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The Cytokine Midkine and Its Receptor RPTPζ Regulate B Cell Survival in a Pathway Induced by CD74

Sivan Cohen,* Or-yam Shoshana,* Einat Zelman-Toister,* Nitsan Maharshak,* Inbal Binsky-Ehrenreich,* Maya Gordin,* Inbal Hazan-Haleyv,† Yair Herishanu,† Lev Shvidel,‡ Michal Haran,‡ Lin Leng,§ Richard Bucala,§ Sheila Harroch,§ and Idit Shachar*

Lasting B cell persistence depends on survival signals that are transduced by cell surface receptors. In this study, we describe a novel biological mechanism essential for survival and homeostasis of normal peripheral mature B cells and chronic lymphocytic leukemia cells, regulated by the heparin-binding cytokine, midkine (MK), and its proteoglycan receptor, the receptor-type tyrosine phosphatase ζ (RPTPζ). We demonstrate that MK initiates a signaling cascade leading to B cell survival by binding to RPTPζ. In mice lacking PTPRZ, the proportion and number of the mature B cell population are reduced. Our results emphasize a unique and critical function for MK signaling in the previously described MIF/CD74-induced survival pathway. Stimulation of CD74 with MIF leads to c-Met activation, resulting in elevation of MK expression in both normal mouse splenic B and chronic lymphocytic leukemia cells. Our results indicate that MK and RPTPζ are important regulators of the B cell repertoire. These findings could pave the way toward understanding the mechanisms shaping B cell survival and suggest novel therapeutic strategies based on the blockade of the MK/RPTPζ-dependent survival pathway. The Journal of Immunology, 2012, 188: 259–269.

Adaptive immunity depends on the production and maintenance of a pool of mature peripheral lymphocytes throughout life. In normal individuals, the pool of peripheral lymphocytes is constant in size. The control of lymphoid homeostasis is the result of a very fine balance between lymphocyte production, survival, and proliferation. Survival factors have been shown to play a critical role in maintaining lymphocyte homeostasis.

For many years, the maintenance of peripheral B cell homeostasis was thought to rely only on two key elements: BCR tonic signals (e.g., Igα and Syk) and BAFF (BLyS/TALL-1/THANK).

Abbreviations used in this article: CLL, chronic lymphocytic leukemia; HGF, hepatocyte growth factor; HPRT, hypoxanthine ribosyltransferase; MIF, macrophage migration inhibitory factor; MK, midkine; PI, propidium iodide; qRT-PCR, quantitative real-time RT-PCR; RPTPζ, receptor-type tyrosine phosphatase ζ.

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progression (19). Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of small, mature CD5+ B lymphocytes in the peripheral blood, lymphoid organs, and bone marrow. In CLL cells, binding of MIF to CD74 initiates a similar signaling cascade that induces NF-κB activation and upregulation of TAp63 expression, resulting in the secretion of IL-8, which in turn promotes cell survival (20, 21). Thus, CD74 expressed on the surface of CLL cells plays a critical role in regulating the survival of these cells.

To determine the identities of the molecules whose expression is modulated by CD74, thereby regulating B cell survival, we investigated CD74 target genes. One striking example that we identify in this study, whose expression is strongly regulated by CD74, is the cytokine midkine (MK).

MK is a heparin-binding cytokine whose activities include antiapoptotic, mitogenic, transforming, angiogenic, and chemotactic functions (22). MK is a basic, cysteine-rich polypeptide with a molecular mass of 13 kDa (23). It is composed of two domains, the N- and C-terminal domains. The domain corresponding to the C-terminal half domain of vertebrate MK is evolutionarily conserved and is important in binding heparin and chondroitin sulfate proteoglycans (24–26). MK tends to form dimers via spontaneous association, and the dimers are stabilized by cross-linking with transglutaminase. This dimerization appears to be required for MK activity (27).

