C/EBPβ Regulates Transcription Factors Critical for Proliferation and Survival of Multiple Myeloma Cells

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Abstract

C/EBPβ, also known as NF-IL6, is a transcription factor, which plays an important role in the regulation of growth and differentiation of myeloid and lymphoid cells. Mice deficient in C/EBPβ show impaired generation of B lymphocytes. We show that C/EBPβ regulates transcription factors critical for proliferation and survival in multiple myeloma. Multiple myeloma cell lines and primary multiple myeloma cells strongly expressed C/EBPβ, whereas normal B cells and plasma cells had little or no detectable levels of C/EBPβ. Silencing of C/EBPβ led to down-regulation of transcription factors such as IRF4, XBP1 and BLIMP1 accompanied by a strong inhibition of proliferation. Further, silencing of C/EBPβ led to a complete down-regulation of anti-apoptotic BCL2 expression. In ChIP assays, C/EBPβ directly bound to the promoter region of IRF4, BLIMP1 and BCL2. Our data indicate that C/EBPβ is involved in the regulatory network of transcription factors which are critical for plasma cell differentiation and survival. Targeting C/EBPβ may provide a novel therapeutic strategy in the treatment of multiple myeloma.
Introduction

Multiple myeloma (MM) is a clonal B cell neoplasia. The disorder is characterized by the accumulation of neoplastic plasma cells in the bone marrow and remains an incurable hematologic malignancy. Dysregulation of genes responsible for apoptosis and survival in plasma cells contributes to the pathogenesis of MM. New areas of research focus on targeting various dysregulated proteins involved in the tumorigenesis of MM. In this study, we investigated the role of the transcription factor (TF) CCAAT/enhancer binding protein β (C/EBPβ) in MM pathology.

The C/EBPs belong to a larger family of leucine zipper TFs termed bZip proteins which have a basic DNA-binding domain linked to a leucine zipper dimerization motif. C/EBPβ, also called NF-IL6, regulates a variety of genes involved in diverse functions such as acute phase response, immune function, inflammation, and cellular differentiation processes including adipogenesis, solid organ development, cell survival, tumor invasiveness, and hematopoiesis. Increased levels of C/EBPβ have been found in different types of tumors like breast, renal and colorectal cancer. Deletion of the C/EBPβ gene in mice results in impaired generation of B lymphocytes, and it has been shown that C/EBPβ contributes to the induction of the anti-apoptotic protein, BCL2, in t(14;18) lymphoma cells. Earlier studies identified C/EBPβ as a regulator of IL-6 and demonstrated that C/EBPβ itself is also induced by IL-6, the most important survival factor for MM cells.

Here we identify C/EBPβ as a critical TF in MM, regulating growth, proliferation and anti-apoptotic responses by regulating the expression of other key TFs.
Material and Methods

Chemicals and antibodies

Cell culture media, sera and penicillin-streptomycin were purchased from Gibco BRL (Gaithersburg, MD). Polyvinyl di-fluoride membranes were purchased from Bio-Rad Laboratories (Hercules, CA) and antibodies from the following vendors: anti-C/EBPβ (C-19) (epitope mapping at C-terminus of C/EBPβ and recommended for the detection of C/EBPβ at 45 kDa) and anti-XBP-1 (C-20) (epitope mapping at C-terminus of XBP-1 of human origin): Santa Cruz Biotechnology (Santa Cruz, CA); anti-IRF4 (recommended for the detection of endogenous levels of IRF4 protein and does not cross react with the other family members), and anti-BCL2 (recommended for the detection of endogenous levels of BCL2 protein and does not cross react with the other BCL2 family members): Cell Signaling (Beverly, MA). Thalidomide and pomalidomide were obtained from Celgene (Warren, NJ). DMSO, dexamethasone and melphalan were purchased from Sigma-Aldrich (St. Louis, MO). PD 98059 and LY 294002 were purchased from Cell Signaling Technology (Beverly, MA). Antibody for the surface expression marker CD19 and CD38 were obtained from BD Biosciences (Franklin Lakes, NJ).

Cell culture and cell selection

The human anaplastic large cell lymphoma cell line SU-DHL-1, the acute monocytic leukemia cell line THP-1 and MM cell lines MM.1S, RPMI-8266, H929, OPM2, INA-6 and U266 were cultured in RPMI-1640 medium with L-glutamine, 1X penicillin / streptomycin and 10% FBS at 37° C and 5% CO₂.
Concentrations for *in vivo* cell culture experiment were as follows: pomalidomide 100 µM, thalidomide 100 µM, dexamethasone 5 µM, PD 98059 50 µM, LY 294002 50 µM, and melphalan 5 µM.

Bone marrow mononuclear cells were obtained by using Ficoll (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and CD138+ cells were selected by using CD138+ antibody-specific micro beads followed by magnetic separation column according to the manufacture’s protocol (Miltenyi Biotec, Auburn, CA). The negative cell population was considered as CD138-.

