at maximum power for 30 minutes or for 8 minutes (hot start). Thereafter, sections were washed in Tris-buffered saline (pH 7.6) containing 5% fetal calf serum (Life Technologies, Grand Island, NY) for 20 minutes. The antibodies were incubated overnight or for 2 hours at room temperature. The rest of the procedure in the DakoCytomation autostainer was performed using a peroxidase based detection system (Envision™+ System DakoCytomation), and the slides were counterstained in Gill's hematoxylin and mounted in Pertex (Histolab GmbH, Göteborg, Germany).

Double stainings for C/EBPβ and CD68 (DakoCytomation), S100 (Biogenex, San Ramon, CA), clusterin (Upstate Biotechnology, Lake Placid, NY) or rabbit monoclonal anti-CD3 (Lab Vision, Fremont, CA) were performed using a peroxidase based system (Envision™+ System, DakoCytomation). The primary antibody, C/EBPβ, was incubated overnight, and the secondary antibody for 30 minutes. The reaction was developed with the Envision™+ System and 3,3’–diaminobenzidine as chromogen. The slides were blocked for 10 minutes with 3% peroxide and the second primary antibody; CD3, CD68 or S100 was incubated for 1 hour, and the secondary antibody for 30 minutes. The reaction was detected again with Envision™+ System and VIP substrate as chromogen (Vector Laboratories, Burlingame, CA), and counterstained with Vector methyl green (Vector Laboratories).

Western blot analysis

Frozen tissue samples and cell line pellets were immersed into phosphate-buffered saline (PBS) containing one protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Mannheim, Germany), and sonicated 3 times, 30 seconds each on ice with
30 second pauses. The protein concentration was determined by the BCA protein assay reagent kit (Pierce, Rockford, IL). A total of 30 µg of protein extracts was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to pure nitrocellulose immobilization membranes (Protran, BA85, Dassel, Germany). Membranes were blocked overnight in 5% non-fat dry milk and incubated with the primary antibody for 2 hours in 3% bovine serum albumin (BSA, Sigma, St. Louis, MO). Subsequently, membranes were washed five times for 5 minutes each in a wash buffer (10 mmol/L Tris, pH 7.6, 100 mmol/L NaCl, and 0.1% Tween), and incubated with a biotinylated secondary antibody for 1 hour. Membranes were washed five times in the same wash buffer, and detection was performed by SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes. The membranes were exposed to a Kodak X-OMAT AR film. All assays were repeated three times and gave similar results. Subcellular fractionation was performed using the NE-PERTM kit (Pierce). The immunoreagents used for Western blot were a rabbit polyclonal antibody against ALK1 (Zymed Laboratories Inc, San Francisco, CA), and rabbit polyclonal anti-C/EBPβ (C-19, Santa Cruz Biotechnology). The C/EBPβ antibody recognizes the two major isoforms: LAP 35kDa and LIP 21kDa. For loading control mouse monoclonal anti-alpha-tubulin (Sigma) was used. Additional antibodies used included monoclonal Stat3 (Transduction laboratories, Lexington, KY), phospho-Tyr705 Stat3 (pStat3) (clone 3E2; Cell Signaling, Beverly, MA).

