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Role of Interleukin 16 in Multiple Myeloma

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- Background** Multiple myeloma is a malignancy characterized by the expansion of a plasma cell clone that localizes to the human bone marrow. Myeloma cells and bone marrow stromal cells produce soluble factors that promote the survival and progression of multiple myeloma. Interleukin 16 (IL-16) is involved in regulating the migration and proliferation of normal leukocytes. However, the role of IL-16 in human cancers, including multiple myeloma, is unclear.
- Methods** We investigated IL-16 expression in cell lines ($n = 10$) and in the bone marrow of myeloma patients ($n = 62$) and healthy bone marrow donors ($n = 12$) by quantitative reverse transcription–polymerase chain reaction, immunoblot analysis, enzyme-linked immunosorbent assay, flow cytometry, and immunohistochemistry. Transfection of two human multiple myeloma cell lines with small interfering RNAs was used to examine the effect of *IL-16* gene silencing on apoptosis by flow cytometry, on proliferation by bromodeoxyuridine incorporation, and on colony formation. Protein neutralization assays were performed by treating multiple myeloma cells with a monoclonal antibody against the carboxyl-terminal fragment of IL-16. All statistical tests were two-sided.
- Results** IL-16 was strongly overexpressed in the bone marrow of myeloma patients compared with healthy donors. Myeloma cell lines as well as primary tumor cells from myeloma patients constitutively expressed IL-16 and its receptors CD4 and/or CD9 and spontaneously secreted soluble IL-16. Silencing of *IL-16* reduced the proliferative activity of myeloma cells by approximately 80% compared with untreated cells (mean relative proliferative activity IL-16 siRNA vs untransfected cells, EJM cells: 20.1%, 95% confidence interval [CI] = 14.3% to 26.0%, $P = .03$; KMS-12-BM cells: 22.8%, 95% CI = 5.5% to 40.0%, $P = .04$), and addition of a recombinant carboxyl-terminal IL-16 peptide reversed that effect. A monoclonal antibody directed against IL-16 or its receptors had a comparably strong growth-inhibiting effect on the tumor cells.
- Conclusions** IL-16 is an important growth-promoting factor in multiple myeloma and a candidate for novel diagnostic, prognostic, and therapeutic applications for this incurable human malignancy.

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Multiple myeloma, the second most common hematological malignancy, is a plasma cell neoplasia characterized by the expansion of a malignant plasma cell clone in the bone marrow with clinical consequences that include anemia, lytic bone lesions, hypercalcemia, renal dysfunction, hypogammaglobulinemia, and an increased risk of infection (1,2). Within the last decade, new therapeutic options for multiple myeloma have been developed. However, even after treatment with drugs such as bortezomib, thalidomide, or lenalidomide, most multiple myeloma patients eventually relapse and succumb to the disease within a median of only 5 years (3).

The interaction of myeloma cells with bone marrow stroma is important for their homing pattern, survival, and proliferation. Myeloma cells and stromal cells both produce a variety of cytokines and chemokines that promote the development, persistence, and

progression of the malignancy (4). It has previously been reported that serum levels of cytokine interleukin 16 (IL-16) are elevated in patients with myeloma compared with healthy subjects (5), particularly in patients with advanced stages of the disease (5–7). However, the exact origin of the elevated IL-16 and the biological role of IL-16 in multiple myeloma have remained unclear.

IL-16, also known as lymphocytic chemoattractant factor, does not appear to have any homology with other cytokines (8). It is produced by a variety of leukocyte subsets, including T cells (9,10), eosinophils (11), mast cells (12,13), monocytes (14), and dendritic cells (15). Moreover, B cells seem to constitutively express the precursor of IL-16—pro-IL-16—at least on the RNA level, and it has been suggested that IL-16 may mediate the cross-talk between B cells and T cells via its chemotactic properties within lymph node follicles (16,17).

IL-16 is synthesized in the form of a precursor protein, pro-IL-16, with an apparent molecular weight of 80 kDa (18). Upon cell activation, pro-IL-16 is cleaved by caspase-3 into a secreted carboxyl-terminal peptide with a molecular weight of 20 kDa and an amino-terminal 60-kDa prodomain (9,10,18–22). The cell surface molecule CD4 is known to be a receptor for IL-16 (8,23); however, another surface molecule, CD9, has also been proposed as an alternate IL-16 receptor (24). Carboxyl-terminal IL-16 acts through these receptors as a chemotactic factor for CD4-positive T cells (25), monocytes (23), immature dendritic cells (15), Langerhans cells (26), and eosinophils (27). In addition, IL-16 seems to have immediate immunoregulatory properties, that is, it suppresses effector T-cell function (23,28–34). Moreover, it has been shown that IL-16 preferentially induces the migration of immunosuppressive CD4- and CD25-positive T regulatory cells and might even facilitate de novo induction of fork head box P3 (*FOXP3*), the key regulatory gene for the development of T regulatory cells (35).

On the basis of these collected findings, we hypothesized that IL-16 might play an important role in the biology of B cell malignancies. We conducted a stringent investigation of the expression and secretion of IL-16 in a human B cell lymphoma, namely multiple myeloma. Moreover, we sought to identify the biological function of this cytokine in myeloma, and we delineated possible diagnostic, prognostic, and therapeutic applications for targeting IL-16 in multiple myeloma patients.

Materials and Methods

Cell Lines

For this study, human myeloma cell lines AMO-1, MOLP-8, RPMI-8226, KMS-12-BM, EJM, IM-9, U-266, NCI-H929, OPM-2, LP-1, and human embryonic kidney cell line HEK-293 were newly obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Two additional human myeloma cell lines—Brown and SK-007—were provided by the New York branch of the Ludwig Institute for Cancer Research. Upon arrival in our laboratory, the authenticity of the cell lines was verified using cytology and flow cytometry. All cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with penicillin–streptomycin (Invitrogen) and 10% fetal calf serum (Lonza, Basel, Switzerland). For evaluation of IL-16 concentrations in cell cultures, supernatants were removed 48 hours after the cells had been plated (unless otherwise indicated), and samples were frozen at -80°C until final analysis. For some experiments, granulocyte-macrophage colony-stimulating factor (GM-CSF; Antigenix, Hamilton Station, NY), interferon alpha ($\text{IFN-}\alpha$), B cell activation factor of the TNF family (BAFF; Biovision, Wehrheim, Germany), a proliferation-inducing ligand (APRIL; R&D Systems, Minneapolis, MN), tumor necrosis factor-alpha ($\text{TNF-}\alpha$), insulin-like growth factor (IGF; Miltenyi Biotec, Bergisch Gladbach, Germany), interleukin1 β (IL-1 β), interleukin 6 (IL-6), interleukin 10 (IL-10) (Randox Life Sciences, Crumlin, UK), and interleukin 17 (IL-17) (Genscript, Piscataway, NJ) were added at different concentrations (10, 50, 250 ng/mL) to the cells 48 hours after cells had been plated and their effects on IL-16 production and cell growth were evaluated 24 hours later.

Patients and Healthy Donors

We studied blood and bone marrow samples from 62 consecutive myeloma patients, one patient with monoclonal gammopathy of undetermined significance (MGUS), 12 healthy bone marrow donors, and six healthy blood donors. Tonsillar tissue was obtained from seven adult patients undergoing tonsillectomy for chronic tonsillitis. Bone marrow samples from multiple myeloma patients were obtained during routine diagnostic procedures. Whole bone marrow samples were obtained from healthy donors who provided bone marrow for allogeneic stem cell transplantation. Multiple myeloma patients had at least 10% bone marrow-infiltrating myeloma cells as defined by CD138 and CD38 coexpression and as confirmed by conventional cytological examination. Healthy subjects and patients, who were admitted for treatment at the University Medical Center Hamburg-Eppendorf, gave written informed consent in accordance with the revised version of the Declaration of Helsinki. The study protocol was approved by the local ethics committee (decision numbers OB-038/06 and WF-015/10).

Preparation of Tissue, Blood, and Bone Marrow Samples

Tonsillar tissue was manually chopped into small pieces and used to prepare a cell suspension by use of a Medimachine (BD Biosciences, San Jose, CA) and density gradient centrifugation. For preparation of plasma samples, whole bone marrow or peripheral blood was centrifuged at 680g and the supernatants (plasma) were frozen at -80°C . Mononuclear cells were isolated from blood and bone marrow samples by density gradient centrifugation, and in some cases CD8-positive T cells were enriched from whole peripheral blood mononuclear cells (PBMC) with the use of a magnetic microbead-based technique (Miltenyi Biotec, Bergisch Gladbach, Germany).

Human Cytokine Array and Enzyme-linked Immunosorbent Assay (ELISA)

The Human Cytokine Array (R&D Systems) allows simultaneous profiling of the relative levels of 36 cytokines and chemokines in a single sample by using nitrocellulose membranes, on which selected capture antibodies are spotted in duplicate. In brief, myeloma EJM and KMS-12-BM cells were plated at 2×10^6 cells per 2 mL complete medium per well in a 24-well plate. Supernatants were harvested 48 hours later and diluted with Standard Array Buffer (R&D Systems). A cocktail of biotinylated detection antibodies was added to the diluted samples, each sample was added to a nitrocellulose membrane bearing the capture antibodies, and the membranes were incubated overnight at 4°C . The membranes were washed, followed by incubation with streptavidin-conjugated horseradish peroxidase (HRP) for 30 minutes. Each membrane was incubated with a chemiluminescent detection reagent. The membranes were exposed to an X-ray film for 10 minutes and chemiluminescence was quantified by scanning the developed X-ray film on a transmission-mode scanner. The array was repeated three times for each cell line to guarantee reproducibility of the results.

Quantitative analysis of IL-16 concentrations in cell culture supernatants and plasma samples derived from bone marrow and peripheral blood was performed using a Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. Briefly, standards and samples were added to a microplate

precoated with a mouse monoclonal antibody specific for IL-16. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-16 was added to the wells. Following a wash to remove any unbound antibody or enzyme reagent, a substrate solution was added to the wells; color development was stopped using Stop Solution; and the resulting absorbance was read at 450nm with the use of a spectrophotometer (Tecan, Mannedorf, Switzerland). IL-16 concentrations were interpolated from a standard curve, which was generated using the recombinant IL-16 protein.

Conventional and Real-time Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

Total RNA was extracted from tonsillar tissue, all myeloma cell lines, bone marrow, PBMC, and CD8-positive T cells with the use of an RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed to complementary DNA (cDNA) with the use of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). RNA derived from a set of 20 different healthy human tissues was obtained from Ambion (Austin, TX). Conventional and quantitative PCR were performed as previously described (36). Primers for qualitative PCR amplification of IL-16 cDNA (Forward: 5'-ATGCCTGACCTCAACTCCACT-3'; Reverse: 5'-GCCACCCAGCTGCAAGATTTC-3') and the cDNA for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Forward: 5'-TGATGACATCAAGAAGGTGG-3'; Reverse: 5'-TTTCTTACTCCTTGGAGGCC-3') were obtained from MWG Biotech (Ebersberg, Germany). Primers for real-time PCR amplification of IL-16 (Cat. # QT00075138), FOS (Cat. # QT00007070), and JUN (Cat. # QT00242956) cDNAs were obtained from Qiagen. Results for real-time PCR experiments are given as the ratio of the number of copies of the target gene (*IL-16*) to the number of copies of *GAPDH*. All RT–PCR experiments were performed at least twice. To assess primer specificity, PCR products were analyzed repeatedly by DNA sequence analysis.

Real-time PCR Array

We used RT² Profiler PCR arrays (SABiosciences, Frederick, MD) to perform quantitative mRNA expression analyses for three human molecular signaling pathways: mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and the phosphatidylinositol 3-kinase (PI3K) (SABiosciences). We used these arrays to analyze the expression of 84 target genes per pathway in RNA samples extracted from myeloma RPMI-8226 cells 72 hours after transfection with an IL-16-specific small interfering RNA (siRNA) (see below) or a scrambled sequence control siRNA. Extraction of RNA and reverse transcription were performed as described above. PCR array analysis was performed according to the manufacturer's instructions with the use of an iCycler System (BioRad, Waltham, MA). Results were analyzed using the comparative C_T method ($\Delta\Delta C_T$), an approach to measure relative gene expression. Gene expression was considered different if the expression of the target gene after siRNA-mediated knockdown of IL-16 expression was at least threefold higher or lower compared with target gene expression in the control siRNA sample.