For the generation of normal plasma cells (CD38+) from the B cells, human peripheral blood buffy coats were obtained from the Pittsburgh Blood Bank. PBMCs were separated by density gradient centrifugation using lymphocyte separation medium. CD19+ B cells were purified using a negative isolation kit according to manufacturer instructions (Miltenyi Biotec). Purified B cells were cultured at 1X 10^6 cells/ml in 24-well cultured plate. The cells were incubated with a combination of human IL-21 (100ng/ml; PeproTech, Rocky Hill, NJ), 1 µg/ml anti-human CD40 (eBiosciences, San Diego, CA) and 5 µg/ml anti-IgM (Pierce Biotechnology Inc., Rockford, IL) according to the protocol described by Ettinger, et al. The B cells were analyzed by flow cytometry before induction and on day 6 of differentiation for the expression of CD19 as well as CD38. All blood samples were obtained as approved by the University of Pittsburgh Institutional Review Board. Informed consent was provided according to the Declaration of Helsinki.
RT-PCR analysis

For the determination of mRNA levels of C/EBPβ, total RNA were isolated by using the Mini RNA isolation II kit (Zymo Research, Orange, CA) according to the manufacturer’s instructions. Total RNA was converted into cDNA using the Superscript III RT (Invitrogen, Carlsbad, California). Quantitative RT-PCR was performed on ABI Prism 7700 Sequence detection System (Applied Biosystems, Foster City, CA). RT-PCR was carried out with the SYBR Green PCR master mix (Bio-Rad, Hercules, CA) using 1 µl cDNA in a 20 µl final reaction mixture (15 minutes at 95º C; 40 cycles of 15 seconds at 95º, 60 seconds at 60º C and 10 min at 79º C). The average threshold cycle (Ct) for each gene was determined from triplicate reactions and data were analyzed by taking the difference between mean thresholds of RT-PCR cycles values for target and control gene ($\Delta$Ct). Target gene expression was normalized to β-actin using the $\Delta$Ct value. This was then calibrated to the control sample in each experiment to give the $\Delta\Delta$Ct value, where the control had a $\Delta\Delta$Ct value of 0. The fold target gene expression, compared with the calibrator value, is given by the formula $2^{-\Delta\Delta CT}$. The following primer sets were used (Real Time Primers, Elkins Park, PA).

C/EBPβ: 5’-AACTCTCTGTGCCCTCCCTCTTG-3’
         5’- AAGCCCGTGGAGACATCTTTT-3’
IRF4: 5’-TTAATTCTCCAAGCGGATGC -3’
      5’-AAGGAATGAGGAAGCCGTTC-3’
BCL2: 5’-AGGAAGTGAACATTTCGGTGAC-3’
      5’- GCT CAG TTC CAG GAC CAG GC -3
BLIMP1: 5’- TGA GAG TGC ACA GTG GAG AA -3’
5'- ATT GCT GGT GCT GCT AAA TC -3'

β-actin: 5'-GGACTTCGAGCAAGAGATGG-3'
5' AGCACTGTGGTGGCGTACAG-3'

**Western Blot Analysis**

Briefly the protein was extracted from the cells by using 1X RIPA buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with the cocktail of protease inhibitor, sodium benzoate and PMSF. Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl di-fluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with respective antibodies for the protein expression and immune complexes were detected using enhanced chemiluminescence (Amersham, Piscataway, NJ).

**Immunohistochemistry**

Bone marrow biopsies from 72 patients with MM infiltrates were selected from the Lymphoma Reference Centre at the Institute of Pathology, Charité, University Medicine Berlin, Germany. Non-neoplastic plasma cells were analyzed in specimens from palatine tonsils removed from 10 patients because of enlargement due to hyperplasia of lymphoid tissue and florid inflammation. For demonstration of C/EBPβ expression in normal and neoplastic plasma cells we used the anti-C/EBPβ (clone H7) monoclonal antibody from Santa Cruz Biotechnology, Inc, Santa Cruz, CA and the anti-CD138 antibody from Dako (Glostrup, Denmark). CD138-C/EBPβ double labeling was necessary to distinguish CD138⁺-C/EBPβ⁺ cells from C/EBPβ⁺-myeloid cells. For this purpose
the dewaxed sections were subjected to an antigen retrieval protocol consisting of a brief, high-temperature heating in citrate buffer (10 mMol, pH 6.0) for 2 min in a high-pressure cooker followed by an incubation with the anti-CD138 and visualization of bound antibody using the streptavidin-biotin-peroxidase method and diaminobenzidine as chromogen. Subsequently the sections were incubated with the anti-C/EBPβ antibody stained by alkaline phosphatase anti-alkaline phosphatase method and FastRed as chromogen (all reagents were obtained by Dako). The result was a brown membrane staining for CD138 and a red nucleus for C/EBPβ. Negative control slides were processed with the control antibody IgG in place of primary antibody followed by incubation with the secondary antibody.

Plasmids for transfection studies

Expression vectors for the full-length wild-type C/EBPβ (pcNF-IL6) (WT-C/EBPβ) and for a truncated dominant-negative C/EBPβ (DN-C/EBPβ), with a deletion of the internal SpII-SpII fragment [pcmNF-IL6 (ΔSpI)], were generated by inserting the respective coding regions into pcDNA3.1 (Invitrogen Corp., San Diego, CA) and provided by Dr. Philip. E. Auron (Duquesne University, Pittsburgh) (Fig. 2A).

Transfection

MM cells (2 x 10⁷) were transfected by electroporation with 30 μg of the empty vector pcDNA3.1 (EV), WT-C/EBPβ, or the DN-C/EBPβ plasmids. Electroporation was performed in serum- and antibiotic-free media with the Gene Pulser II apparatus (Bio-Rad) using 0.4 cm gene pulser cuvettes (Bio-Rad) with
settings of 250 V and 950 μF. Twenty-four h after transfection, transfected cells were selected for resistance to G418 by 10 days of continuous G418 (500 μg/ml) treatment. Protein of the transfected cells was extracted using 1X RIPA buffer with a cocktail of protease inhibitors, sodium benzoate and phenylmethylsulphonyl fluoride (Santa Cruz Biotechnology). Protein extracts were analyzed for the expression of C/EBPβ by western blot analyses.