**Cell culture and DNA transfections**

The murine pro-B lymphoid cell line Ba/F3 and the murine 32Dc13 myeloid cell line (referred to hereafter as “32D cell line”) were maintained in RPM1 1640 (GIBCO-BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom KG, Berlin Germany), and 1ng/mL murine recombinant interleukin-3 (mIL-3; R&D Systems, DPC Bierman GmgH, Wiesbaden, Germany). BaF3 is a murine lymphoid cell line that requires IL-3 for proliferation and survival. It expresses only B-220 and contains immunoglobulin genes in germ-line configuration. Overexpression of NPM-ALK in BaF3 cells converted cells from IL-3 dependent to IL-3 independent growth.28 32D cell line is a myeloid progenitor, IL-3 dependent, diploid cell line that undergoes normal
granulocytic differentiation in response to G-CSF. DNA transfections in Ba/F3 and 32D cell lines were performed by electroporation, as described previously.\textsuperscript{28,29} Ba/F3 Mig-NPM-ALK, Ba/F3-Mig-NPM-ALK-ATP-Abl and Ba/F3-Mig (empty vector used as control) are derived from parental Ba/F3 cells modified by retroviral infection with the respective constructs. Ba/F3 Mig-NPM-ALK, Ba/F3-Mig-NPM-ALK-ATP-Abl and 32D-Mig-NPM-ALK are mIL-3 independent. In the Mig-NPM-ALK-ATP-Abl (Mig-NAAA) construct, the ATP-binding site of ALK was replaced by the corresponding domain of the Abl-kinase. This substitution renders NPM-ALK protein responsive to imatinib, while retaining the NPM-ALK kinase substrate phosphorylation pattern (manuscript in preparation). The protein band produced by this construct is 85kDa, whereas the one produced by the Mig-NPM-ALK is of 75kDa. For Western blot analysis, cells growing exponentially were pelleted and lysed, as described above. Ba/F3 Mig-NAAA cells were either treated with 5\textmu M imatinib for 12 hours, to inhibit the NPM-ALK kinase activity or were left untreated before lysis, and used as control. Imatinib mesylate was kindly provided by Novartis Pharma (Basel, Switzerland).

**Real-Time Quantitative RT-PCR**

Real-Time quantitative RT-PCR analysis was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of C/EBP\beta and TBP (= TATA box-binding protein, as house keeping gene), we used gene expression assays from Applied Biosystems (C/EBP\beta: Mm00843434_s1; TBP: Mm00446973_m1). Two \mu g of RNA were transcribed into cDNA using Supercript II-reverse transcriptase (Invitrogen, Carlsbad, CA), and a mix of Oligo(dT) primer and random hexamers (Roche, Penzberg, Germany) in a final volume of 50 \mu l following manufacturer`s directions. PCR was carried out with the Taqman Universal Master Mix (Applied Biosystems) using 4 \mu l of diluted cDNA in a 20 \mu l final reaction mixture (10 min 95°C; 40 cycles of 15sec 95°C / 60sec 60°C). Before starting the experiments the linear range of the assays was validated via a dilution curve with transcribed embryonic mouse RNA. Data were analysed using the \(\Delta C_T\) method. Target gene expression was normalized to TBP by taking the difference between mean threshold PCR cycle values for target and control genes (\(\Delta C_T\) value). This was then calibrated to
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the control sample in each experiment to give the $\Delta \Delta C_T$ value, where the control had a $\Delta \Delta C_T$ value of 0. The fold target gene expression, compared to the calibrator value, is given by the formula $2^{-\Delta \Delta CT}$. All reactions were performed in duplicates, at least two times.

RESULTS:

C/EBPβ protein expression in normal lymphoid tissue

C/EBPβ was evaluated by immunohistochemistry performed on paraffin sections of reactive lymph node, tonsil and thymus. In tonsils and lymph nodes C/EBPβ was strongly expressed in the nuclei of scattered cells in the germinal centers, mantle zone and in the paracortical region (Figure 1A). The morphology of the C/EBPβ positive cells in the germinal centers corresponded to that of follicular dendritic cells (FDCs) (Figure 1A-insert), whereas the tingible body macrophages were negative or weakly positive (Figure 1A). In contrast, histiocytes in the sinuses, interdigitating cells and monocyte-like cells were strongly positive for C/EBPβ. Double stainings demonstrated that CEBPβ positive cells were also positive for clusterin, a marker of follicular dendritic cells (Figure 1B), whereas T- and the B-cells were negative for C/EBPβ (Figure 1C-D). In the tonsils, the epithelial cells of the surface epithelium were strongly positive for C/EBPβ (Figure 1A). In the thymus, neither the cortical nor the medullary T-cells showed expression of C/EBPβ. CEBPβ staining was seen only in thymic epithelial cells and in rare macrophage-like cells.