Immunoblot Analysis

Whole cell protein extracts were prepared from all myeloma cell lines, bone marrow mononuclear cells, and CD8-positive T cells enriched from whole PBMC in RIPA buffer containing a cocktail of protease inhibitors (Sigma, Steinheim, Germany). Cell culture supernatants, as well as bone marrow and peripheral blood plasma samples from myeloma patients and healthy donors, were used either undiluted or diluted 1:100 for immunoblot analysis. Protein concentrations were determined using the Bradford assay, and immunoblot analysis was performed as previously described (16) applying 30 µg of protein per lane. The primary antibodies were a mouse monoclonal antibody against human IL-16 that recognizes the carboxyl-terminal part of the mature protein (clone 70719; R&D Systems) used at a dilution of 1:1000 and a mouse anti-human monoclonal antibody against β-actin (Santa Cruz Biotechnology) used at a dilution of 1:3000. The secondary antibody was HRP-labeled anti-mouse monoclonal antibody (R&D Systems) used at a dilution of 1:3000. Equivalent protein loading of plasma samples was visualized using an HRP-labeled goat anti-human IgG heavy chain antibody (Sigma-Aldrich). For the detection of c-Fos and c-jun, appropriate rabbit anti-human monoclonal antibodies (Cell Signaling Technology, Danvers, MA) were used at a dilution of 1:1000. Specific antibody binding was visualized by chemiluminescence (Amersham Biosciences).

For measurement of caspase-3 activation by immunoblot analysis, we used a mouse monoclonal anti-human caspase-3 antibody (clone 3G2; Cell Signaling Technology). This antibody detects the full-length (35 kDa) protein as well as the large 17-kDa fragment of caspase-3 resulting from cleavage at aspartic acid 175.

Immunohistochemistry

Prior to staining with a primary murine anti-human IL-16 (clone 70719; R&D Systems) and the plasma cell-specific mouse anti-human CD138 (clone MI15; Dako, Denmark) antibodies, slides prepared from bone marrow biopsy samples were deparaffinized, followed by pretreatment in citrate buffer (Zymed, San Francisco, CA) with autoclaving at 121°C for 5 minutes (for IL-16 detection) or in Bond Epitope Retrieval Solution (Leica, Germany) for 30 minutes (for CD138 detection) using a BOND-MAX processing module (Menarini, Firenze, Italy). The slides were further pretreated with REALT Peroxidase-Blocking Solution (Dako), followed by incubation with goat serum diluted 1:5 in 50 mmol/L Tris–HCl to block nonspecific binding. The mouse monoclonal anti-human IL-16 antibody (clone 70719; R&D Systems) and the plasma cell-specific mouse monoclonal anti-human CD138 antibody (clone MI15; Dako) were applied to the slides at 1:100 and 1:25 dilutions, respectively. The slides were washed and incubated with a polyclonal goat HRP-conjugated anti-mouse antibody (Dako) for 30 minutes. The slides were washed, and antibody binding was detected with the use of the REALT EnVisionT Detection System (Dako) according to the manufacturer's instructions.

Colorimetric Caspase-3 Activity Assay

We used a Caspase-3/CPP32 Colorimetric Assay kit (BioVision, Mountain View, CA) to measure the activity of caspases that recognize the amino acid sequence DEVD. This assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA)

after cleavage from the labeled substrate DEVD-pNA. HEK-239, Amo-1, EJM, Im-9, KMS-12-BM, LP-1, Molp-8, OPM-2, RPMI-8226, and U226 cells (5×10^6 cells) were incubated for 16 hours in the absence or presence of 50 nM bortezomib (Janssen-Cilag, Neuss, Germany), followed by incubation with Cell Lysis Buffer on ice for 10 minutes. The lysates were centrifuged for 2 minutes at 10000g, and the resulting supernatants (cytosolic extracts) were transferred into a microtiter plate; Reaction Buffer and DEVD-pNA (final concentration 200 μ M) were added to each well. The plates were incubated for 90 minutes at 37°C, and the absorbance (optical density [OD]) at 405 nm was read with the use of a spectrophotometer. In parallel, we used the Bradford assay to determine the protein concentration of the cytosolic extracts. Background-subtracted fluorescence values were normalized by protein concentration (37) according to the following formula:

In Vitro Differentiation and Expansion of CD138-positive Plasma Cells

In vitro generation and expansion of plasma cells was performed as previously described (38). Briefly, bone marrow-derived CD19-positive B cells (6×10^6) were mixed with non-B cells isolated from autologous PBMC (5.7×10^6) in 2 mL of complete medium. These cells were seeded into one well of a six-well plate and incubated with a combination of CpG 2006 (6 μ g/mL, a 24-mer DNA oligonucleotide containing a phosphorothioated optimized human CpG motif; 5'-TCGTCGTTTGTGCGTTTGTGCGTT-3'; DNA Technology, Risskov, Denmark), human CD40 Ligand (CD40L, 500 ng/mL; GenWay Biotech, San Diego, CA), and interleukin 21 (IL-21, 50 ng/mL; PeproTech, Hamburg, Germany). The cells were cultured for 6 days at 37°C with 5% CO₂ until phenotypic analysis by flow cytometry.

IL-16 Gene Silencing by Transfection with siRNA

For the transient transfection of myeloma cells, anon targeting GFP-coupled siRNA, scrambled control siRNA, and three different siRNAs potentially targeting IL-16 were purchased from Invitrogen. Specific knockdown of IL-16 expression was achieved with only two of the three potentially IL-16-targeting siRNAs (HSS142654 and HSS142656). Myeloma EJM and KMS-12-BM cells were transfected using Lipofectamine 2000 (Invitrogen). For each condition, 3×10^5 cells were washed and resuspended in 100 μ L Optimem I medium (Gibco, Karlsruhe, Germany). SiRNA (50 pmol) with or without 1 μ L Fluorescent Control (Invitrogen) was added to the cells and incubated for 10 minutes at room temperature. Lipofectamine 2000 was gently mixed before being used and was diluted 1:40 in Optimem I medium without serum followed by incubation at room temperature for 10 minutes. The Lipofectamine 2000 dilution (100 μ L) was then added to the cells and incubated at room temperature for 20 minutes. Next, cell suspensions were transferred to a 24-well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated at 37°C for 4 hours. Afterwards, 1.5 mL of complete medium was added and cells were cultured at 37°C for another 72 hours. The cells were stained with

Dead Cell Stain (RNAi Basic Control Kit-Human; Invitrogen) and the transfection efficiency was evaluated at $\times 40$ magnification using bright-field microscopy. Images were obtained with the use of a digital camera (Canon, Krefeld, Germany) and Adobe Photoshop CS3 imaging software (Adobe Systems Inc., San Jose, CA). The transfection efficiency was generally 70%–80%, and cell death was less than 5% at 24 hours after transfection as determined by fluorescent microscopy.

Flow Cytometry

Cell surface staining of myeloma cell lines and patient-derived mononuclear cells was performed using mouse monoclonal antibodies against human CD4, CD9 (both from BD Biosciences), and CD138 (Beckmann Coulter, Krefeld, Germany). For the analysis of cytoplasmic IL-16 protein expression, myeloma cell lines or bone marrow mononuclear cells were fixed using FACS Lysing Solution, followed by permeabilization with Permeabilizing Solution (both from BD Biosciences). The cells were incubated with aphycoerythrin-conjugated mouse monoclonal anti-human IL-16 antibody (clone 14.1; BD Biosciences) or an appropriate isotype control antibody. Samples were analyzed by fluorescence-activated cell sorting using a FACSCalibur cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

We used two different flow cytometric assays to determine levels of apoptosis. The terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay was done according to the manufacturer's recommendations (Millipore, Billerica, MA). Briefly, myeloma EJM or KMS-12-BM cells (5×10^6 cells) were fixed for 1 hour in 1% paraformaldehyde followed by incubation in 70% ethanol for 18 hours at -20°C . The cells were washed and incubated in staining solution containing 8 μ L of Fluorescein-dUTP at 37°C for 1 hour, followed by incubation with a propidium iodide-RNase A solution for 30 minutes at room temperature. Analysis by flow cytometry was performed within the next 3 hours. To detect cells that are undergoing apoptosis, myeloma EJM or KMS-12-BM cells were incubated with Annexin V-FITC in Annexin Binding Buffer (both from BD Biosciences). Analysis by flow cytometry was performed within the next 3 hours.

Analysis of Cell Proliferation

Proliferation of myeloma cells was evaluated with or without knockdown of IL-16 expression. Following transfection of myeloma EJM and KMS-12-BM cells with IL-16-targeting siRNA or control siRNA, IL-16 knockdown was confirmed by immunoblotting for each of three independently performed experiments. The effect of IL-16 knockdown on the proliferation of myeloma cells was assessed with the use of a Biotrak cell proliferation ELISA assay (Amersham Biosciences), which is a colorimetric assay that quantitates cell proliferation based on the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) into the DNA of proliferating cells cultured in a multiwell plate. Myeloma cells were transferred to 96-well plates (1×10^5 cells/well; Greiner Bio-One) and cultured for 72 hours. The cells were pulsed with 10 μ M BrdU for the last 18 hours of culture. The cells were then fixed and incubated with an HRP-labeled anti-BrdU antibody, which binds to the BrdU incorporated into newly synthesized cellular DNA. Substrate was added and the

resulting absorbance was read at 450 nm using a microtiter plate spectrophotometer (Tecan). Relative proliferative activity in percent was calculated using the following formula: OD treated cells/OD untreated cells $\times$ 100. In addition, cell numbers were counted every 24 hours for the duration of the cell culture and used to construct growth curves.

In some experiments, we added blocking antibodies targeting IL-16 (clone 70719; R&D Systems), CD4 (clone 10C12; Abcam, Cambridge, MA), and/or CD9 (clone MEM61; Abcam) at a concentration of 1 μ g/mL 6 hours before pulsing the myeloma cells with BrdU. Rescue experiments were performed by adding recombinant 20-kDa IL-16 (R&D Systems) or a control protein (glutathione *S*-transferase) at a concentration of 5 μ g/mL to the cell cultures at the same time as the blocking antibodies.

Colony Formation Assay

Myeloma cell lines EJM and KMS-12-BM were plated at 1000 cells/mL of methylcellulose medium (StemCell Technologies, Cologne, Germany) and 1 mL/well in a 6-well culture dish (Nunc, Langensfeld, Germany). The plates were incubated at 37°C, and colonies consisting of more than 40 cells were counted at 10 days after culture initiation.

Statistical Analysis

Statistical analyses were performed using SPSS statistical software (version 17; SPSS, Inc., Chicago, IL). The Mann–Whitney *U* test was used to determine the statistical significance of differences between experimental conditions. All *P* values are two-sided, and *P* values less than .05 were considered statistically significant.