**Cell proliferation assays**

MM.1S (3x10^5/well), RPMI-8266 cells (3x10^4/well) or H929 cells (3x10^4/well) were incubated in 96-well plates in the presence of RPMI-1640 medium containing 10% FCS at 37°C/5% CO2 for 48 h. DNA synthesis was measured by ^3^H-thymidine (^3^H-TdR) incorporation (NEN Products, Zaventem, Belgium). Cells were pulsed with ^3^H-TdR (1 Ci/well [0.037 MBq]) for the last 8 h of culture, harvested onto glass-fiber filter mats (Wallac, Gaithersburg, MD) using an automatic cell harvester (Tomtec Harvester 96, Mach III, Tomtec, Inc., Hamden, CT), and counted using a Wallac TriLux Beta plate scintillation counter (PerkinElmer, Waltham, MA). All experiments were performed in triplicate.

**Apoptosis assays**

Apoptosis was analyzed by annexin V-FITC staining using the Alexa Fluor 488 annexin V kit (Invitrogen Corporation, Carlsbad, CA). Briefly, 0.5x10^6/ml cells were harvested, washed once with cold PBS and then resuspended in 1x annexin-binding buffer. Cell survival was determined by annexin V-
FITC/propidium iodide (PI) double staining. Samples were analyzed on FACSCalibur (Becton-Dickinson) using the software program CellQuest.

**Chromatin immunoprecipitation assay and RT-PCR**

H929 human MM cells were cultured as described above. Cells were treated with formaldehyde, nuclei isolated and chromatin sheared by sonication as per the protocol previously described by Shell et al.\(^{19}\) Sheared chromatin was immunoprecipitated by rabbit antibodies: anti-RNA polymerase II (N20, Santa Cruz), anti-C/EBPβ (Abcam Inc, Cambridge, MA) or normal IgG (Sigma, St. Louis, MO) using the magnetic bead ChIP-IT™ express kit from Active Motif (Carlsbad, CA). DNA was isolated from the immunoprecipitated chromatin on Protein G linked magnetic beads using directions supplied by the ChIP-IT™ kit manufacturer except that the de-crosslinking step was conducted at 65° C overnight, the proteinase K digestion was increased to 2 μl of 10 mg/ml proteinase K at 37° C for 2 hrs and the DNA isolated on QIAquick® PCR purification kit (Qiagen, Gaithersburg, MD). The DNA was analyzed by quantitative RT-PCR using an Applied Biosystems instrument with SYBR® GREEN PCR master mix (Applied Biosystems, Warrington, UK). Probes for RT-PCR were purchased from Integrated DNA Technologies (Coralville, IA). Error bars on the graph (Fig. 6) represent standard deviation from at least 4 determinations. The oligos used as RT-PCR primers were as follows.

C/EBP

AGAAGTCGGTGACAAAGACAGCA
ATTGTCACTGGTCAGCTCCAGCA

CCR5 (intron) TCCTGCCTCATAAGGTGCCCTAA
AGGGCACATACTGGATGCCAATCA
IRF4 a
TCACCACTGCCAGCTGCTA
AAACTCCGGATGGCCTCAT
IRF4 b
AGGGAGCTGGGCCATTTCCTATTT
TGTAACGGAAGACGGAGGAATGGT
BLIMP a
GGACAGAGGCTGAGTTTGAAGA
CGCCATCAGCACCAGAAATC
BLIMP b
AGAGCCCAAGTAAGCGTTGAGGTT
AGAGCTTCTCCTCTTCGCATGTGT
BCL2 exon 1
TGTGTACAGGGAAACGCACCTGAT
CCCTTGGCATGAGATGCAGGAAAT
XBP1
TCTATCTCGACTTTCGGCTCCACT
TCCAAACCGAGAGCTTTCCAGACT
DHFR
ACCTGGTCGGGCTGCACCT
TTGCCCTGCCATGTCTCG

The midpoint Ct values in quantitative RT-PCR were converted to ΔCt relative to the total DNA standard and converted to a % by taking the power of 2 to the negative ΔCt as previously described. The data were normalized to the value obtained with RNA polymerase II set as 100%, resulting in the percentages shown in Fig. 6B.
Results

C/EBPβ is highly expressed in MM cells.

In our previous work we found in gene array studies that C/EBPβ RNA is highly expressed in the MM cell line MM.1S. Therefore, we first analyzed C/EBPβ protein expression in MM cell lines, primary MM cells, and MM tissue. Our data showed that C/EBPβ LAP isoform at 45 kDa (Fig. 1A, upper panel) and mRNA (Fig. 1B) are highly expressed in all tested MM cell lines (MM.1S, U266, OPM2, H929 and RPMI-8266). Expression of C/EBPβ was also induced by IL-6 treatment in serum-starved MM cells, suggesting that C/EBPβ is up-regulated in response to cytokines critical for MM cell growth such as IL-6 (Fig. 1A, lower panel). We found a heterogeneous expression pattern of the inhibitory form (LIP) in untransfected MM cells. Certain myeloma cells such as INA-6 show no expression of LIP and other cell lines such as MM.1S only expressed LIP after stimulation with IL-1β (Supplemental Fig. S1). Immunohistochemical analysis of MM cell lines showed the following: U226: 90% C/EBPβ+ cells; OPM2: 80-90% C/EBPβ+ cells; MM.S1: 60-70% C/EBPβ+ cells; NCL-H929: 100% C/EBPβ+ cells; RPMI: 80% C/EBPβ+ cells. In contrast to MM cells, we could not detect C/EBPβ transcripts in normal B cells (CD19+) and only very low levels of C/EBPβ-mRNA in in vitro generated plasma cells by quantitative real-time-PCR (RT-PCR) (Fig. 1B).