C/EBPβ is highly expressed in ALK positive anaplastic large cell lymphomas.

To assess C/EBPβ expression in malignant lymphoid neoplasms, a total of 229 well-characterized lymphomas, including 93 B-cell lymphomas, 107 T-cell lymphomas (33 PTCL and 74 ALCL), and 29 Hodgkin lymphomas were immunostained for C/EBPβ. Intratumoral histiocytes, present in all cases, served as internal positive controls (Figure 1E). The results are summarized in Table 2.
Of the 93 B-cell NHL, only one case of multiple myeloma with anaplastic features stained positively for C/EBPβ (1 of 93; 1%). All 9 cases of nodular lymphocyte predominant Hodgkin lymphoma (9 cases) were negative for C/EBPβ, whereas five of 20 cases of classical HL showed faint nuclear staining within the Reed-Sternberg cells (Figure 1F-G). Of the 33 cases of PTCL, only one showed weak staining for C/EBPβ, in a small number of tumor cells (Figure 1H-J). In some cases, the presence of numerous C/EBPβ positive cells made it difficult to evaluate whether these cells corresponded to the neoplastic T-cell population or to the reactive myelomonocytic cells. In these cases, double stainings with CD3 revealed that the neoplastic T-cells were negative for C/EBPβ (Figure 1 K), and that the vast majority of the C/EBPβ+ cells were positive for CD68 or S100 (Figure 1 J).

Of the 74 cases of CD30 positive ALCL, 45 were ALK-positive, and 29 cases were ALK-negative (Figure 2). C/EBPβ was strongly positive in the majority of the tumor cells in 40 of 45 cases (88%) of the ALK-positive ALCL. The expression of C/EBPβ was independent of the cytoplasmic or nuclear localization of ALK (Figure 2). ALK-negative cases were C/EBPβ negative, or were weakly positive in eight of the 29 cases. (Figure 1L and Figure 2). Because of the striking association of CEBPβ with ALK-positive ALCL the remainder of the study focuses on these lymphomas.

**C/EBPβ Western blot analysis: LIP is expressed in excess over LAP.**

Overexpression of the LIP isoform of C/EBPβ has been associated with high proliferative states in normal tissues and has been observed in several types of epithelial tumors. For these reasons, we were particularly interested to investigate the LIP/LAP ratio in the ALK-positive ALCL. To assess the LIP/LAP ratio, 4 ALK-positive ALCL cell lines, and 4 primary ALK-positive ALCL cases were studied by Western blot analysis using the C-terminal specific anti-C/EBPβ (C-19) antibody that recognizes both LIP and LAP. For comparison, we also assessed expression in a variety of non-ALCL T and B-cell lines (4 & 11 cell lines, respectively), and several non-ALCL T-cell neoplasms. The positive control epithelial cell lines, Hela and MCF7 demonstrated two bands of the approximate expected sizes for the LIP and LAP isoforms (21 and 35 kDa,
respectively) (Figure 3). The anaplastic large cell lymphoma cell lines, Ki-JK, Karpas 299, and SUDHL-1 expressed both isoforms of C/EBPβ, with an excess of LIP over the LAP isoform. SR786 showed only weak expression of LIP isoform (Figure 3B). Immunocytochemistry of the four cell lines showed strong nuclear staining for total CEBPβ in Ki-JK, Karpas 299, and SUDHL-1, with weaker nuclear staining observed in SR786 (data not shown). Subcellular fractionation of the SUDHL-1 cell line demonstrated that both isoforms were exclusively expressed in the nucleus (Figure 3A). Other B- and T-cell lymphoma cell lines in this survey either expressed LIP weakly (SUDHL-4, SUDHL-6, SUDHL-10, OPM2, KMM1, Jim3, ST 486, MOLT4), or were negative for the two isoforms (CEM, Jurkat, Hut102, Granta, NCEB, KMS12) (Figure 3B-C and data not shown). The weak expression of C/EBPβ in the myeloma and B-cell lymphoma lines may be linked to endogenous production of interleukin-6, a known inducer of C/EBPβ.