Results

IL-16 Expression in Myeloma Cell Lines

To search for new diagnostic and therapeutic targets for myeloma, we used an antibody array to simultaneously screen culture supernatants of myeloma cell lines EJM and KMS-12-BM for the expression of 36 different cytokines and chemokines. We detected substantial concentrations of a number of soluble factors, such as Regulated on Activation, Normal T Expressed and Secreted (RANTES), macrophage migration inhibitory factor (MIF), soluble intercellular adhesion molecule-1 (sICAM-1), stromal cell-derived factor-1 (SDF-1), macrophage inflammatory protein 1 alpha (MIP-1 α), serpin-E1, IL-13, interferon-inducible protein 10 (IP-10), and IL-17 (representative results for myeloma line EJM in Supplementary Figure 1, available online). Most of these cytokines

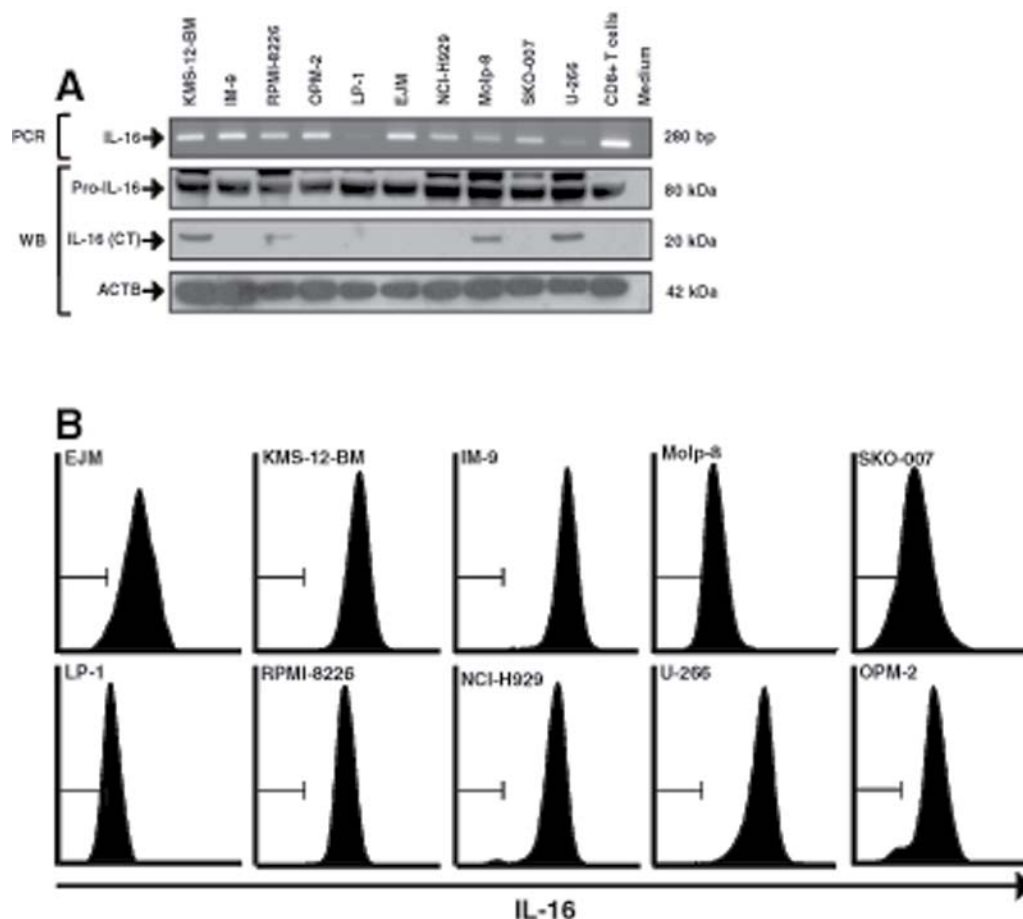


Figure 1. Interleukin 16 (IL-16) expression in myeloma cell lines. **A)** Expression of IL-16 in myeloma cell lysates. Expression of IL-16 was examined in 10 myeloma cell lines by conventional reverse transcription–polymerase chain reaction (PCR; upper row) and by immunoblot analysis (WB; lower rows). Unstimulated CD8-positive T cells were used as positive controls and housekeeping gene beta actin (ACTB) served as a loading control. CT = carboxyl-terminal. **B)** Single-cell analysis of IL-16 expression in myeloma cell lines. Myeloma cell lines were analyzed for IL-16 expression on a single-cell level applying cytoplasmic staining followed by flow cytometry. **Black lines** indicate staining intensity with an irrelevant isotype control; histograms show staining with anti-IL-16 antibody.

and chemokines have previously been shown to be produced by myeloma cells. Although cytokine IL-16 has never been assigned a clear role in multiple myeloma, we found that this cytokine was also produced at comparably high levels by myeloma cell lines EJM and KMS-12-BM (Supplementary Figure 1, available online, and data not shown).

We next examined IL-16 expression in 10 different multiple myeloma cell lines. Conventional RT-PCR revealed that IL-16 RNA was present in varying amounts in all myeloma cells tested (Figure 1, A). Immunoblot analysis revealed that all 10 cell lines strongly expressed the 80-kDa precursor peptide, pro-IL-16. Moreover, in four of the 10 myeloma cell lines, we also observed constitutive expression of the “mature” and bioactive 20-kDa carboxyl-terminal portion of IL-16 (Figure 1, A). By contrast, the 20-kDa fragment was not expressed in resting (ie, CD8-positive) T cells (Figure 1, A) and has previously been described as being undetectable in lysates of most cell types that express IL-16 (14,15,39), an observation that has usually been attributed to the quick and complete release of mature IL-16 from the cytoplasm into the culture supernatant.

To confirm constitutive expression of IL-16 protein in myeloma cell lines at a single-cell level, we performed intracellular staining with a different IL-16 monoclonal antibody followed by flow cytometry. We observed that all 10 multiple myeloma cell lines expressed intracellular IL-16 protein (Figure 1, B), which was consistent with the RT-PCR and immunoblot findings. Furthermore, we found that six of the 10 cell lines showed surface expression of the IL-16 receptor CD9 and that myeloma line Molp-8 also expressed CD4, the second known receptor for IL-16 (data not shown).

IL-16 Expression in Primary Tumor Cells From Myeloma Patients

To gain further insight into which tissues express IL-16, we used real-time PCR to analyze a large variety of human organs for the presence of IL-16 RNA. Previous reports (9,18) have indicated that expression of IL-16 RNA is primarily restricted to normal lymphoid tissues. We found that most of the tissues we examined showed only low levels of IL-16 RNA and that only lymphatic organs such as thymus, spleen, and tonsils had substantial expression of this cytokine (Figure 2, A). As expected, we observed comparably high expression of IL-16 in PBMC from healthy donors.

Human bone marrow is another type of lymphatic tissue and is the body compartment where most of the tumor load in multiple myeloma patients resides. We first asked whether IL-16 expressed in the bone marrow of multiple myeloma patients was primarily produced by the malignant cells or by the bone marrow stroma by arbitrarily grouping the myeloma patients into those with less than 30% bone marrow-infiltrating plasma cells ($N = 18$) versus those with 30% or more bone marrow-infiltrating plasma cells ($N = 18$). We observed that patients with less than 30% bone marrow-infiltrating plasma cells had higher levels of IL-16 RNA in their bone marrow compared with eight healthy donors, but the difference was not statistically significant (mean normalized copy number: 1.59 vs 0.96, $P = .15$) (Figure 2, B). However, multiple myeloma patients with higher levels of bone marrow-infiltrating plasma cells had statistically significantly higher IL-16 RNA expression compared with healthy bone marrow donors (mean

normalized copy number: 2.78 vs 0.96, $P = .01$). This finding suggested that the local malignant plasma cells may be primarily responsible for the elevated expression of this cytokine within the bone marrow compartment of these patients.

Immunohistochemical analysis of IL-16 expression in bone marrow biopsy samples from randomly selected patients with multiple myeloma or MGUS and from healthy subjects provided further support for this idea: We observed overexpression of IL-16 in myeloma cells compared with other bone marrow cells. Expression of IL-16 protein was associated with the presence of plasma cells, which were detected by staining with the plasma cell-specific anti-CD138 antibody, and accordingly the number of bone marrow-infiltrating IL-16-positive cells increased in the following order: healthy subject < MGUS patient < multiple myeloma patient (Figure 2, C). We also noticed some expression of IL-16 in other types of fully differentiated leukocytes in MGUS patients and healthy donors. Importantly, however, IL-16 was always absent from hematopoietic progenitors.

We next examined whether the expression of IL-16 protein was dependent on the developmental stage of a given B cell subtype. Although it has been suggested (16) that low levels of IL-16 protein are constitutively present in the cytoplasm of normal CD19-positive (nonplasma cell) B cells, we found that normal resting B cells were usually negative for intracellular IL-16 protein when analyzed by flow cytometry (Figure 3, A). This finding suggested that IL-16 might specifically become overexpressed in B cells following differentiation into CD138-positive plasma cells. By applying an optimized method for the in vitro generation and expansion of plasma cells (38), we found that the cytoplasmic expression of IL-16 in CD19-positive B cells increased after stimulation with CD40L, CpG 2006 oligonucleotide, and IL-21 (Figure 3, A). However, IL-16 expression was sometimes even stronger in the in vitro expanded CD138-positive plasma cells.

We next examined IL-16 protein expression in bone marrow-derived CD138-positive plasma cells from six myeloma patients and three healthy donors. Flow cytometry revealed that CD138-positive plasma cells from the healthy donors and the myeloma patients expressed IL-16 (Figure 3, B and C). However, it seemed that the patient-derived plasma cells expressed higher levels of IL-16 compared with plasma cells from the healthy donors (Figure 3, C). Indeed, the mean fluorescence intensity of staining for IL-16 in CD138-positive plasma cells (in arbitrary units) was statistically significantly higher in the patients compared with the donors (37.0 vs 7.9, $P = .004$).

To further confirm constitutive overexpression of IL-16 by myeloma cells in the human bone marrow, we used flow cytometry to analyze the cytoplasmic expression of IL-16 in bone marrow mononuclear cells in 25 myeloma patients with at least 10% bone marrow-infiltrating myeloma cells and in one patient with MGUS. Remarkably, myeloma cells of all patients showed strong and homogenous expression of IL-16 independent of disease stage or treatment status (data not shown). Importantly, whereas primary myeloma cells were usually negative for the IL-16 receptor CD4, flow cytometry showed cell surface expression of the IL-16 receptor CD9 in the majority (74%) of these 26 patient samples (Figure 3, D).