The expression of MK is significantly upregulated in various malignant tumors and plays crucial roles in carcinogenesis. Its expression has been shown to be strongly correlated with poor prognosis in patients with neuroblastomas, astrocytomas, pancreatic head carcinomas, or gastrointestinal stromal tumors. In accordance with its high expression in various malignant tumors, MK exerts cancer-related activities in the process of carcinogenesis, including transformation, fibrolysis, cell migration, cell survival, antiapoptosis, and angiogenesis (22, 30, 31).

Several cell-surface receptors were shown to bind to MK, including members of the syndecan family, syndecan-1, -3, and -4 (32), a proteoglycan receptor–type tyrosine phosphatase ζ (RPTPζ) (26), a transmembrane low-density lipoprotein, a receptor-related protein (LRP) (33), the anaplastic lymphoma kinase (34), and the ε4β1- and αβ1-integrins (35). After MK stimulation, activation of the PI3K/Akt and MAPK signaling pathways takes place, resulting in suppression of caspase activation (36). The signaling cascade involved in this pathway is not well understood, although it likely involves Src, as PPI, an inhibitor of Src, inhibits MK activity (22).

Little is known about the role of MK in B cells; however, it was recently shown that transfection of MK cDNA into the IL-3–dependent pro-B cell line Ba/F3 promotes cell cycle progression and partially inhibits apoptosis of these cells (37).

In the current study, we followed the role of MK and its receptor, RPTPζ, in normal and CLL B cells. We show that MK and RPTPζ are expressed in normal B and CLL cells and play an important role in the MIF/CD74–induced survival cascade. These findings establish a key and novel role for MK and RPTPζ in the regulation of B cell survival during health and disease.

Materials and Methods

Cells

Mature B cells. B cells were obtained from spleen, lymph nodes, bone marrow, and peritoneal cavity of C57BL/6, CD74−/− (38), 129 SvEv, and RPTPζ−/− (39) mice, at 6–8 wk of age. All animal procedures were approved by the Animal Research Committee at the Weizmann Institute of Science. Splenic B cells were purified using CD45R beads (BD Bio-

Human B cells. B lymphocytes were obtained from spleen, lymph nodes, bone marrow, and peritoneal cavity of C57BL/6, CD74−/−, and 129 SvEv mice. B cells were purified using anti-B220 magnetic beads (StemCell, Vancouver, BC, Canada), as described previously (20).

Stimulation of cells

MIF stimulation. Recombinant murine MIF was prepared in its native sequence form and purified from an expression system, as previously described (14). Contaminating endotoxin was removed by C8 affinity chromatography prior to protein renaturation, and the experimental preparations had an activity of 1800 EU/mg MIF. For MIF stimulation, 1 × 10⁶ primary B cells or CLL cells were incubated in Iscove’s medium containing 0.1% (v/v) FCS at 37°C for 1 h. Next, cells were resuspended in medium containing recombinant MIF (600 ng/ml). The cells were then incubated at 37°C for the indicated periods.

Hepatocyte growth factor stimulation. Primary B cells (1 × 10⁶) were incubated in Iscove’s medium containing 3% FCS with 20 ng/ml recombinant hepatocyte growth factor (HGF) (R&D Systems) and incubated at 37°C for the indicated periods.

MK stimulation. Primary B cells or CLL cells (1 × 10⁶) were incubated in Iscove’s medium containing 10% FCS with 100 ng/ml recombinant MK (endotoxin level is <1 ng/μg; Peprotech) and maintained at 37°C for the indicated periods.

MK and MIF injections

MIF injections. C57BL/6 mice were i.p. injected with 1200 ng MIF or PBS. After 24 h, spleens were collected and splenocytes analyzed for their MK expression.

MK injections. C57BL/6 mice were i.p. injected daily with 400 ng MK or PBS for 2 d. Spleens were collected, and splenocytes were analyzed for their B cell repertoire and survival.