Analysis of CD138+ MM cells from bone marrow of MM patients showed high levels of C/EBPβ-mRNA in all samples measured by quantitative RT-PCR whereas CD138- cells from these same patients had barely detectable levels of
C/EBPβ-mRNA (Fig. 1C). In accordance with these results we detected a strong expression of C/EBPβ in primary MM samples by western blot (Fig. 1C). Additional immunohistochemical staining of 72 MM tissue samples showed variable results. In order to distinguish C/EBPβ-positive myeloid cells from C/EBPβ-positive plasma cells in the bone marrow we performed CD138/C/EBPβ double labeling. A significant up-regulation of C/EBPβ was detected in 29% of MM cases by immunohistochemistry. In 7 cases C/EBPβ expression was detected in more than 50% of the neoplastic cells, in additional 9 cases C/EBPβ was present in 10–50% of the neoplastic cells while 5 cases exhibited few C/EBPβ+ tumor cells (<10% of the neoplastic population) (Fig. 1D: a–d). In contrast, analysis of non-neoplastic plasma cells of palatine tonsils was completely negative in all analyzed samples for C/EBPβ-positive plasma cells (Fig. 1D: d).

C/EBPβ regulates expression of IRF4

The fact that C/EBPβ is not expressed in normal tissues, but is rapidly and drastically induced by cytokines such as IL-1, TNF and IL-6 which support MM cell growth,21 raises the question of the role of C/EBPβ in MM and effects on other TFs involved in plasma cell development. To explore the interactions between C/EBPβ and other TFs critical for MM survival and proliferation, the MM cell lines MM.1S, RPMI-8266 and H929 were transfected with expression vectors containing cDNA encoding WT-C/EBPβ, or DN-C/EBPβ (ΔSpl) lacking the internal Spl-I-SplII fragment encoding amino acids
41 to 205 (Fig. 2A), or EV. The DN-C/EBPβ lacks the transactivation and regulatory domains, leaving intact the DNA binding and dimerization domains, which makes it capable of binding to DNA but with no transcriptional activation capacity. The plasmids contain the neomycin-resistance gene, and transfected clones were selected with G418 (500 µg/ml) for 10 days. After selection, protein expression levels of C/EBPβ were determined by western blot. When compared with the EV, transfection of MM cell lines with the DN-C/EBPβ resulted in significant inhibition of endogenous C/EBPβ (Fig. 2B upper panel). Inhibition of endogenous C/EBPβ expression by transfection of the DN-C/EBPβ suggests that there is autoregulation of C/EBPβ in MM cells. Auto-regulation has been described as a common mechanism for the transcriptional control of all C/EBP family members and it has been shown that C/EBPβ can stimulate its own transcription.22,23 On the other hand, transfection with WT-C/EBPβ increased the amounts of C/EBPβ protein expression in all tested MM cell lines (Fig. 1 and Fig. 2C upper panel).

It has been reported that IRF4 is a protooncogene in MM, playing a critical role in survival and proliferation of MM cells.24 We found that all tested MM cell lines expressing high levels of C/EBPβ concomitantly express high levels of IRF4 protein (Fig. 2D upper panel). In addition, induction of C/EBPβ by IL-6 was also associated with increased expression of IRF4 (Fig. 2D lower panel), raising the possibility that C/EBPβ regulates IRF4 protein levels. Intriguingly, overexpression of WT-C/EBPβ also increased expression of IRF4 protein (Fig. 2C),
whereas expression of the DN-C/EBPβ resulted in down-regulation of IRF4 expression (Fig. 2B), suggesting that IRF4 is under control of C/EBPβ.

**C/EBPβ affects TFs critical for B/plasma cell development such as XBP1 and BLIMP1**

IRF4 is critical for the generation of plasma cells by regulating various TFs such as XBP1 and BLIMP1, which are essential for the development and maturation of the B/plasma cells. IRF4 acts upstream of XBP1, an activator required for plasma cell formation. We investigated how regulation of C/EBPβ/IRF4 impacts XBP1 and BLIMP1 protein levels. Down-regulation of C/EBPβ by DN-C/EBPβ induced an almost complete abrogation of XBP1 expression in all tested MM cell lines (Fig. 3A). In contrast, over-expression of C/EBPβ induced the expression of XBP1 (Fig. 3B). BLIMP1 has been reported to be a master regulator of plasma cell differentiation by repressing TFs which are important for the expression of B cell-associated genes, such as PAX5, or for cell cycle progression, like c-myc. Over-expression of C/EBPβ up-regulated the expression of BLIMP1 (Fig. 3C), suggesting that the expression of XBP1 and BLIMP1 is regulated by C/EBPβ in MM cells.

**C/EBPβ induces MM proliferation**

It has been shown that C/EBPβ plays an important role in the regulation of cell differentiation and proliferation. We therefore analyzed the effect of C/EBPβ on proliferation and apoptosis induction in MM cells. Over-expression of WT-
C/EBPβ significantly \((p \leq 0.001)\) induced proliferation in all tested MM cell lines (MM.1S, H929 and RPMI-8266) as compared to cells transfected with the control vector (Fig. 4A). In contrast, transfection with DN-C/EBPβ resulted in significant inhibition of proliferation measured by thymidine uptake of MM cells (Fig. 4A).