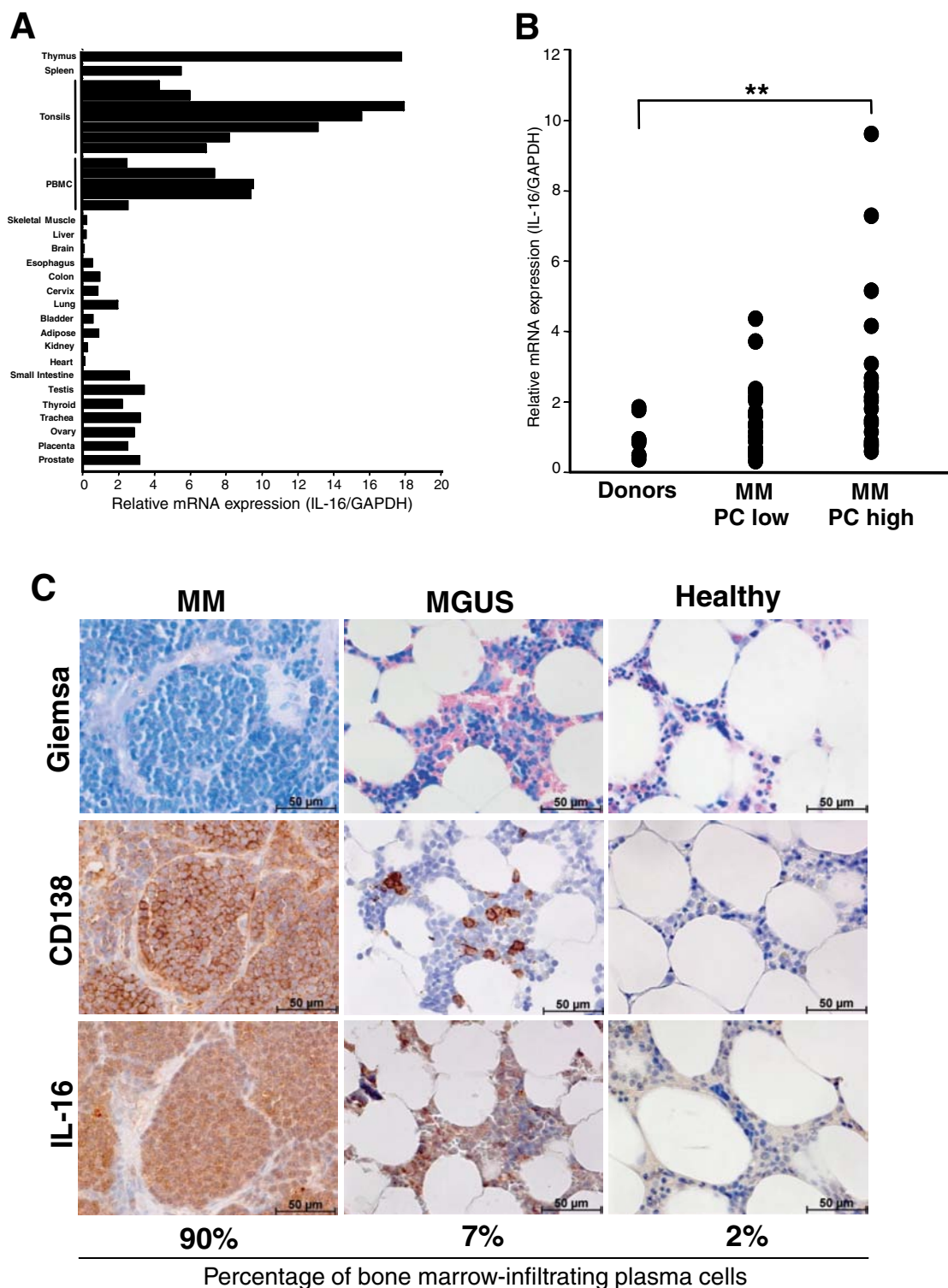


Figure 2. Interleukin 16 (IL-16) expression in the bone marrow of myeloma patients. **A)** IL-16 expression in normal human tissues. Expression of IL-16 RNA was evaluated in a wide variety of human tissues by quantitative polymerase chain reaction (PCR); results are shown as the number of copies of IL-16 mRNA in relation to the number of copies of mRNA for the housekeeping gene GAPDH. **B)** Expression of IL-16 RNA in the bone marrow of multiple myeloma (MM) patients. Bone marrow samples of myeloma patients with <30% (N = 18) or ≥30% (N = 18) bone marrow-infiltrating plasma cells (PC) and bone marrow samples of healthy donors (N = 18) were analyzed for IL-16 expression by real-time PCR. ***P* = .01 (two-tailed Mann-Whitney U test). **C)**

Immunohistochemical analysis of IL-16 protein expression in myeloma. Selected bone marrow biopsy samples of monoclonal gammopathy of undetermined significance (MGUS; N = 4), myeloma patients (MM; N = 4), and four healthy control subjects were analyzed by immunohistochemistry (magnification ×400). Consecutive cuts are shown from the block of one representative subject per category. In addition to a routine Giemsa stain (**upper row**), staining was performed using a plasma cell-specific antibody raised against CD138 (**middle row**) and an anti-IL-16 antibody (**lower row**). The percentage of bone marrow-infiltrating plasma cells is given for each sample. PBMC = peripheral blood mononuclear cells.

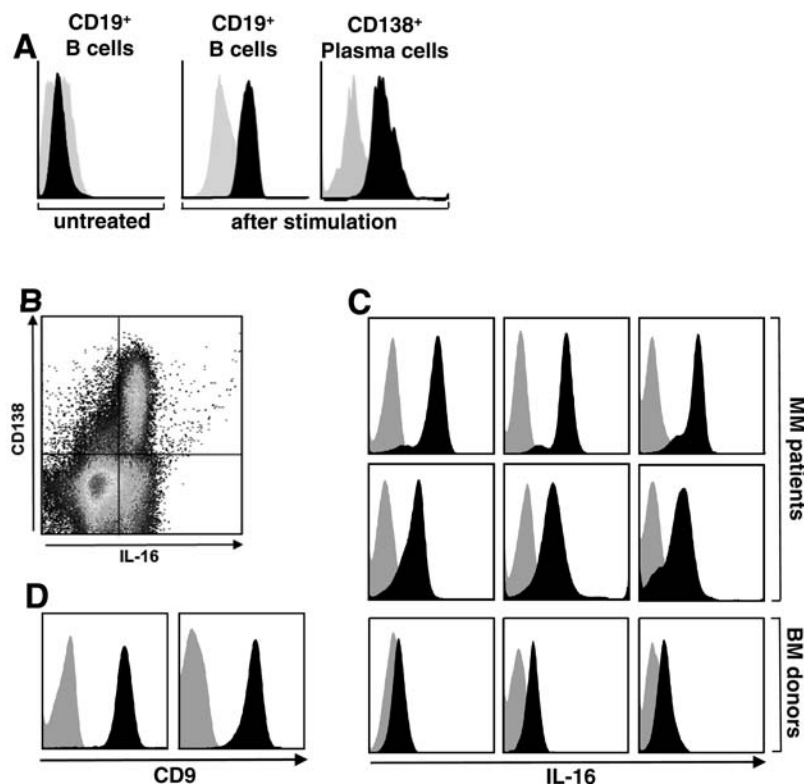


Figure 3. Flow cytometric analysis of interleukin 16 (IL-16) protein expression in in vitro differentiated plasma cells and patient-derived myeloma cells. **A)** Expression of IL-16 in in vitro differentiated plasma cells. Cytoplasmic expression of IL-16 was analyzed by flow cytometry in untreated CD19-positive B cells, and in CD19-positive B cells and fully differentiated CD138-positive plasma cells, both stimulated with a mixture of CD40L, CpG, and IL-21. **B)** Flow cytometric analysis of mononuclear cells derived from the bone marrow of a myeloma patient following staining for plasma cell surface marker CD138 and intracellular protein IL-16. The density plot gives the number of CD138-positive plasma (upper right quadrant) and nonplasma (lower right quadrant) cells expressing intracellular IL-16

protein. The two quadrants on the left side show IL-16-negative plasma and nonplasma cells. **C)** Flow cytometric analysis of IL-16 expression in patient-derived myeloma cells. Bone marrow-residing plasma cells of 21 myeloma patients, one MGUS patient, and three healthy bone marrow donors were analyzed by flow cytometry for intracellular expression of IL-16. Histograms show results of six representative multiple myeloma (MM) patients and three healthy bone marrow (BM) donors after gating on CD138-positive bone marrow plasma cells. **Gray areas** indicate staining with an irrelevant isotype control; **black areas** indicate staining with anti-IL-16 antibody. **D)** Flow cytometry analysis of IL-16 receptor CD9 expression on malignant CD138-positive plasma cells from two myeloma patients.

Secretion of Soluble IL-16 by Myeloma Cell Lines and Primary Myeloma Cells in the Patients' Bone Marrow

We next asked whether malignant cells spontaneously secrete IL-16 protein. We subjected the culture supernatants from myeloma cell lines at 48 hours after culture initiation to a quantitative ELISA and found that all 10 cell lines examined secreted soluble IL-16, albeit at varying degrees (Figure 4, A). We examined two myeloma cell lines that showed strong expression of IL-16 in a more detailed manner and observed that IL-16 concentrations in the culture supernatants of KMS-12-BM and EJM cells increased continuously up to 96 hours after culture initiation (Figure 4, B), proving that myeloma cells spontaneously secrete high levels of soluble IL-16.

We next subjected supernatants of myeloma cell lines to immunoblot analysis to determine which form of IL-16 was constitutively produced. Most of the cell lines (6 of 10) secreted the 80-kDa pro-IL-16 peptide into the culture medium. However, we also detected substantial amounts of the bioactive 20-kDa carboxyl-terminal fragment of IL-16 in the culture supernatants of the myeloma cell lines (Figure 4, C). The latter finding seems remarkable given that detectable amounts of the carboxyl-terminal IL-16 have only rarely been demonstrated in supernatants of IL-16-secreting cells (9). It

has been suggested that, even when its bioactivity is detectable by functional assays, mature IL-16 often remains undetectable by less sensitive assays, such as immunoblots, due to its limited production under most physiological conditions (39).

Production and secretion of bioactive carboxyl-terminal IL-16 usually requires cell activation followed by caspase-3-mediated cleavage of the pro-IL-16 precursor protein (9,10,18–22). Therefore, we next examined whether myeloma cells possess a sufficient constitutive caspase-3 activity to enable the continuous generation of soluble IL-16. To this end, we used a quantitative assay that measured the absorbance of a specific substrate cleaved by the caspase-3 enzyme in cell lysates from myeloma cell lines. Of the nine myeloma cell lines tested, eight showed spontaneous caspase-3 activity (Figure 4, D). Treatment of the cells with the apoptosis-inducing antimyeloma agent bortezomib further increased the activity of caspase-3 (Figure 4, D). Immunoblot analysis of AMO-1 and EJM cells with and without bortezomib treatment revealed that bortezomib-mediated activation of caspase-3 led to the simultaneous decrease in the level of pro-IL-16 and increase in the level of the bioactive carboxyl-terminal fragment of IL-16 in whole cell lysates and an increase in the level of soluble IL-16 in cell culture supernatants (Figure 4, E). These findings suggest that