HGF, c-Met, and RPTPζ blocking

HGF blocking. B cells (1 × 10⁶) were incubated in 1 ml RPMI 1640 medium containing 1–3% (v/v) FCS in the presence of 5 μg/ml anti-murine HGF (R&D Systems) or 5 μg/ml anti-isotype control Ab for 16 h at 37°C.

c-Met blocking. B cells (1 × 10⁶) were incubated in 1 ml RPMI 1640 medium containing 10% (v/v) FCS in the presence of 0.1 ng/ml c-Met inhibitor, PH-665752 (a kind gift from Pfizer) at 37°C.

RPTPζ blocking. CLL cells (1 × 10⁶) were incubated in 1 ml RPMI 1640 medium containing 10% (v/v) FCS in the presence of anti-human RPTPζ (2 μg/ml; rabbit IgG raised against extracellular domain residues 141–440; Santa Cruz Biotechnology) or an isotype control Ab (2 μg/ml; Jackson ImmunoResearch Laboratories) for 24 h at 37°C.

RNA isolation and reverse transcription

Total RNA was isolated from cells using the Tri Reagent (Sigma). Reverse transcription was carried out using Superscript II RT (Life Technologies-BRL). Primers used included the following: hypoxanthine ribosyltransferase (HPRT): 5′-GGAGGTAGTGGCGC-TATGGC-3′; 5′-GTTGGACAGCGCAGTTGTTG-3′; MK: 5′-CCCCCAGCAGCAAGGACTG-3′; 5′-GGGCTCTGGGGAGGGG-3′; RPTPζ: 5′-CACAGGGGACTATAACATAAGGA-3′; 5′-GGACTTCTTGTGATTCTCTGAA-3′.

Real-time RT-PCR analysis

Levels of mRNA of MK and Bcl-2 were analyzed by quantitative real-time RT-PCR using a Light-Cycler instrument (Roche Diagnostics, Mannheim, Germany). The reaction volume (10 μl) contained 3 mM MgCl₂, LightCycler HotStart DNA SYBR Green 1 mix (Roche Diagnostics), specific primer pairs, and 2.5 μl cDNA. β-Actin or RP2 levels were used to normalize samples for calculation of the relative expression levels of all of the genes. Primer sequences were as follows:

Actin: 5′-GGGCGAGGTTACCTTTGGTC-3′; 5′-CAGTACAGTCCGCT-3′; Bcl-2 (murine): 5′-GCTACCCGCTGACTT-3′; 5′-GCGGGTTCAGG-3′;
MK: 5′-CCAGGAGACCATCCGCG-3′, 5′-TCCTTTCTTCTTCTGGC-3′; RP2: 5′-GTGGCGGCTTCCTCTAATA-3′, 5′-GCACAGTCCAATGACAT-3′; Bcl-2 (human): 5′-GGA CAT CGC CCT GTG-3′, 5′-GACTGTCACAACGAGGAA-3′.

Preparation of cell extracts

Stimulated cells were lysed in buffer containing 25 mM Tris, pH 7.4; 2 mM vanadate; 75 mM β-glycerophosphate, pH 7.2; 2 mM EDTA; 2 mM EGTA; 10 mM Na3P; and 0.5% Nonidet P-40 in the presence of the following protease inhibitors: 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 10 µg/ml chymostatin (Roche), 1 mM PMSF (Sigma), and 20 mM N-ethylmaleimide (Sigma Aldrich).

Immunoprecipitation

Protein-G Sepharose beads (Pharmacia) were conjugated to Tyr(P) (Santa Cruz Biotechnology), anti-RPTPα (Santa Cruz Biotechnology), anti–p-Akt (Cell Signaling Technology), anti-Syk (LR; Santa Cruz Biotechnology), anti–RPTPζ (directed against the C-terminal domain of the protein; Santa Cruz Biotechnology), and anti-Bcl-2 (C-2; Santa Cruz Biotechnology) overnight. The protein G bound material was washed three times with PBS containing 0.1% SDS and 0.5% Nonidet P-40. Immunoprecipitates were separated by 10% (w/v) SDS-PAGE.