We next analyzed whether C/EBPβ is implicated in the induction of MM cell apoptosis. MM cells (H929), transfected with the EV, WT-C/EBPβ or DN-C/EBPβ, were analyzed by flow cytometry using annexin V staining to measure apoptosis [Annexin V (+) and PI (-)]. We detected a significant induction of apoptosis by DN-C/EBPβ (mean 20% ± SD 5%) relative to EV (13% ± 2) and decreased apoptosis in the presence of over-expressed full-length C/EBPβ (7% ± 3) \((n = 4)\) experiments (Fig. 4B). Fig. 4C shows a representative experiment out of 4 experiments. Our data suggest that inhibition of C/EBPβ in MM cells decreases proliferation and to a lesser extent induces apoptosis. In contrast, over-expression of WT-C/EBPβ in MM cells enhances proliferation and in part protects the cells from cell death by apoptosis, suggesting that C/EBPβ is involved in proliferation and survival.

**C/EBPβ regulates BCL2**

BCL2 has previously been reported to have anti-apoptotic effects in MM.\(^{28}\) In Fig. 5A we show that inhibition of C/EBPβ by DN-C/EBPβ resulted in complete down-regulation of BCL2 protein expression (Fig. 5A). On the other hand, over-expression of WT-C/EBPβ induced the expression of BCL2 in all tested MM cell lines (Fig. 5B). These data suggest that BCL2 protein levels are regulated in MM
cells at least in part by C/EBPβ and thereby might play a potential role in the survival of the MM cells.

C/EBPβ binds to the regulatory gene regions of IRF4, BLIMP1 and BCL2

In order to determine whether inhibition or overexpression of C/EBPβ affects the RNA levels of the TFs critical for MM cells, we analyzed the mRNA expression of C/EBPβ, IRF4, BCL2 and BLIMP1 in MM cell lines and found that overexpression of WT-C/EBPβ significantly induces the gene expression of IRF4, BCL2 and BLIMP1. In contrast, transfection with DN-C/EBPβ resulted in downregulation of the mRNAs of these TFs. (Fig. 6A, S2). To further determine whether C/EBPβ protein interacts with the regulatory regions of MM-relevant TF genes, we conducted chromatin immunoprecipitations with anti-C/EBPβ antibody in MM cells. The DNA from the immunoprecipitated material was analyzed by RT-PCR using primers that span potential regulatory sequences which contain a CAAT site and could therefore serve as a binding site for C/EBPβ. In parallel, an antibody to RNA polymerase II was used in a ChIP reaction as a positive control, while an irrelevant antibody was used as a negative control. The amount of DNA of particular genes precipitated by anti-C/EBPβ antibody relative to that precipitated by anti-RNA polymerase II antibody is plotted (Fig. 6B). As a positive control we took advantage of the observation that the C/EBPβ gene itself has a C/EBPβ binding site in an intron.22 Here, we observed binding of C/EBPβ at about 12% the level of RNA polymerase II. C/EBPβ has been reported to bind to an intronic region of the CCR5 gene in T-lymphocytes.29 Using primers to that
region we observed binding of C/EBPβ at about 37% that of RNA polymerase II. Two sets of primers separated by 500 nt for the IRF4 5’ end were used, near closely spaced CAAT sites in the upstream region of the gene; these primers showed 30% and 20% C/EBPβ binding relative to RNA polymerase II. Primers 200 nt upstream of the PRDM-1 gene (encoding BLIMP1), where several CAAT sites are found were used and also showed C/EBPβ binding at about 20% relative to RNA polymerase II. Primers in exon 1 of the BCL2 gene were used and also showed C/EBPβ binding to BCL2 at about 25% relative to RNA polymerase II. Meanwhile, XBP1 and dihydrofolate reductase showed little or no binding of C/EBPβ at the upstream sites examined. We conclude that C/EBPβ directly binds to potential regulatory DNA sequences within the IRF4, BLIMP1 and BCL2 genes.

**Down-regulation of C/EBPβ by anti-MM agents**

Because our studies indicated that C/EBPβ is a TF regulating growth and survival of MM cells, we next analyzed whether anti-MM agents known for their anti-proliferative and apoptotic effect on MM cells inhibit expression of C/EBPβ. We cultured MM.1S MM cells with thalidomide, the IMiD compound pomalidomide, dexamethasone, the MEK kinase inhibitor PD 98059, the PI3 kinase inhibitor LY 294002 and melphalan as described in Material and Methods. DMSO was used as control. Western blot analysis revealed that treatment with agents which significantly inhibited DNA synthesis (pomalidomide, dexamethasone, LY 294002, and melphalan), resulted in decreased C/EBPβ protein expression (Fig. 7A, B). Of note, thalidomide that has less strong
inhibitory effects on proliferation of MM cells induced only a weak down-regulation of C/EBPβ (Fig. 7B). Taken together, this suggests that C/EBPβ is an important regulator of proliferation, survival and apoptosis of MM cells.