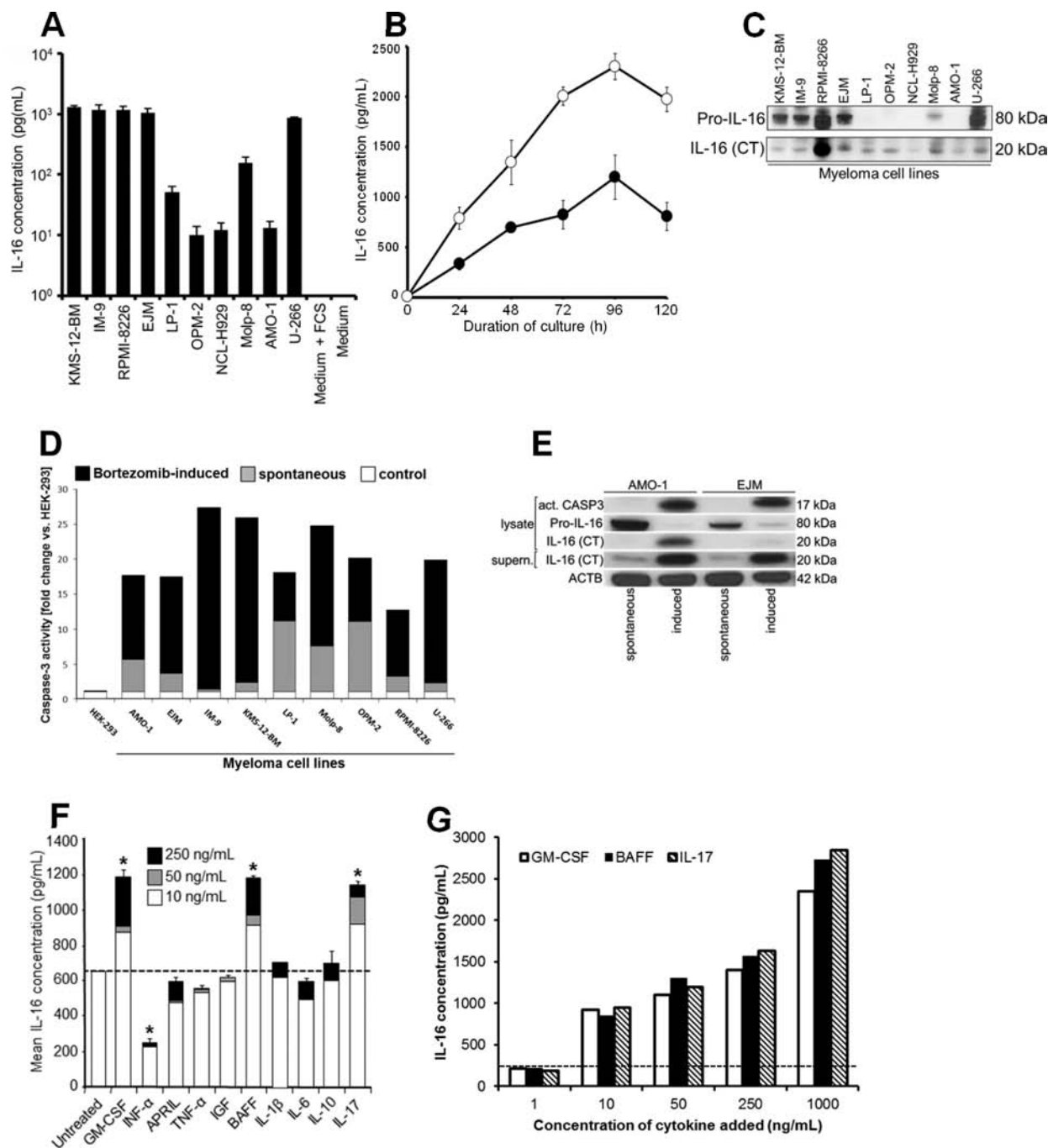


Figure 4. Analysis of interleukin 16 (IL-16) secretion by myeloma cell lines. Cell culture supernatants were analyzed for soluble IL-16 by an enzyme-linked immunosorbent assay (ELISA). **A**) Concentration of soluble IL-16 in the supernatants of myeloma cell lines. Bars represent the mean value for three replicate samples. **B**) Longitudinal analysis of IL-16 secretion by myeloma cell lines. Data are shown for one of two separate experiments. Each ELISA assay was performed in duplicates and mean values are indicated. **C**) Immunoblot analysis of IL-16 secretion by myeloma cell lines. Supernatants of 10 myeloma lines were analyzed by immunoblotting for the expression of the 80kDa pro-IL-16 and the smaller 20kDa C-terminal (CT) IL-16. **D**) Caspase-3 activity in myeloma cell lines. A colorimetric caspase-3 activity assay was used to analyze untreated (spontaneous) and bortezomib-treated myeloma cell lines (N = 9) and control cell line HEK-293. Bars represent a single measurement taken 16 hours after treatment with bortezomib and indicate the fold increase in caspase activity compared with HEK-293. **E**) Effect of caspase-3 activation on pro-IL-16 cleavage in myeloma cells. Whole cell lysates and culture supernatants of myeloma cell lines AMO-1 and EJM with or without treatment with anti-myeloma agent bortezomib were subjected to immunoblot analysis with antibodies

specific for activated caspase 3, pro-IL-16, and the bioactive carboxyl-terminal (CT) portion of IL-16. Housekeeping protein beta-actin (ACTB) was assessed as a control for equal protein loading. **F**) Effect of different myeloma-associated cytokines on the production of IL-16 protein by myeloma cell lines. Ten different cytokines were separately added to cultures of myeloma cell line EJM, and resulting IL-16 concentrations were evaluated 24 hours later. Data are shown for one of two separate experiments. Each ELISA assay was performed in triplicates; bar graphs indicate mean values for both experiments, and colors of stacked bars indicate concentrations of the respective cytokines added (white: 10 ng/mL; gray: 50 ng/mL; black: 250 ng/mL). The dotted line indicates IL-16 concentration in the supernatant of untreated cells, and asterisks mark statistically significant differences (all $P = .03$) compared with untreated cells as indicated by a two-tailed Mann-Whitney U test. **G**) Effects of GM-CSF, BAFF, and IL-17 on the production of soluble IL-16 by myeloma cell line EJM. Cells were treated with the respective cytokines for 24 hours before the read-out assay was performed. Data represent a single measurement. Dotted line represents concentrations of IL-16 in the supernatant of untreated myeloma cells. All error bars correspond to 95% confidence intervals. FCS = fetal calf serum.

caspase-3 is responsible for the generation of the carboxyl-terminal IL-16 peptide in myeloma cells and that a constant basal activity of caspase-3 in these cells guarantees a permanent supply of the bioactive form of the IL-16 protein.

We next examined whether any of the cytokines known to be involved in the pathophysiology of multiple myeloma influence the production of IL-16 by myeloma EJM cells. We added 10 different soluble factors separately to cultures of EJM cells and evaluated resulting IL-16 concentrations in culture supernatants 24 hours later by ELISA. Cytokines were added to the cultures at three different concentrations (10, 50, and 250 ng/mL) to cover the range of concentrations at which such soluble factors typically exert their effects on myeloma cells. We found that three cytokines—GM-CSF, BAFF, and IL-17—increased the amount of secreted IL-16 compared with untreated cells at the three tested concentrations (Figure 4, F). By contrast, INF- α led to diminished secretion of IL-16 protein by EJM cells. To examine whether these effects simply reflected differences in cell numbers for each culture condition rather than actual changes in the production of IL-16 per cell, we determined the effect of each cytokine on the numbers of cells within the respective cultures. We found that GM-CSF, BAFF, and IL-17 were the only cytokines that stimulated myeloma cell growth, whereas INF- α inhibited cell growth (data not shown). Therefore, we conclude that the effects of GM-CSF, BAFF, IL-17, and INF- α on observed levels of secreted IL-16 are best explained by the growth-modulating effects of these cytokines on myeloma cells.

It was previously shown that GM-CSF (40–42), BAFF (43–46), and IL-17 (47,48) typically exert their effects on myeloma and plasma cells in particular at a concentration range of 10–1000 ng/mL. We examined whether lower (ie, more physiological) concentrations of these three cytokines would also have an effect on the amount of IL-16 secreted by myeloma cells and/or the cell number present in

the culture. As expected, when applied at a concentration less than 10 ng/mL, these cytokines had no such effect (Figure 4, G).

Next, by using an ELISA assay, we asked if we could detect soluble IL-16 protein in bone marrow or peripheral blood plasma samples from 10 myeloma patients and 10 healthy donors. The myeloma patients had somewhat higher concentrations of IL-16 in their peripheral blood compared with healthy donors, but the difference was not statistically significant (mean IL-16 concentration = 384.8 vs 23.3 pg/mL, $P = .33$) (Figure 5, A). By contrast, mean IL-16 concentrations in bone marrow were statistically significantly higher in the myeloma patients compared with healthy donors (13376.2 vs 1469.5 pg/mL, $P = .003$). Moreover, among the myeloma patients, the absolute concentration of IL-16 was approximately 40 times higher in bone marrow compared with peripheral blood from the same subjects (mean bone marrow: peripheral blood ratio = 42.5, 95% CI = -0.5 to 89.8) (Figure 5, A).

We next conducted immunoblot analyses of bone marrow plasma samples from four myeloma patients to examine which forms of IL-16 were present. Both the 20-kDa mature version of IL-16 and pro-IL-16 were detectable in the bone marrow of the patients (Figure 5, B); also present was a 40-kDa carboxyl-terminal form of IL-16, which has not previously been described (Figure 5, B). It has been suggested that alternative IL-16 cleavage sites exist (9); however, the physiological relevance of the alternative cleavage fragments for the function of IL-16 is not yet known. Importantly, bone marrow plasma from healthy donors showed no evidence of any form of IL-16 protein by immunoblot analysis (Figure 5, B).

Effect of IL-16 on Myeloma Cell Proliferation

Because IL-16 was present in all of the multiple myeloma cell lines that were available to us, we could not investigate the functional consequences of its overexpression in IL-16-negative lines. Therefore, we performed knockdown experiments using

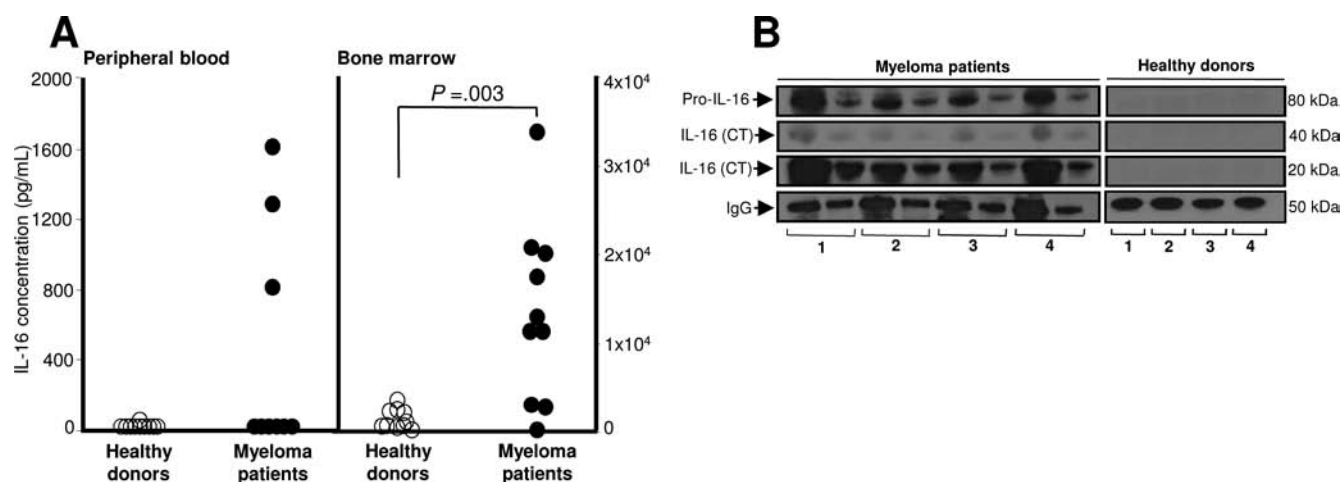


Figure 5. Production of soluble interleukin 16 (IL-16) in the bone marrow environment of myeloma patients. **A)** IL-16 concentrations in the peripheral blood and bone marrow. Absolute concentrations of IL-16 in the peripheral blood (left box) and in the bone marrow (right box) were compared between myeloma patients (black dots; $N = 10$) and healthy donors (open circles; $N = 10$) in an enzyme-linked immunosorbent assay. The P value indicates a statistically significant difference in IL-16 concentration in bone marrow between patients and healthy donors (two-tailed Mann–Whitney U test). **B)** Immunoblot analysis of IL-16

expression in the bone marrow plasma. Bone marrow plasma samples of four myeloma patients and four healthy donors were analyzed for the presence of the 80-kDa pro-IL-16, the 20-kDa carboxyl-terminal (CT) IL-16, and a 40-kDa IL-16 variant by immunoblotting. An anti-IgG heavy chain antibody was used to confirm equal protein loading. In the case of the myeloma patients (who have IL-16 in their plasma), the samples were analyzed undiluted and diluted 1:100. In the case of the healthy control subjects (who do not have IL-16 in their plasma), the samples were only analyzed undiluted.

IL-16-specific siRNA. Myeloma EJM and KMS-12-BM cells were transiently transfected with IL-16-targeting siRNA or scrambled sequence control siRNA and the effects on proliferation and apoptosis were evaluated. We observed that two of the three siRNAs we had obtained for these experiments resulted in decreased expression of both pro-IL-16 and the mature 20-kDa fragment of IL-16 (Figure 6, A). Knockdown efficiency peaked at 96 hours after transfection and lasted for approximately 2 days thereafter. For all subsequent experiments, one of the two IL-16-targeting siRNAs (HSS142656) and scrambled sequence control siRNA were used.

Next, we examined the effect of siRNA knockdown of IL-16 on the secretion of soluble IL-16 protein into the culture supernatant of EJM and KMS-12-BM cells. Following the typical increase in IL-16 concentrations during the first days after culture initiation, IL-16 was no longer detectable by ELISA in the supernatant of both cell lines starting on day 5 after transfection with IL-16-specific siRNA but not after transfection with the control siRNA (Figure 6, B). IL-16 remained undetectable by ELISA in the cell culture medium for approximately 2 days and then became detectable again at 7 days after transfection with IL-16-specific siRNA.

The fact that IL-16 levels recovered 7 days after transfection with IL-16 siRNA argues against an immediate cytotoxic or pro-apoptotic effect on the myeloma cells. However, we noticed a substantial growth delay in cultures of both myeloma cell lines after transfection with IL-16-specific siRNA (Figure 6, C). To examine whether this growth delay reflected a pro-apoptotic effect of IL-16 silencing, we subjected EJM and KMS-12-BM cells after IL-16 knockdown to a TUNEL assay and to annexin V staining followed by flow cytometry. Importantly, in neither apoptosis assay did we observe any effect of siRNA knockdown of IL-16 on the number of apoptotic cells (Figure 6, D).