Western blot analysis

To detect changes in protein phosphorylation, lysates or immunoprecipitates were separated by 8–12% (w/v) SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti–p-Tyr (pTyr99; Santa Cruz Biotechnology) followed by 1-h incubation with HRP-conjugated anti-mouse (Jackson ImmunoResearch Laboratories), anti-rabbit (Jackson ImmunoResearch Laboratories), or anti-goat (Santa Cruz Biotechnology) and peroxidase visualization by ECL (Amersham). The membrane was then stripped and reprobed with anti-tubulin Ab (Sigma), followed by peroxidase-conjugated anti-mouse (Jackson ImmunoResearch Laboratories).

Flow cytometry

Magic Red apoptosis detection kit. Cells were incubated with Magic Red (Immunochemistry Technology), according to the manufacturer’s instructions, at 37°C for 1 h. Magic Red staining was measured by FACS analysis. The positive population was identified by comparison of the staining with that of the negative stained population.

Annexin/propidium iodide staining. Cells were washed and stained with annexin (BD Biosciences) and propidium iodide (PI; Bender MedSystems, Vienna, Austria) for 30 min at 4°C. Annexin and PI staining were analyzed by FACS. The positive population was identified by comparison of the staining with that of the negative stained population.

Phenotypic characterization of B cells. Cells were stained for anti-CD45R/ B220, anti-CD21 (CR2/CR), anti-CD24 (heat stable Ag), anti-CD23, anti-IgM, anti-IgD, anti–Mac-1, and anti-CD5 (all from ebioscience) and analyzed by FACS.

Intracellular staining for MK. Cells were permeabilized with 4% PFA for 20 min at 4°C and washed twice with 0.1% saponin buffer. They were then stained for 30 min on ice with anti-MD Ab (Santa Cruz Biotechnology) or anti-isotype control, followed by 30-min staining with PE–anti-rabbit (Jackson ImmunoResearch Laboratories). Cell staining was assessed in a FACScalibur flow cytometer (BD Biosciences).

Staining of RPTPζ. Human B cells were stained for anti-CD19 (BioLegend) and anti-PTPζ (Santa Cruz Biotechnology), and analyzed by FACS.

ELISA

MK in the serum of CLL patients and in conditioned media of stimulated human B cells was determined by ELISA according to the manufacturer’s instructions (Peprotech).

FIGURE 1. MIF elevates MK expression in B cells in a CD74-dependent manner. A–C, B cells derived from C57BL/6 (A, B) or CD74-deficient (C) mice were incubated in the presence or absence of MIF (600 ng/ml) for 8 h. Total RNA was then isolated. A, RT-PCR using primers for MK or HPRT was performed. The intensity of the MK band after each treatment was normalized by dividing by the measured intensity of the HPRT band from the same treatment. The fold activation ratio in the absence of any treatment was normalized to 1, and the ratio for each treatment was calculated as the intensity of the treatment sample relative to 1. The results presented are representative of at least six different experiments. B and C, Quantitative real-time PCR was performed using primers for MK and β-actin. β-Actin levels were used to normalize samples for calculation of the relative expression levels of MK. Results are expressed as fold change in MK expression in stimulated cells compared with non-stimulated cells, which was defined as 1. The results presented are representative of at least five different experiments. D, B220+ cells derived from C57BL/6 and CD74−/− mice were incubated in the presence or absence of MIF (600 ng/ml) for 16 h, and intracellular MK protein levels were analyzed by intracellular staining as described in Materials and Methods. Gray line, isotype control; black line, MK staining. The graphs show an average of five independent experiments. Gray line, isotype control; black line, MK staining.

E, Intracellular staining with that of the negative stained population.

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Statistical analysis

Comparisons between groups were evaluated by Student t test. Data are expressed as mean ± SD and are considered statistically significant at p values ≤0.05.