Discussion

In this study we demonstrate a critical role for C/EBPβ in MM. It has been shown before that C/EBPβ is important for proliferation and differentiation in a variety of cellular systems and its aberrant expression has been implicated in the development of various epithelial tumors. In addition, C/EBPβ plays an important role in lymphopoiesis. Expression and DNA binding activity of C/EBPβ increase during B cell differentiation. Furthermore, C/EBPβ-deficient mice demonstrate an impaired generation of bone marrow B cells and, after 25 to 30 weeks of age, a peripheral lymphoproliferative disease, presumably due to deregulated cytokine expression and a dysregulation of the Th2 response. The fact that enforced expression of C/EBPβ in immature or mature B cells can induce transdifferentiation into macrophages indicates that precise control of C/EBPβ expression levels in B lymphocytes is critical to avoid interference with normal lymphoid differentiation. Recently, it has been demonstrated that C/EBPβ is over-expressed in anaplastic lymphoma kinase positive (ALK+) anaplastic large cell lymphoma (ALCL). This is in accordance with our results showing high expression of C/EBPβ in the ALK+ anaplastic large cell lymphoma cell line SU-DHL-1 (Fig. S1). Expression of C/EBPβ is nucleophosmin-ALK dependent in
ALK+ ALCL and plays a critical role in the pathogenesis and unique phenotype of this lymphoma.\textsuperscript{35}

Since C/EBP\(\beta\) has been shown to be involved in the regulation of B cell proliferation and survival, and no information is available on the role of C/EBP\(\beta\) in MM, we investigated the role of C/EBP\(\beta\) in relation to MM tumor biology. Herein, we describe the expression of C/EBP\(\beta\) in MM cells. We found no expression of C/EBP\(\beta\) in all non-malignant plasma cell samples, including non-neoplastic plasma cells from palatine tonsil samples and \textit{in vitro} generated plasma cells. In contrast, up to 30\% of primary MM bone marrow samples expressed C/EBP\(\beta\) as determined by immunohistochemistry. In addition, C/EBP\(\beta\) was detected in all MM cell lines and in all primary MM patient samples tested by RT-PCR or western blot. The discrepancies in the frequency of C/EBP\(\beta\) detection in malignant plasma cells might reflect different sensitivity of the methods with higher sensitivity of RT-PCR and western blot in comparison to immunohistochemistry. In addition the difference of C/EBP\(\beta\) expression might reflect the higher proliferative capacity of MM cell lines in contrast to primary myeloma samples since we found an inverse correlation of C/EBP\(\beta\) expression with doubling times (DT) of MM cell lines, with the slowest DT\textsuperscript{5} and the lowest rate of C/EBP\(\beta\) positive cells by immunohistochemistry for MM.1S cells.

By interacting with other TFs, C/EBP\(\beta\) stimulates its own transcription,\textsuperscript{2,34,36,37} and auto-regulation was reported as one of the main mechanism for regulation of C/EBP\(\beta\) activity.\textsuperscript{22} In this study we show that DN-C/EBP\(\beta\) significantly inhibited endogenous C/EBP\(\beta\), which resulted in a potential block of C/EBP\(\beta\) regulation of
other TF expression in MM cell lines. Our studies of C/EBPβ detected the activating isoform (LAP) in MM cells. Other cell lines such as SU-DHL-1 a large anaplastic lymphoma cell line expressed high levels of LIP. We have found a heterogeneous expression pattern of the inhibitory isoform (LIP) in untransfected MM cells. Certain MM cells such as INA-6 show no expression of LIP and other cell lines such as MM.1S only expressed LIP after stimulation with IL-1β. (Fig. S1). Unfortunately due to the limited number of MM cells after transfection and selection we were not able to obtain sufficient whole cell lysate to allow for unambiguous detection of LIP by western blot analysis.

MM is a malignancy of terminally differentiated B cells and the maturation into non-dividing, Ig-secreting plasma cells is controlled by a network of TFs. Initially, IRF4 was identified as an oncogene associated with the chromosomal translocation t(6;14) (p25;q32) in MM, but it also is a well defined factor for normal plasma cell differentiation. Recently, IRF4 has been reported as a critical factor controlling MM survival and as a prognostic marker in patients with MM associated with poor survival. We found that IRF4 shows a similar expression pattern as C/EBPβ in B cells, plasma cells and MM cells. It is highly expressed in all MM cell lines expressing C/EBPβ. In contrast, normal plasma and B cells showed very low or no detectable levels of IRF4 expression. Recently IRF4 has been described as “a master regulator of the MM specific gene expression program.” In our studies, induction of C/EBPβ increased IRF4, whereas inhibition decreased IRF4 expression, suggesting that C/EBPβ can act as an upstream regulator of IRF4. Hence, the fact that C/EBPβ is up-regulated in MM cells and regulates the expression of MM-critical IRF4 makes it a main
regulator in MM. In addition, the induction of IRF4 indicates an important role of C/EBPβ in MM. Decreased XBP1 expression was found in DN-C/EBPβ transfected MM cells, suggesting that C/EBPβ also controls XBP1, which in turn regulates the growth of MM cells.\textsuperscript{25,41,43}

We further observed that overexpression of C/EBPβ induces proliferation and in part protects against spontaneous apoptosis of MM cells. Interestingly only drugs that significantly decreased expression of C/EBPβ inhibited proliferation of MM cells, suggesting that C/EBPβ is critical for proliferation. Immunohistochemical analysis of MM cell lines indicated an inverse correlation of C/EBPβ expression with doubling times (DT) of MM cell lines described in the literature, with the longest DT of 72h\textsuperscript{44} and the lowest rate of C/EBPβ positive cells (60-70\%) by immunohistochemistry for MM.1S cells. In contrast cell lines with a shorter DT of 48 hours\textsuperscript{45-48} displayed a higher positivity of C/EBPβ with NCI-H929: 100\%; U226: 90\%; OPM2: 80-90\%; RPMI: 80\% C/EBPβ\textsuperscript{+} cells.