We next examined whether siRNA-mediated knockdown of IL-16 expression in EJM and KMS-12-BM cells, both of which express the IL-16 receptor CD9, had an anti-proliferative effect. We observed that transfection of cells with IL-16 siRNA markedly reduced proliferation of EJM and KMS-12-BM cells compared with untransfected cells (mean relative proliferative activity IL-16 siRNA vs untransfected cells; EJM cells: 20.1%, 95% CI = 14.3% to 26.0%, $P = .03$; KMS-12-BM cells: 22.8%, 95% CI = 5.5% to 40.0%, $P = .04$), whereas transfection of cells with scrambled control siRNA did not (mean relative proliferative activity control siRNA vs untransfected cells; EJM cells: 89.4%, 95% CI = 76.9% to 102.0%, $P = .12$; KMS-12-BM cells: 87.3%, 95% CI = 85.9% to 88.8%, $P = .1$) (Figure 6, E).

We next examined the effect of adding exogenous IL-16 to myeloma cell cultures. We found that recombinant IL-16 protein had no effect on the proliferation of myeloma EJM and KMS-12-BM cells (Supplementary Figure 2, available online), probably because these tumor cells constitutively secrete soluble IL-16. Indeed, exogenous IL-16 seemed to inhibit the proliferation of myeloma cells only when added at high (ie, nonphysiological) concentrations (Supplementary Figure 2, available online). However, we found that the anti-proliferative effect of IL-16 knockdown was, at least in part, abrogated by the addition of the recombinant 20-kDa carboxyl-terminal fragment of IL-16 protein, but not by addition of control protein, to the cell culture (Figure 6, E). These

findings suggested for the first time that IL-16, in particular its carboxyl-terminal fragment, might be an important growth factor for myeloma cells.

It has long been proposed (49) that in the case of multiple myeloma, a small number of precursors with the ability to undergo cell division generates and replenishes a large number of nondividing myeloma cells. Clonogenic precursors, which are also present among myeloma cell lines, are thought to be resistant to chemotherapy and responsible for relapses commonly seen in this malignancy (50). We used a colony formation assay to examine whether silencing of IL-16 production would not only reduce the proliferation of end-stage myeloma cells but also inhibit the outgrowth of colony-forming myeloma precursors. Importantly, in myeloma EJM and KMS-12-BM cells, we observed a substantial reduction in the number of cell colonies in a standard assay measuring clonogenic growth of myeloma precursors after siRNA-mediated silencing of IL-16 expression compared with untreated controls or myeloma cells transfected with control siRNA (Figure 6, F). These results suggest that IL-16 not only promotes the survival of common myeloma cells but may also exert a growth-enhancing effect on precursor cells that replenish the bulk of myeloma cells.

Finally, we performed several preliminary studies to analyze the effects of IL-16 silencing on the expression of three signaling pathways (MAPK, NF κ B, and PI3K) central to the biology of myeloma cells. To this end, we used pathway-focused, real-time PCR-based arrays to compare the relative RNA expression of 84 genes per pathway in myeloma RPMI-8226 cells transfected with IL-16-specific siRNA with that of cells transfected with a scrambled sequence control siRNA. We observed a marked increase in expression of FOS and JUN, two genes that were represented in all three arrays, after IL-16 knockdown (Supplementary Figure 3, A and B, available online). Increased RNA expression of JUN and FOS in RPMI-8226 cells following IL-16 knockdown was confirmed by real-time PCR with different primers than the ones used for the PCR-based array (Supplementary Figure 3, C, available online). We observed increased expression of JUN protein after siRNA-mediated knockdown of IL-16 in RPMI-8226 cells (Supplementary Figure 3, D, available online); however, we have not been able to reliably detect FOS protein by immunoblotting (data not shown).

Effect of an IL-16 Monoclonal Antibody on Myeloma Cell Growth

Based on our findings of a growth-promoting effect of IL-16 on myeloma cells (Figure 6, E), we next investigated whether the application of a monoclonal antibody directed against IL-16, in particular the carboxyl-terminal part, would have an inhibitory effect on myeloma cell proliferation comparable to the one we observed with siRNA-induced knockdown of IL-16 expression (Figure 6, E). We found that the addition of increasing concentrations of anti-IL-16 antibody to cultures of myeloma KMS-12-BM and EJM cells resulted in declining cell growth compared with untreated cells (Figure 6, G). By contrast, addition of the highest concentration of an isotype control antibody (10 μ g/mL) to the myeloma cells had no effect on their proliferation. This finding suggests that treatment with an anti-IL-16 antibody may be a promising therapeutic option for patients with multiple myeloma.

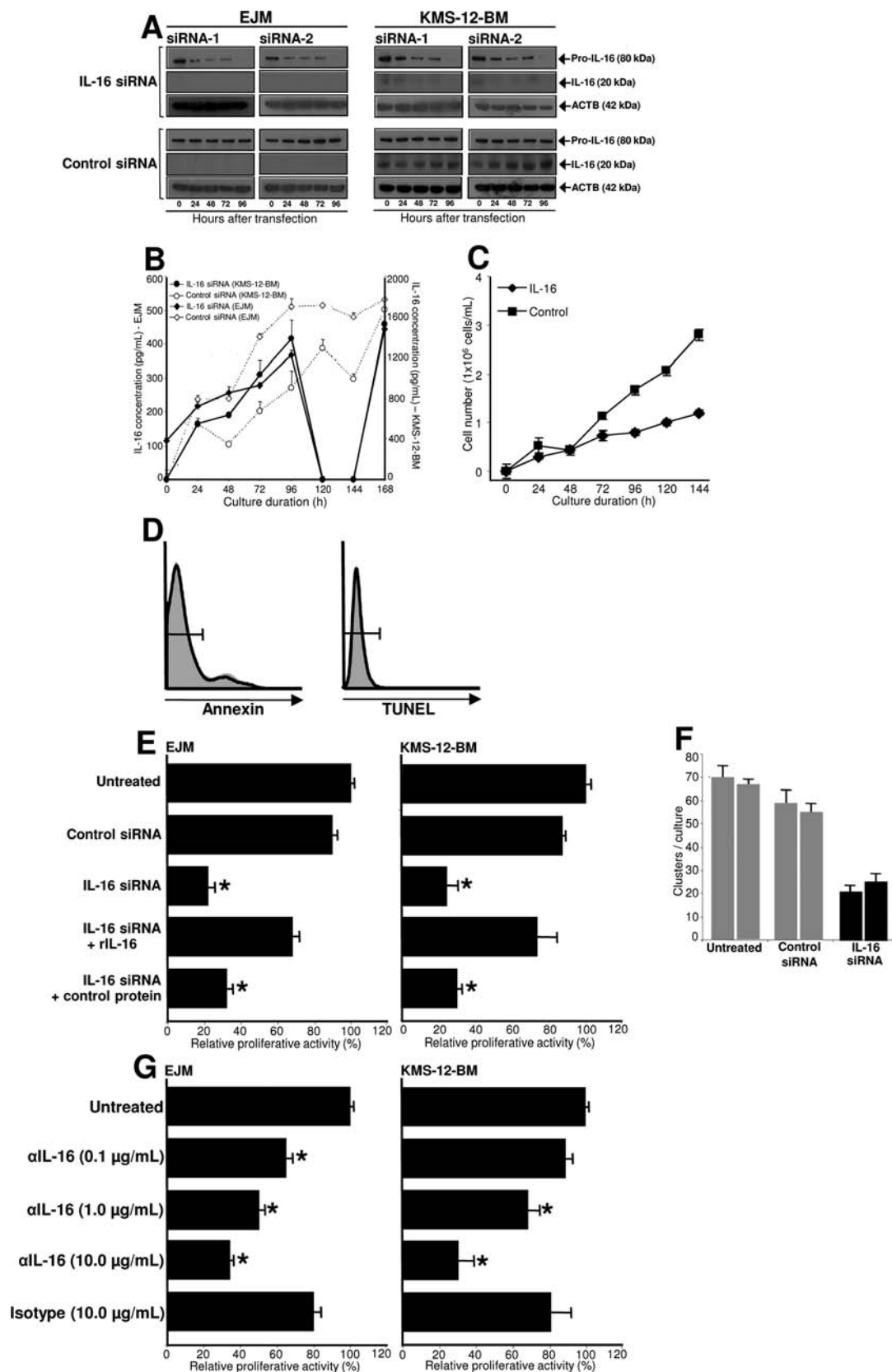


Figure 6. Functional consequences of IL-16 expression in myeloma cell lines. **A**) Immunoblot analysis of IL-16 expression in small interfering RNA (siRNA)-transfected myeloma cells. Myeloma cell lines EJM (left panels) and KMS-12-BM (right panels) were transfected with one of two siRNAs specific for IL-16 (siRNA-1 or siRNA-2) or with a scrambled-sequence control siRNA. IL-16 protein expression was analyzed

by immunoblotting with two IL-16-specific antibodies at the indicated times after transfection; an anti-beta actin antibody (ACTB) was used to confirm equal protein loading. **B**) Effect of IL-16 knockdown on the production of soluble IL-16 by myeloma cell lines. The effect of IL-16 RNA knockdown on the secretion of soluble IL-16 protein by myeloma cell lines EJM and KMS-12-BM was examined by an enzyme-linked

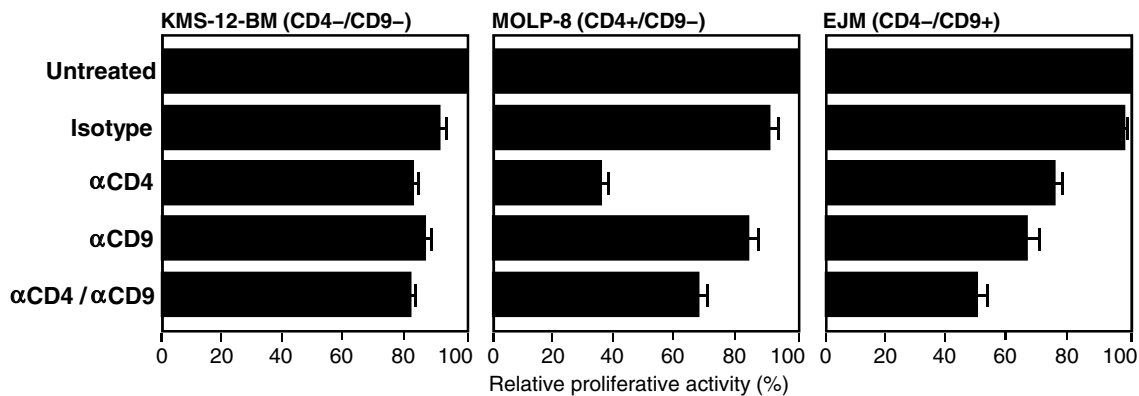


Figure 7. Effect of blocking interleukin 16 (IL-16) receptors CD4 and CD9 on myeloma cell proliferation. Myeloma cell lines KMS-12-BM (CD4 and CD9 negative), MOLP-8 (CD4 positive and CD9 negative), and EJM (CD4 negative and CD9 positive) were treated with blocking antibodies targeting CD4 and/or CD9 or an isotype control antibody (each at 1 µg/mL) 6 hours before the cells were pulsed with 5'-bromo-2'-deoxyuridine in an enzyme-linked immunosorbent assay of cell proliferation. **Bars** show mean values of proliferative activity, as a percentage, relative to untreated cells for three independent experiments; error bars represent 95% confidence intervals.

Role of Cd4 and Cd9 in the Growth-Promoting Effects of IL-16 in Myeloma Cells

The cell surface protein CD4 has long been suggested to be the most relevant receptor for IL-16 (8,23). More recently, the cell surface protein CD9 has been proposed as an alternate IL-16 receptor (24). We therefore investigated the possible involvement of CD4 and CD9 in mediating the growth-promoting effects of IL-16 by treating myeloma cells that expressed CD4 only (MOLP-8 cells), CD9 only (EJM cells), or neither CD4 nor CD9 (KMS-12-BM cells) with antibodies against CD4 and/or CD9. Treatment of myeloma KMS-12-BM cells with an antibody against either CD4 or CD9 had no effect on cell proliferation compared with untreated cells (Figure 7). By contrast, treatment of MOLP-8 cells with an anti-CD4 antibody, alone or in combination with an anti-CD9 antibody, inhibited proliferation compared with untreated cells, whereas treatment with an anti-CD9 antibody alone had no effect. Likewise, treatment of EJM cells with an anti-CD9 antibody alone or in combination with an anti-CD4 antibody inhibited

proliferation compared with untreated cells (Figure 7). These preliminary data suggest that the positive effect of IL-16 on the proliferation of myeloma cells might, at least in part, be mediated by IL-16 receptors CD4 and CD9. However, the fact that myeloma KMS-12-BM cells, which do not express CD4 or CD9, responded to siRNA-mediated knockdown of IL-16 and rescue by exogenous IL-16 (Figure 6, E) suggests that alternative IL-16 receptors might be responsible for the effect of this cytokine on myeloma cell proliferation.