The strong expression of C/EBPβ and high levels of LIP were also confirmed by Western blot in four primary ALK-positive ALCL cases (Figure 3D). The reactive lymph node, the acute lymphoblastic lymphoma, the peripheral T-cell lymphoma and the two cases of adult T-cell lymphoma/leukemia were negative for C/EBPβ.

C/EBPβ is induced in NPM-ALK-transformed Ba/F3 and 32D cell lines.

To determine the role of NPM-ALK in the expression of C/EBPβ in ALK-positive ALCL cases, Ba/F3 and 32D cells were transfected with NPM-ALK. Both the transfected Ba/F3 cells and the 32D cells showed strong expression of both NPM-ALK and activated Stat3 (pStat3), a known downstream target of the NPM-ALK kinase (Figure 4A). The parental Ba/F3 and 32D cell lines were negative for NPM-ALK and pStat3, as expected. All cell lines were positive for total Stat3, however, the transfected cell lines expressed higher amounts of Stat3. Western blot analysis of C/EBPβ revealed that parental Ba/F3 cells expressed no C/EBPβ, whereas parental 32D myeloid cell line expressed a modest level of C/EBPβ. The latter finding is not unexpected because myeloid cells and macrophages express C/EBPβ normally. Significantly, both NPM-
ALK transformed cell lines showed strong induction of C/EBPβ LIP and LAP isoforms to levels comparable with the ALCL cell line Karpas 299.

**C/EBPβ expression is induced through the kinase activity of NPM-ALK**

To demonstrate whether the expression of C/EBPβ in ALK+ALCL primary cases and cell lines is dependent upon NPM-ALK kinase activity, we transformed Ba/F3 cells with an NPM-ALK-ATP-Abl construct. This construct is identical to the NPM-ALK construct with the exception that the ATP-binding site of ALK has been replaced by the corresponding domain of the Abl-kinase, rendering NPM-ALK responsive to imatinib. As a result, the NPM-ALK kinase activity can be inhibited completely. Western Blot analysis demonstrated the expression of NPM-ALK in both treated and untreated Ba/F3 NPM-ALK-ATP-Abl cells (Figure 4A). In contrast, while pStat3 was expressed in the untreated Ba/F3 NPM-ALK-ATP-Abl cells, pStat3 was undetectable in the corresponding imatinib treated cells, confirming that the NPM-ALK kinase activity has been switched off. Significantly, C/EBPβ was expressed in the untreated Ba/F3 NPM-ALK-ATP-Abl cells, but was greatly reduced in the imatinib treated Ba/F3 NPM-ALK-ATP-Abl cells, indicating dependence of C/EBPβ expression upon NPM-ALK kinase activity.