To our knowledge, we are the first to report that C/EBPβ is involved in regulation of proliferation and survival in MM. Our findings on the role of C/EBPβ in MM cells are in agreement with a previous report describing its role in the proliferation and maintenance of lymphoma cells by regulating the promoter activity of BCL2.\textsuperscript{8}

In our study, we observed that over-expression of C/EBPβ significantly induced the expression of anti-apoptotic protein BCL2, suggesting that C/EBPβ positively regulates BCL2 in MM cells. Piva \textit{et al} showed that ALK induced C/EBPβ and BCL2 are involved in the proliferation and anti-apoptotic actions in anaplastic
lymphomas.\textsuperscript{36} In contrast, the expression of DN-C/EBP\(\beta\) significantly decreased BCL2 expression accompanied by increased sensitivity of MM cells to apoptosis. Analysis of mRNA expression of IRF4, BCL2 and BLIMP1 in MM cells with overexpressed or silenced C/EBP\(\beta\) as well as in B cells and plasma cells confirmed the expression pattern that we observed. That led us to investigate the protein-promoter interaction of C/EBP\(\beta\) by CHiP revealing that C/EBP\(\beta\) protein is capable of binding to the promoter regions of IRF4 and BLIMP1, and to a site in exon 1 of BCL2, potentially resulting in increases in their transcription (Fig. 6B). The observation that C/EBP\(\beta\) also binds to the CCR5 gene is consistent with its up-regulation in MM;\textsuperscript{49} C/EBP\(\beta\) binding to this site in CCR5 was previously seen in T-lymphocytes.

The mechanism that controls the activation of C/EBP\(\beta\) in MM cells is presently unclear. But our data suggest that C/EBP\(\beta\) regulates the expression of various TFs associated with proliferation and survival of malignant plasma cells. Our results may help to explore the critical role of C/EBP\(\beta\) in MM and may offer future therapeutic targets in plasma cell tumorigenesis.

**Acknowledgments**

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Authorship and Conflict of Interest Statements

RP performed experiments and wrote the manuscript. MJ performed gene array studies and helped to design the experiments. DLG provided plasmids and helped to design experiments. MG and SLi performed western blot. KJ and IA performed immunohistochemical analysis of primary MM samples. BD and GDR provided advice on design of experiments. MYM, LB, LK, and CM helped to design the experiments. CM performed ChIP assays. LK and LB provided B cells and non-malignant plasma cells. SL conceived and developed the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the manuscript. The authors have no competing financial interests.
References


45. German Collection of Microorganisms and Cell Cultures. Braunschweig, Germany.


Figure legends

Figure 1. C/EBPβ is highly expressed in MM cells.

(A) Upper panel: Expression of C/EBPβ in MM cell lines was detected by western blotting as described in material and methods. β-actin was used as a loading control for all samples. Lower panel: Induction of expression of C/EBPβ by IL-6. Cells were cultured in 1% FBS with or without IL-6 for 48h, and analyzed for C/EBPβ protein expression. (B) C/EBPβ-mRNA expression in MM.1S and H929 MM cell lines, B and non-malignant plasma cells were analyzed by quantitative RT-PCR. Data were analyzed according to the ΔC\textsubscript{T} method. The results are expressed as C/EBPβ mRNA expression relative to β-actin. B cells were cultured in a cocktail of CD40 and human IgM for 6 days for the differentiation into plasma cells. Expression of CD19 of B cells as well as CD38 for plasma cells was confirmed by flow cytometry. (C) Mononuclear cells of bone marrow samples of four MM patients were collected by Ficoll. CD138\textsuperscript{+} and CD138\textsuperscript{−} cells from the same patient were separated using MACS Beads selection. The total RNA was extracted, subjected to cDNA synthesis and used for quantitative RT-PCR. Results are depicted as C/EBPβ mRNA fold expression in CD138\textsuperscript{+} cells as compared to CD138\textsuperscript{−} cells. The level of mRNA was normalized to β-actin expression. Expression of C/EBPβ and IRF4 was detected by western blotting with anti-C/EBPβ and anti-IRF4 in CD138\textsuperscript{+} cells and CD138\textsuperscript{−} cells from the same patient. β-actin was used as a loading control. Error bars indicate SD of the mean. (D) Immunohistochemical double staining was
performed on paraffin embedded MM bone marrow trephines to detect C/EBPβ expression in CD138+ cells. Positive staining shows a red nucleus for C/EBPβ and brown membrane for CD138. Positive double staining is indicated in (a) and (b) by arrows, negative double staining is shown in (c). Non-neoplastic plasma cells in palatine tonsils did not show a C/EBPβ expression (d). Vertical lines have been inserted to indicate a repositioned gel lane.

Figure 2. Regulation of IRF4 by C/EBPβ.