Discussion

We have shown here that myeloma cells constitutively express IL-16 and spontaneously secrete the soluble form of this cytokine. We have demonstrated for the first time that IL-16 supports the proliferation of myeloma cells, possibly through involvement of transcription factors FOS and JUN, and that targeting IL-16 has a strong growth-inhibiting effect on these tumor cells.

Figure 6 (Continued).

immunosorbent assay (ELISA). Effects of transfection with a scrambled-sequence control siRNA are shown. Note the different scales on the left x-axis (EJM) and on the right axis (KMS-12-BM). Data are shown for one of two separate experiments. Each ELISA assay was performed in duplicate and mean values are indicated. **C)** Effect of IL-16 knockdown on cell number. Numbers of cells in culture were counted at different times after transfection of cell line EJM with IL-16-specific siRNA or scrambled-sequence control siRNA. Data are shown for one of three separate experiments. Each assay was performed in duplicate and mean values are indicated. **D)** Effect of IL-16 knockdown on the number of apoptotic cells in myeloma cell cultures. KMS-12-BM cells transfected with IL-16-specific or control siRNA were stained with Annexin V-FITC (**left histogram**) or subjected to the terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay (**right histogram**), followed by flow cytometry. **Black peaks** indicate KMS-12-BM cells transfected with IL-16-specific siRNA; **gray areas** represent KMS-12-BM cells transfected with control siRNA. Results of staining with appropriate isotype controls are indicated by the horizontal bars. **E)** Effect of IL-16 knockdown on myeloma cell proliferation. The proliferative rate of myeloma cell lines EJM and KMS-12-BM was assessed 72 hours after transfection with IL-16-specific or control siRNA in an ELISA-based proliferation assay that measured incorporation of 5'-bromo-2'-deoxyuridine. Rescue experiments were

performed by adding recombinant carboxyl-terminal IL-16 protein (rIL-16) or a control protein (glutathione S-transferase) to the cell culture at a final concentration of 5 µg/mL at 72 hours after siRNA transfection. **Bars** show mean values of proliferative activity, as a percentage, relative to untreated cells for three independent experiments and the asterisks indicate statistically significant differences compared with untreated cells ($P = .03$ for each of the four conditions). **F)** Colony formation assay after IL-16 knockdown. The effect of IL-16 knockdown on clonogenic myeloma precursor cells was assessed 72 hours after transfection with IL-16-specific or control siRNA in a standard assay measuring clonogenic growth. Colonies consisting of more than 40 cells were counted at 10 days after culture initiation. **Bars** show mean colony numbers for three independent experiments with EJM (left bar in each pair) and KMS-12-BM (right bar in each pair) cells. **G)** Effect of an anti-IL-16 antibody on myeloma cell proliferation. Cultures of myeloma KMS-12-BM and EJM cells were incubated for 6 hours with increasing amounts of a monoclonal antibody against IL-16 and subjected to an ELISA-based proliferation assay that measured incorporation of 5'-bromo-2'-deoxyuridine. **Bars** show mean values for three independent experiments and the asterisks indicate statistically significant differences compared with untreated cells ($P = .03$ for each of the five conditions). Error bars represent 95% confidence intervals. All P values are two-sided (Mann-Whitney U tests).

The expression and function of IL-16 in human malignancies has not received broad attention. Richmond et al. (51) have recently shown production of IL-16 by malignant T cells from patients with Sezary syndrome, a common form of cutaneous T cell lymphoma, and another study (52) demonstrated IL-16 expression by B cell lines derived from human lymphomas. Both of these studies support our observation of substantial IL-16 expression in a hematological malignancy, such as multiple myeloma. Furthermore, another study (53) revealed that IL-16 expression is increased in human gliomas, indicating that this cytokine might not only be overexpressed by hematological malignancies but also by solid tumors. It is interesting that two large studies (54,55) have recently demonstrated that distinct single-nucleotide polymorphisms of the *IL16* gene are associated with the susceptibility to colorectal, gastric, and prostate cancers, suggesting that IL-16 may be involved in cancer development and/or progression.

We have demonstrated that myeloma cells not only secrete IL-16 in vitro but also in the immediate microenvironment of the tumor, namely, the bone marrow. It has previously been reported that serum levels of IL-16 are increased in patients with solid tumors, particularly in patients with advanced stages of the disease (54,56). It was also shown that IL-16 concentrations were higher in pleural effusions caused by lung cancer than in pleural effusions caused by nonmalignant conditions (57). Furthermore, it has been reported that IL-16 levels might be elevated in the peripheral blood of multiple myeloma patients compared with healthy control subjects, and that IL-16 concentrations might increase with progression of the disease (5,6). In this study, we found that peripheral blood serum levels of IL-16 were only moderately elevated in myeloma patients compared with healthy blood donors, whereas the concentration of this cytokine was markedly increased in the bone marrow of the same patients when compared with the bone marrow of healthy control subjects. Moreover, local cytokine levels were positively associated the number of IL-16-expressing plasma cells in the patient's bone marrow. These findings suggest that: 1) myeloma cells, and not stromal cells, are responsible for the increased IL-16 production in myeloma; 2) myeloma cells or other cell types might use an IL-16 concentration gradient to specifically migrate from the periphery into the bone marrow; and 3) IL-16 might, in general, play an important role in creating a favorable local environment for human malignancies, such as multiple myeloma.

To our knowledge, nothing is known about a possible function of IL-16 in human cancers, and among nonmalignant cell types, B cells have received relatively little attention with regard to a possible biological function of this cytokine. Although IL-16 is thought to facilitate the development of B cells in T cell-deficient mice (58), an immediate effect on differentiated human and nonhuman plasma cells has not been observed. Here we described a direct effect of IL-16 on the proliferation of malignant human plasma cells that may be related to the activity of transcription factors FOS and JUN, two proteins that are known to interact with each other and are involved in the transformation and progression of cancer as well as in cellular proliferation (60,61). Specifically, we found that the proliferative activity of myeloma cells was markedly reduced after treatment with an IL-16-specific inhibitory RNA or an anti-IL-16 monoclonal antibody.

Hitherto, the biological function of IL-16 has been thought to consist of two main aspects, that is, its immunomodulatory effects on T cells and its activity as a chemoattractant for various leukocytes. This restricted view of the function of IL-16 was probably based on the fact that, initially, the biological activity of IL-16 was exclusively ascribed to the smaller carboxyl-terminal IL-16 peptide, which is secreted after cleavage (8,18). However, it was later shown in a monkey kidney (COS) cell expression system that after cleavage of pro-IL-16, the amino-terminal 60-kDa prodomain of pro-IL-16 translocates into the nucleus, where it induces a G₀/G₁ arrest in the cell cycle (62). Comparable findings have been obtained in human T cells (63). In this study, we found that the 20-kDa carboxyl-terminal IL-16 peptide at least partly reversed the inhibitory effects of anti-IL-16 siRNA on myeloma cell proliferation. Furthermore, we observed that treatment of myeloma cells with an antibody directed against the carboxyl-terminal fragment of IL-16 reduced their proliferation. These findings, as well as the fact that we also detected the 20-kDa IL-16 peptide in myeloma cell culture supernatants and in bone marrow plasma from myeloma patients, argue that the carboxyl-terminal protein has an important role in promoting the proliferation of multiple myeloma. However, we also found that exogenous IL-16 was not able to completely rescue the effect of IL-16 knockdown on myeloma cell proliferation. This finding may indicate that other versions of IL-16, perhaps in addition to the small carboxyl-terminal fragment that we have used for our rescue experiments, are involved in supporting the proliferation of myeloma cells. Experiments are currently underway in our laboratory to delineate the exact physiological role(s) of the different IL-16 fragments.

Myeloma precursors have been shown to be resistant to standard chemotherapeutic agents (49) and, as a consequence, most myeloma patients eventually relapse and succumb to the disease even after they have been treated with strategies that incorporate new drugs such as bortezomib, thalidomide, or lenalidomide (64). In this study, anti-IL-16 reagents not only inhibited the proliferation of end-stage myeloma cells but also suppressed the outgrowth of their clonogenic precursors. This finding suggests that IL-16 might represent a promising target for novel myeloma therapies that inhibit the bulk of end-stage myeloma cells as well as their dormant progenitors.

The glycoprotein CD4 is the first cell surface molecule to be described as a functional receptor for IL-16 (8,23,27,31,65–67). However, reports that PBMC (68) and Langerhans cells (26) isolated from CD4 knockout mice are as responsive to IL-16 as the corresponding cells from wild-type mice suggest that at least one alternate receptor for this cytokine must exist. More recent evidence suggested that human mast cells use the tetraspanin family protein CD9, rather than CD4, as an IL-16 receptor (24). It is interesting that CD9 is a cell surface molecule associated with the early stages of B cell differentiation and that polyclonal stimulation of myeloma cells results in increased cell surface expression of CD9 (69). Increased expression of CD9 has been found on myeloma cells that are in close physical contact with bone marrow endothelial cells, and it has been suggested that CD9 might be involved in the homing of myeloma cells to bone marrow (70). However, CD9 expression seems to disappear in more advanced stages of the disease, and a complete loss of CD9 surface from the patients' myeloma cells seems to result in a reduced survival (71). Our preliminary data suggest that, in addition to a possible chemotactic

effect of IL-16 on myeloma cells, the soluble form of IL-16 might stimulate myeloma cell proliferation through CD4 and CD9. We believe that both of these receptors should be further evaluated as possible therapeutic targets for multiple myeloma.

This study has a few limitations. First, we have not yet deciphered the exact contribution of the different IL-16 fragments to the control of myeloma cell proliferation, and we need to further delineate the molecular mechanisms mediating its effects. Second, we have so far only analyzed the function of IL-16 in multiple myeloma in in vitro experiments, and we need to prove its value as a therapeutic target in a reliable myeloma animal model. We believe that, in addition to its role as a proliferation-inducing factor, IL-16 may contribute in additional ways to the creation of the typical myeloma environment, that is, by regulating migration of myeloma cells and/or regulatory immune cells or by directly suppressing immunity. We plan to analyze these IL-16-related disease-promoting networks in future studies.