**C/EBPβ is transcriptionally regulated by NPM-ALK**

To see whether the induction of C/EBPβ by NPM-ALK is transcriptionally or posttranscriptionally regulated, we analyzed in parallel C/EBPβ expression at the protein and mRNA level of parental Ba/F3 and NPM-ALK transformed cells. Western blot analysis showed induction of C/EBPβ in the NPM-ALK transformed Ba/F3 cells, while the amount of C/EBPβ mRNA increased approximately two-fold (Figure 5). To confirm that C/EBPβ is transcriptionally regulated by NPM-ALK, we also analyzed C/EBPβ protein and mRNA expression in the NPM-ALK-ATP-Abl transformed Ba/F3 cells, following treatment with imatinib or control buffer after 6 and 20 hours. Western blot analysis confirmed the strong expression of C/EBPβ in the untreated NPM-ALK-ATP-Abl cell line, weak expression in the imatinib treated NPM-ALK-ATP-Abl cells after 6 hours, and complete LIP inhibition after 20 hours. (Figure 5B) Interestingly,
pStat3, used as control for the kinase activity of NPM-ALK, was undetectable in the imatinib treated NPM-ALK-ATP-Abl cells after 6 hours, signifying that the inhibition of pStat3 occurs shortly after treatment with imatinib and before the inhibition of C/EBP\(\beta\). C/EBP\(\beta\) mRNA level were 5.2 fold higher in the untreated NPM-ALK-ATP-Abl cell line as compared to the parental BaF3 cell line, and steadily decreased with an ongoing blockade of NPM-ALK-ATP-Abl by imatinib; 3.5 fold after 6 hours and 1.2 fold after 20 hours treatment with imatinib. Importantly, there was a very good correlation between the C/EBP\(\beta\) protein expression and the mRNA level. Together both results indicate that the induction of C/EBP\(\beta\) expression by NPM-ALK occurs at the transcriptional level.

**DISCUSSION:**

C/EBP\(\beta\) is a multifunctional transcription factor that plays important roles in the proliferation and differentiation of a variety of cell types, including B and T-cells, and its aberrant expression has been implicated in the pathogenesis of several epithelial tumors.\(^{16-19,30}\) There is limited information available concerning its expression in normal lymphoid tissues, and virtually no information on its expression in lymphoid neoplasms. For this reason, we investigated C/EBP\(\beta\) expression in normal lymphoid tissues and in a large number of primary lymphomas and lymphoma cell lines, including examples from most major subgroups. Normal non-neoplastic lymphoid cells from thymus, tonsil and lymph node, and the vast majority of B-cell and T-cell NHL did not express immunohistochemically detectable C/EBP\(\beta\). In striking contrast, we found that most cases of ALK-positive ALCL (88%) strongly expressed C/EBP\(\beta\). Significantly, the LIP isoform of C/EBP\(\beta\) was preferentially expressed in ALK-positive ALCL cell lines and in the primary cases examined. Furthermore, we have shown in two cell line systems that enforced expression of both NPM-ALK and a kinase inhibitable modified NPM-ALK results in the inductions of C/EBP\(\beta\) only when the kinase activity is intact, demonstrating not only that NPM-ALK is capable of inducing C/EBP\(\beta\) expression, but also that its induction is transcriptionally dependent upon the NPM-ALK kinase activity.

ALCL represents a distinct type of NHL of T or null phenotype with unique morphological features and expression of the CD30 antigen.\(^{31}\) Recently it has become
evident that there are two forms of ALCL based on the presence or absence of the characteristic cytogenetic abnormality, the \( t(2,5) \) chromosomal translocation, which juxtaposes the anaplastic lymphoma kinase (ALK) gene at 2p23 to the nucleophosmin (NPM) gene at 5q35, resulting in the expression of a chimeric protein called NPM-ALK.\(^{32} \) Other variant translocations involving fusion of the ALK gene with other partner genes have also been described.\(^{33} \) All of the translocations generate ALK fusion proteins that are capable of autodimerization leading to constitutive activation of the ALK-tyrosine kinase, believed to initiate the process of lymphomagenesis.\(^{29,34,35} \)

NPM-ALK interacts with many adaptor proteins and activates several key signaling pathways involved in cell proliferation, transformation and survival. These include the p85 regulatory subunit of type 1A Phosphatidylinositol 3-Kinase (PI3K), which activates AKT and other signaling intermediates,\(^{28,36} \) and the JAK/STAT pathway, which plays an important role in the mitogenic signaling initiated by NPM-ALK and protects hematopoietic cells from apoptosis.\(^{37,38} \) In addition, prosurvival PLC-gamma pathways and MAPK pathways have been implicated in NPM/ALK signaling.\(^{39,40} \)