(A) The human WT-C/EBPβ (also known as NF-IL6) cDNA is depicted as well as the region (an Sp/1 fragment encoding amino acids 41-205) deleted to generate the DN-C/EBβ cDNA.[54] These cDNAs inserted into empty vector (EV) pCDNA3.1 were used for the transfection studies. Transfection of the WT-C/EBPβ cDNA in MM cells results in over-expression. MM cell lines, MM.1S, H929 and RPMI-8266, were transfected with the EV, (B) DN-C/EBPβ, or (C) WT-C/EBPβ followed by G418 (500 μg/ml) selection for 10 days. Transfected MM cells were selected, and expression of C/EBPβ and IRF4 was detected by western blotting with anti-C/EBPβ and anti-IRF4 antibody. β-actin was used as a loading control. (D) Upper panel: Expression of IRF4 in MM cell lines and primary MM cells were detected by western blotting as described above. Lower panel: Cells were cultured in 1% FBS with or without IL-6 for 48h, and analyzed for induction of IRF4 protein expression. β-actin was used as a loading control for all samples. Vertical lines have been inserted to indicate a repositioned gel lane.
Figure 3. C/EBPβ regulates IRF4 dependent transcription factors such as XBP1 and BLIMP1.

MM cells were transfected with EV, (A) DN-C/EBPβ or (B) WT-C/EBPβ. Transfected cells were selected for 10 days by G418 (500 μg/ml). The selected cells were analyzed for expression of XBP1 by western blotting with the anti-XBP1 antibody. (C) EV and WT-C/EBPβ transfected and selected cells were analyzed for the protein expression of BLIMP1. β-actin was used as a loading control. Vertical lines have been inserted to indicate a repositioned gel lane.

Figure 4. C/EBPβ induces MM cell proliferation and is involved in apoptosis.

Proliferation and apoptosis of transfected MM cells with EV, WT- C/EBPβ, or DN-C/EBPβ were analyzed. (A) DNA synthesis was measured by ³H-thymidine incorporation and results are presented as mean of quadruplicates. EV □, WT-C/EBPβ plasmid, ■ or DN-C/EBPβ □. (B) The graph represents the mean (n=4) of the percentage of transfected MM cells that have undergone apoptosis measured by Annexin V staining. Error bars indicate SD of the mean. (C) Percentage of apoptotic cells of transfected cells was analyzed by Annexin V binding/ PI staining using flow cytometry. The figure shows a representative experiment of 4B.
**Figure 5. BCL2 is regulated by C/EBPβ.**

MM cells were transfected with EV, (A) DN-C/EBPβ or (B) WT-C/EBPβ. Transfected cells were selected for 10 days with G418 (500 μg/ml). Lysates of transfected cells were subjected to western blotting using anti-BCL2 antibody. β-actin was used as a loading control. Vertical lines have been inserted to indicate a repositioned gel lane.

**Figure 6. C/EBPβ binds to the promoter regions of IRF4, BLIMP1 and in exon 1 of BCL2.**

(A) mRNA expression of C/EBPβ, IRF4, BCL2 and BLIMP1 in H929 MM cells transfected with EV, WT- C/EBPβ, or DN-C/EBPβ were analyzed by quantitative RT-PCR. Data were analyzed according to the ΔCₜ method. The results are expressed as mRNA fold change as compared to control pcDNA group. (B) Chromatin immunoprecipitation with anti-C/EBPβ antibody and RT-PCR on the resulting genomic DNA was conducted on H929 MM cells. PCR probes to the various promoters are indicated and described further in the materials and methods section. The a and b probes for IRF4, BLIMP1, and BCL2 represent two areas of CAAT sequence in the promoter regions, respectively. The C/EBP and CCR5 probes were to intron regions previously shown to bind the protein. DNA that was immunoprecipitated by RNA polymerase II was used as the standard (set to 100 %) and the amount of DNA immunoprecipitated by C/EBPβ antibody was normalized to it. Control represents the amount of DNA immunoprecipitated by normal rabbit IgG (a negative control). Error bars represent the SD of the mean from at least four determinations.
Figure 7. Down-regulation of C/EBPβ by various anti-MM agents is associated with inhibition of proliferation.

MM.1S MM cells were incubated with various anti-MM agents such as pomalidomide, thalidomide, dexamethasone, PD98059, LY294002 and melphalan for 48 h. DMSO 0.1% was used as control treatment. (A) Whole cell extracts were analyzed for C/EBPβ expression by western blotting using anti-C/EBPβ antibody. β-actin was used as a loading control. (B) DNA synthesis was measured by ³H-thymidine incorporation and results presented as the mean from triplicates. Error bars indicate SD.
Figure 1

A

B

C

D
Figure 2

Diagram of C/EBPβ (NF-IL6) with regions labeled as Trans-activation, Regulation, Basic Region, and Leucine Zipper. The Δ(41-205) region is indicated as Dominant Negative.
Figure 3

A

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Figure 4

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Cell Proliferation

- MM.1S
- H929
- RPMI

B

% Annexin V positive cells

- EV
- WT-C/EBPβ
- DN-C/EBPβ

C

Annexin V

- EV
- WT-C/EBPβ
- DN-C/EBPβ

PI

13%

6%

25%
Figure 5

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Figure 7

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control pomalidomide thalidomide dexamethasone PD 98059 LY 294002 melphalan

CEBPβ (45 kDa)

β-actin (45 kDa)

B

\[ \text{[^3]H}(dT) \text{ uptake (% of control)} \]

control pomalidomide thalidomide dexamethasone PD 98059 LY 294002 melphalan
C/EBPβ regulates transcription factors critical for proliferation and survival of multiple myeloma cells

Rekha Pal, Martin Janz, Deborah L. Galson, Margarete Gries, Shirong Li, Korinna Johrens, Ioannis Anagnostopoulos, Bernd Dorken, Markus Y. Mapara, Lisa Borghesi, Lela Kardava, G. David Roodman, Christine Milcarek and Suzanne Lentzsch