References

- Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med*. 2004;351(18):1860–1873.
- Dispenzieri A, Lacy MQ, Greipp PR. Multiple Myeloma. In: Greer JP, Foerster J, Lukens JN, Rodgers GM, Paraskevas F, Glader B, ed. *Wintrobe's Clinical Hematology*. 11th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004:2583–2636.
- Palumbo A, Facon T, Sonneveld P, et al. Thalidomide for treatment of multiple myeloma: 10 years later. *Blood*. 2008;111(8):3968–3977.
- Aggarwal R, Ghobrial IM, Roodman GD. Chemokines in multiple myeloma. *Exp Hematol*. 2006;34(10):1289–1295.
- Alexandrakis MG, Passam FH, Kyriakou DS, et al. Serum level of interleukin-16 in multiple myeloma patients and its relationship to disease activity. *Am J Hematol*. 2004;75(2):101–106.
- Koike M, Sekigawa I, Okada M, et al. Relationship between CD4(+)/CD8(+) T cell ratio and T cell activation in multiple myeloma: reference to IL-16. *Leuk Res*. 2002;26(8):705–711.
- Bellomo G, Allegra A, Alonci A, et al. Serum levels of interleukin-16 in lymphoid malignancies. *Leuk Lymphoma*. 2007;48(6):1225–1227.
- Cruikshank WW, Center DM, Nisar N, et al. Molecular and functional analysis of a lymphocyte chemoattractant factor: association of biologic function with CD4 expression. *Proc Natl Acad Sci U S A*. 1994;91(11):5109–5113.
- Chupp GL, Wright EA, Wu D, et al. Tissue and T cell distribution of precursor and mature IL-16. *J Immunol*. 1998;161(6):3114–3119.
- Wu DM, Zhang Y, Parada NA, et al. Processing and release of IL-16 from CD4+ but not CD8+ T cells is activation dependent. *J Immunol*. 1999;162(3):1287–1293.
- Lim KG, Wan HC, Bozza PT, et al. Human eosinophils elaborate the lymphocyte chemoattractants. IL-16 (lymphocyte chemoattractant factor) and RANTES. *J Immunol*. 1996;156(7):2566–2570.
- Lorentz A, Schwengberg S, Sellge G, et al. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *J Immunol*. 2000;164(1):43–48.
- Rumsaeng V, Cruikshank WW, Foster B, et al. Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16. *J Immunol*. 1997;159(6):2904–2910.
- Ellsner A, Doseff AI, Duncan M, et al. IL-16 is constitutively present in peripheral blood monocytes and spontaneously released during apoptosis. *J Immunol*. 2004;172(12):7721–7725.
- Kaser A, Dunzendorfer S, Offner FA, et al. A role for IL-16 in the cross-talk between dendritic cells and T cells. *J Immunol*. 1999;163(6):3232–3238.
- Kaser A, Dunzendorfer S, Offner FA, et al. B lymphocyte-derived IL-16 attracts dendritic cells and Th cells. *J Immunol*. 2000;165(5):2474–2480.
- Kramer MF, Mack B, Rasp G. Immunohistological expression of interleukin 16 in human tonsils. *Arch Otolaryngol Head Neck Surg*. 2001;127(9):1120–1125.
- Baier M, Bannert N, Werner A, et al. Molecular cloning, sequence, expression, and processing of the interleukin 16 precursor. *Proc Natl Acad Sci U S A*. 1997;94(10):5273–5277.
- Sciaky D, Brazier W, Center DM, et al. Cultured human fibroblasts express constitutive IL-16 mRNA: cytokine induction of active IL-16 protein synthesis through a caspase-3-dependent mechanism. *J Immunol*. 2000;164(7):3806–3814.
- Ludwiczek O, Kaser A, Koch RO, et al. Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death. *Eur Cytokine Netw*. 2001;12(3):478–486.
- Zhang Y, Center DM, Wu DM, et al. Processing and activation of pro-interleukin-16 by caspase-3. *J Biol Chem*. 1998;273(2):1144–1149.
- Gianoukakis AG, Martino LJ, Horst N, et al. Cytokine-induced lymphocyte chemoattraction from cultured human thyrocytes: evidence for interleukin-16 and regulated upon activation, normal T cell expressed, and secreted expression. *Endocrinology*. 2003;144(7):2856–2864.
- Cruikshank WW, Berman JS, Theodore AC, et al. Lymphokine activation of T4+ T lymphocytes and monocytes. *J Immunol*. 1987;138(11):3817–3823.
- Qi JC, Wang J, Mandadi S, et al. Human and mouse mast cells use the tetraspanin CD9 as an alternate interleukin-16 receptor. *Blood*. 2006;107(1):135–142.
- Laberge S, Ghaffar O, Boguniewicz M, et al. Association of increased CD4+ T-cell infiltration with increased IL-16 gene expression in atopic dermatitis. *J Allergy Clin Immunol*. 1998;102(4 pt 1):645–650.
- Stoitzner P, Ratzinger G, Koch F, et al. Interleukin-16 supports the migration of Langerhans cells, partly in a CD4-independent way. *J Invest Dermatol*. 2001;116(5):641–649.
- Rand TH, Cruikshank WW, Center DM, et al. CD4-mediated stimulation of human eosinophils: lymphocyte chemoattractant factor and other CD4-binding ligands elicit eosinophil migration. *J Exp Med*. 1991;173(6):1521–1528.
- Matsumoto Y, Fujita T, Hirai I, et al. Immunosuppressive effect on T cell activation by interleukin-16- and interleukin-10-cDNA-double-transfected human squamous cell line. *Burns*. 2009;35(3):383–389.
- Klimiuk PA, Goronzy JJ, Weyand CM. IL-16 as an anti-inflammatory cytokine in rheumatoid synovitis. *J Immunol*. 1999;162(7):4293–4299.
- Newell MK, Haughn LJ, Maroun CR, et al. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. *Nature*. 1990;347(6290):286–289.
- Theodore AC, Center DM, Nicoll J, et al. CD4 ligand IL-16 inhibits the mixed lymphocyte reaction. *J Immunol*. 1996;157(5):1958–1964.
- Cruikshank WW, Lim K, Theodore AC, et al. IL-16 inhibition of CD3-dependent lymphocyte activation and proliferation. *J Immunol*. 1996;157(12):5240–5248.
- Ogasawara H, Takeda-Hirokawa N, Sekigawa I, et al. Inhibitory effect of interleukin-16 on interleukin-2 production by CD4+ T cells. *Immunology*. 1999;96(2):215–219.
- Parada NA, Center DM, Kornfeld H, et al. Synergistic activation of CD4+ T cells by IL-16 and IL-2. *J Immunol*. 1998;160(5):2115–2120.
- McFadden C, Morgan R, Rahangdale S, et al. Preferential migration of T regulatory cells induced by IL-16. *J Immunol*. 2007;179(10):6439–6445.
- Atanackovic D, Luetkens T, Hildebrandt Y, et al. Longitudinal analysis and prognostic effect of cancer-testis antigen expression in multiple myeloma. *Clin Cancer Res*. 2009;15(4):1343–1352.
- Berens HM, Tyler KL. The proapoptotic Bcl-2 protein Bax plays an important role in the pathogenesis of reovirus encephalitis. *J Virol*. 2011;85(8):3858–3871.
- Cao Y, Gordic M, Kobold S, et al. An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body. *J Immunol Methods*. 2010;358(1–2):56–65.
- Yoshimoto T, Wang CR, Yoneto T, et al. Role of IL-16 in delayed-type hypersensitivity reaction. *Blood*. 2000;95(9):2869–2874.
- Zhang XG, Bataille R, Jourdan M, et al. Granulocyte-macrophage colony-stimulating factor synergizes with interleukin-6 in supporting the proliferation of human myeloma cells. *Blood*. 1990;76(12):2599–2605.
- Till KJ, Burthem J, Lopez A, et al. Granulocyte-macrophage colony-stimulating factor receptor: stage-specific expression and function on late B cells. *Blood*. 1996;88(2):479–486.

42. Villunger A, Egle A, Kos M, et al. Functional granulocyte/macrophage colony stimulating factor receptor is constitutively expressed on neoplastic plasma cells and mediates tumour cell longevity. *Br J Haematol*. 1998;102(4):1069–1080.
43. Avery DT, Kalled SL, Ellyard JJ, et al. BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J Clin Invest*. 2003;112(2):286–297.
44. Moreaux J, Legouffe E, Jourdan E, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood*. 2004;103(8):3148–3157.
45. Tai YT, Li XF, Breitkreutz I, et al. Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. *Cancer Res*. 2006;66(13):6675–6682.
46. Schneider P, MacKay F, Steiner V, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*. 1999;189(11):1747–1756.
47. Prabhala RH, Pelluru D, Fulciniti M, et al. Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. *Blood*. 2010;115(26):5385–5392.
48. Xie S, Li J, Wang JH, et al. IL-17 activates the canonical NF-kappaB signaling pathway in autoimmune B cells of BXD2 mice to upregulate the expression of regulators of G-protein signaling 16. *J Immunol*. 2010;184(5):2289–2296.
49. Matsui W, Wang Q, Barber JP, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res*. 2008;68(1):190–197.
50. Matsui W, Huff CA, Wang Q, et al. Characterization of clonogenic multiple myeloma cells. *Blood*. 2004;103(6):2332–2336.
51. Richmond J, Tuzova M, Parks A, et al. Interleukin-16 as a marker of Sezary syndrome onset and stage. *J Clin Immunol*. 2011;31(1):39–50.
52. Sharma V, Sparks JL, Vail JD. Human B-cell lines constitutively express and secrete interleukin-16. *Immunology*. 2000;99(2):266–271.
53. Liebrich M, Guo LH, Schluesener HJ, et al. Expression of interleukin-16 by tumor-associated macrophages/activated microglia in high-grade astrocytic brain tumors. *Arch Immunol Ther Exp (Warsz)*. 2007;55(1):41–47.
54. Gao LB, Rao L, Wang YY, et al. The association of interleukin-16 polymorphisms with IL-16 serum levels and risk of colorectal and gastric cancer. *Carcinogenesis*. 2009;30(2):295–299.
55. Thomas G, Jacobs KB, Yeager M, et al. Multiple loci identified in a genome-wide association study of prostate cancer. *Nat Genet*. 2008;40(3):310–315.
56. Kovacs E. The serum levels of IL-12 and IL-16 in cancer patients. Relation to the tumour stage and previous therapy. *Biomed Pharmacother*. 2001;55(2):111–116.
57. Qin XJ, Shi HZ, Huang ZX, et al. Interleukin-16 in tuberculous and malignant pleural effusions. *Eur Respir J*. 2005;25(4):605–611.
58. Szabo P, Zhao K, Kirman I, et al. Maturation of B cell precursors is impaired in thymic-deprived nude and old mice. *J Immunol*. 1998;161(5):2248–2253.
59. Trudelle A, El Bassam S, Pinsonneault S, et al. Interleukin-16 inhibits immunoglobulin e production by B lymphocytes. *Int Arch Allergy Immunol*. 2007;143(2):109–118.
60. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer*. 2003;3(11):859–868.
61. Vesely PW, Staber PB, Hoefler G, et al. Translational regulation mechanisms of AP-1 proteins. *Mutat Res*. 2009;682(1):7–12.
62. Zhang Y, Kornfeld H, Cruikshank WW, et al. Nuclear translocation of the N-terminal prodomain of interleukin-16. *J Biol Chem*. 2001;276(2):1299–1303.
63. Wilson KC, Cattel DJ, Wan Z, et al. Regulation of nuclear Prointerleukin-16 and p27(Kip1) in primary human T lymphocytes. *Cell Immunol*. 2005;237(1):17–27.
64. Huff CA, Matsui W. Multiple myeloma cancer stem cells. *J Clin Oncol*. 2008;26(17):2895–2900.
65. Ryan TC, Cruikshank WW, Kornfeld H, et al. The CD4-associated tyrosine kinase p56lck is required for lymphocyte chemotactant factor-induced T lymphocyte migration. *J Biol Chem*. 1995;270(29):17081–17086.
66. Bandeira-Melo C, Sugiyama K, Woods LJ, et al. IL-16 promotes leukotriene C(4) and IL-4 release from human eosinophils via CD4- and autocrine CCR3-chemokine-mediated signaling. *J Immunol*. 2002;168(9):4756–4763.
67. Cruikshank WW, Greenstein JL, Theodore AC, et al. Lymphocyte chemotactant factor induces CD4-dependent intracytoplasmic signaling in lymphocytes. *J Immunol*. 1991;146(9):2928–2934.
68. Mathy NL, Bannert N, Norley SG, et al. Cutting edge: CD4 is not required for the functional activity of IL-16. *J Immunol*. 2000;164(9):4429–4432.
69. Shallal S, Kornbluth J. CD9 expression enhances the susceptibility of myeloma cell lines to cell-mediated cytotoxicity. *Blood*. 2000;96(1):224–233.
70. De Bruyne E, Andersen TL, De Raevé H, et al. Endothelial cell-driven regulation of CD9 or motility-related protein-1 expression in multiple myeloma cells within the murine 5T33MM model and myeloma patients. *Leukemia*. 2006;20(10):1870–1879.
71. De Bruyne E, Bos TJ, Asosingh K, et al. Epigenetic silencing of the tetraspanin CD9 during disease progression in multiple myeloma cells and correlation with survival. *Clin Cancer Res*. 2008;14(10):2918–2926.

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