In the current study, we have shown that C/EBP\( \beta \) expression is dependent upon NPM-ALK kinase activity. However, it is unclear how this signal is transduced. The complex signaling networks activated by NPM-ALK suggest several possibilities. Among the possible activators is Stat3. Stat3 has been shown to be directly activated in the majority of ALK-positive ALCL.\(^{37,38,41} \) We also have found Stat3 activated in a large subset of the current cases (data not shown). Like C/EBP\( \beta \), this transcription factor has a role in the control of inflammatory and native immune responses.\(^{3} \) Although there are no Stat3 specific binding sites in the C/EBP\( \beta \) promoter region, a novel tethering mechanism is believed to link Stat3 to the C/EBP\( \beta \) promoter.\(^{42} \)

A second possible pathway of C/EBP\( \beta \) activation in ALCL is through MAPK signaling.\(^{40} \) Several adaptor proteins involved in the MAPK pathway, including SHC, GRB-2 and IRS-1 have been co-immunoprecipitated with NPM-ALK. Furthermore, normal ALK has been shown to activate the MAPK pathway in PC12 pheochromocytoma cells.\(^{43} \) Independently, other studies have shown that C/EBP\( \beta \) activity can be stimulated by phosphorylation mediated through the RAS/MAPK pathway.\(^{44,45} \) Once activated (phosphorylated) C/EBP\( \beta \) can bind to its own promoter and
participate in a positive feedback loop, potentially resulting in the high levels of C/EBPβ seen in ALCL.

Finally, several investigators have shown that NPM-ALK activates the PI3K/AKT pathway. Among the many targets of AKT is mTOR (mammalian target of rapamycin), a critical regulator of several translational control proteins, including S6K-1 and translation elongation factors eIF-2α and eIF-4E. Activation of these factors through mTOR signaling has recently been shown to affect C/EBPβ expression and increase the LIP/LAP ratio through regulation of translation initiation in both ALCL and Hodgkin lymphoma cell lines. Given the association of LIP with proliferation in other tumor models, the authors suggested that translationally deregulated expression of C/EBPβ could play an important role in ALCL contributing to sustained cellular proliferation, and that translational control of C/EBPβ isoforms could be used as a target of proliferation control and therapeutic intervention. Our data not only corroborates their finding that ALCL cell lines express high levels of LIP, but it also extends this finding to primary ALK-positive ALCL cases. Although it appears likely that mTOR plays a role in the modulation of C/EBPβ isoforms in ALCL cell lines, this still needs to be confirmed in primary cases. Whether the PIK3/AKT/mTOR pathway is also involved in the induction of C/EBPβ or only in its modulation is still unclear.

The consequences of C/EBPβ expression in ALCL are as yet unknown. In addition to its effect on proliferation, C/EBPβ expression has been shown to promote TH2 responses in T-cells. It has recently been shown that ALCL cells display a chemokine profile most similar to TH2 cells. Thus, it is possible that the expression of C/EBPβ plays a role in the cytokine and chemokine profile of ALCL cells. C/EBPβ has also been associated with activation and/or terminal differentiation in the monocyte-macrophage lineages. In this regard, it is of interest that ALCL shows a loss of important T-cell antigens such as CD3 and ZAP-70, and expresses some proteins shared with monocyctic and dendritic cells like clusterin. CD4 and CD68. A recent report indicates that enforced expression of either C/EBPα or C/EBPβ in differentiated B-cells leads to their rapid and efficient reprogramming into macrophages, with loss of B-cell markers and upregulation of macrophage markers. Similar transdifferentiation effects
Quintanilla-Martinez, et al. have been preliminarily reported in thymocytes, where stage-specific enforced expression of C/EBPα and β induced downregulation of T-cell markers and upregulation of myelomonocytic lineage markers. These experiments suggest that the aberrant expression of C/EBPβ could be responsible for some of the phenotypic characteristics seen in ALCL T-cells.

In conclusion, we report for the first time the constitutive activation of the transcription factor C/EBPβ in ALK-positive ALCL cases. In addition, we demonstrate that NPM-ALK is capable of inducing C/EBPβ and that the induction is dependent upon NPM/ALK kinase activity. The link between CEBPβ LIP overexpression and proliferation, and the role of C/EBPβ in TH2 T-cell commitment and in myelomonocytic differentiation suggests that C/EBPβ is likely to play an important role in the pathogenesis and unique phenotype of this lymphoma.

**Acknowledgments**

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REFERENCES:


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Table 1. Lymphoma cell lines analyzed for C/EBPβ

<table>
<thead>
<tr>
<th>Non-Hodgkin lymphoma</th>
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<tr>
<td>Anaplastic large cell lymphoma</td>
<td>SUDHL-1, Ki-JK, SR786, Karpas 299</td>
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Table 2. Primary lymphoma cases analyzed for C/EBPβ expression by immunohistochemistry

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<tr>
<th>Lymphoma subtype</th>
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LEGENDS:

Figure 1.- Immunohistochemical analysis of C/EBPβ expression in normal lymphoid tissue and in different lymphoid neoplasias. A: C/EBPβ protein expression in normal lymphoid tissue (tonsil). In the germinal center, mantle zone and in the interfollicular T-cell area C/EBPβ is strongly expressed in the nuclei of scattered cells, whereas most surface epithelial cells express high levels of C/EBPβ. Immunoperoxidase (IP); x100 C/EBPβ positive cells within the germinal center correspond to follicular dendritic cells (insert, IP; x400). B: Double staining for C/EBPβ (brown) and clusterin (purple) reveals that the CEBPβ positive germinal center cells express the dendritic cell marker clusterin. Note also that the clusterin stain highlights the dendritic cell processes. IP; x650. C-D: Double staining for C/EBPβ (brown) and CD3 (purple) (C) or CD20 (purple) (D) in the germinal center demonstrates that the CD3+T-cells and CD20 positive B-cells are negative for C/EBPβ. IP; x650. E: C/EBPβ expression in a case of mantle cell lymphoma. The neoplastic cells are negative, whereas the intratumoral histiocytes are positive and serve as an internal positive control. IP; x100. F-G: C/EBPβ in two cases of classic Hodgkin lymphoma. Faint nuclear reactivity is seen in the Reed-Sternberg cells of one case (G), whereas the second case (F) shows no C/EBPβ expression. In both cases abundant histiocytes are strongly positive for C/EBPβ. IP; x650. H: C/EBPβ expression in a case of peripheral T-cell lymphoma, unspecified. The neoplastic cells are negative whereas the intratumoral histiocytes are positive. IP; x100. I-J: C/EBPβ expression in a second case of peripheral T-cell lymphoma, unspecified. I: Double staining for C/EBPβ
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(brown) and CD3 (purple) demonstrates that a small population of the neoplastic T-cells (arrows) show weak nuclear staining for C/EBPβ. In contrast, compare the strong nuclear positivity of the reactive histiocytes. IP; x400. **J:** Double staining for C/EBPβ (brown) and CD68 (purple) confirm the histiocytic origin of the strong C/EBPβ positive cells. IP; x 650. **K:** Peripheral T-cell lymphoma, unspecified with abundant histiocytes. Double staining for C/EBPβ (brown) and CD3 (purple) demonstrates that CEBPβ is not expressed in the CD3 positive tumor cells. IP; x100. **L:** C/EBPβ expression in a case of anaplastic large cell lymphoma, ALK1 negative, with weak nuclear staining in tumor cells. In contrast the reactive histiocytes show strong nuclear positivity with C/EBPβ. IP; x250.

**Figure 2.-** **ALK1 and C/EBPβ immunostaining in anaplastic large cell lymphoma.** The two ALK1-positive cases, with nuclear (above) and cytoplasmic (middle) expression show strong nuclear positivity in the majority of tumor cells for C/EBPβ. The ALCL, ALK- negative case (below) is negative for C/EBPβ; however, the reactive histiocytes are strongly positive and serve as internal control. Immunoperoxidase x400.

**Figure 3.-** **Western Blot analysis for C/EBPβ and ALK1 in lymphoid cell lines and primary lymphoma cases.** **A:** Subcellular fractionation of the SUDHL-1 cell line. Tubulin is used as control for loading and for the cytoplasmic fraction. **B:** KiJk, Karpas 299, SUDHL1 and SR786 represent anaplastic large cell lymphoma (ALCL) cell lines with the t(2;5) translocation. The non-t(2;5) T-cell lines are Molt4, CEM and Jurkat. Tubulin is used as loading control. **C:** Western blot analysis in B-cell lymphoid cell lines including two mantle cell lymphoma lines (Granta 519 and NCEB-1), two multiple myeloma cell lines (KMS12 and OPM2) and two follicular lymphoma cell lines (SUDHL4 and 10). The ALCL cell line Karpas 299 and the two carcinoma cell lines MCF7 and Hela are used as controls. Tubulin is used as loading control. **D:** Western blot analysis in primary T-cell lymphoma cases including one normal lymph node (LN), four primary ALK1-positive (ALCL) lymphomas, one acute lymphoblastic leukemia (ALL),
two cases of adult T-cell leukemia/lymphoma (ATLL) and one peripheral T-cell lymphoma, unspecified (PTCL). Tubulin is used as loading control.

**Figure 4.- ALK1, Stat3 phosphorylated and C/EBPβ expression in NPM-ALK and NPM-ALK-ATP-Abl transformed Ba/F3 and 3D cells.** A: Western blot analysis of NPM-ALK transformed Ba/F3 and 32D cell lines. Each lane contains 30µg of protein extract. The ALCL cell line Karpas 299 is used as control. The NPM-ALK construct produces a band of 75kd. Tubulin is used as loading control. B: Western blot analysis of Ba/F3 NPM-ALK-ATP-Abl transformed cell line untreated or treated with 5µM imatinib for 12 hours. The NPM-ALK-ATP-Abl construct produces a positive protein band of 85kd, 5kd larger than the protein band detected in the SUDHL-1 cell line used as control. Tubulin is used as loading control.

**Figure 5.- QRT-PCR analysis of C/EBPβ mRNA levels and protein expression in NPM-ALK and NPM-ALK-ATP-Abl transformed Ba/F3 cells.** A: Western blot analysis of transformed Ba/F3 cells. Each lane contains 30 µg of protein extract. The ALCL cell line Karpas 299 is used as control. Stat3 phosphorylated is used as control for the kinase activity of NPM-ALK. The NPM-ALK-ATP-Abl construct produces a positive protein band of 85kd. Tubulin is used as loading control. B: QRT-PCR analysis of C/EBPβ was performed relative to the TBP housekeeping gene. Data were analyzed using the ΔC_T method. The results are depicted as mRNA fold induction in comparison to Ba/F3 parental cells.
Figure 2
Figure 3
Figure 4

A

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<tr>
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<tr>
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<td>Stat3 phospho</td>
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<td>LAP</td>
<td>-35kd</td>
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<td>CEBPβ</td>
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B

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Figure 5
NPM-ALK dependent expression of the Transcription Factor CCAAT/enhancer binding protein (C/EBP) β in Alk-positive Anaplastic Large cell Lymphomas

Leticia Quintanilla-Martinez, Stefania Pittaluga, Cornelius Miething, Margit Klier, Martina Rudelius, Theresa Davies-Hill, Natasa Anastasov, Antonio Martinez, Angelica Vivero, Justus Duyster, Elaine S Jaffe, Falko Fend and Mark Raffeld

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