Results

The MIF/CD74-induced cascade elevates MK mRNA and protein expression

CD74 stimulation by its ligand MIF induces cell survival (5, 12). To follow further the CD74-induced survival cascade, we sought to identify genes whose expression is modulated by CD74 and are involved in regulation of B cell survival. Hence, gene expression profiles of cells treated in the presence or absence of MIF were compared using the Affymetrix GeneChip expression analysis system (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33352; GSE33352).

Many genes were found to be differentially expressed in these two populations. One striking example was MK, whose expression was markedly elevated in the MIF-stimulated cells. To confirm this result, splenic B cells were stimulated with or without MIF for 8 h, and MK mRNA levels were analyzed by quantitative real-time PCR (Fig. 1). As shown in Fig. 1A and 1B, MK mRNA levels were elevated after stimulation with MIF. No elevation in MK mRNA levels was observed in CD74-deficient B cells, indicating that this elevation was specific for MIF binding to its receptor, CD74 (Fig. 1C). We next followed

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MK intracellular protein levels after MIF stimulation in control or CD74-deficient B cells by intracellular staining (Fig. 1D). A specific elevation in MK protein was observed in wild-type B cells after MIF stimulation, whereas no change was observed in CD74-deficient cells. MK protein was further followed in vivo after MIF injection to C57BL/6 mice. As demonstrated in Fig. 1E, a significant elevation in MK protein levels was detected in B cells derived from MIF-injected mice compared with those in B cells derived from PBS-treated mice. Thus, MIF binding to CD74 induces transcription and expression of MK in vitro and in vivo.

The expression of the c-Met receptor and its ligand, HGF, was recently demonstrated to be regulated by MIF and CD74. Both c-Met and HGF were shown to be involved in the CD74-induced survival cascade (13). We therefore examined whether the modulation of MK expression by CD74 is HGF/c-Met dependent. Wild-type and CD74-deficient B cells were stimulated with HGF for 8 h, and MK expression levels were analyzed. Stimulation of c-Met with its ligand elevated MK mRNA (Fig. 2A) and protein (Fig. 2B) levels in both wild-type and CD74-deficient B cells. Moreover, blocking the c-Met activity using the c-Met inhibitor PHA-665752, a selective small-molecule, active-site inhibitor of the catalytic activity of c-Met kinase (Ki 4 nM) that competes with its ATP binding (41) (Fig. 2C), or using anti-HGF blocking Ab (13) (Fig. 2D) decreased intracellular protein levels of MK. Thus, c-Met activation by its ligand, HGF, elevates MK expression. To determine whether c-Met activation is essential for the MIF-induced expression of MK, we next stimulated B cells with MIF in the presence or absence of anti-HGF blocking Ab or an isotype control Ab, and MK levels were analyzed by intracellular staining. As shown in Fig. 2D, treatment with HGF blocking Ab significantly reduced the MIF-induced elevation of MK expression. Together, these results suggest that MIF elevates MK expression in a c-Met/HGF dependent manner.

**MK induces survival cascades in B cells**

To follow the role of MK in B cells, we analyzed the MK-induced signaling cascade. Syk and Akt phosphorylation were shown to be involved in the B cell survival cascade (42, 43) and in the MIF-induced signaling cascade (5, 12). We therefore followed the phosphorylation levels of Syk and Akt upon MK stimulation. As shown in Fig. 3A and 3B, MK stimulation induced Akt and Syk phosphorylation in B cells.

We further studied the survival cascade by following the expression of the antiapoptotic protein Bcl-2 in MK-stimulated or...
control B cells. As shown in Fig. 3, MK stimulation elevated Bcl-2 mRNA (Fig. 3C) and protein (Fig. 3D) levels.

MK was recently shown to act as an antiapoptotic factor in the human hepatoma cell line, HepG2, by downregulation of caspase 3 activity (36). Therefore, caspase 3 and 7 activity was analyzed in B cells after MK stimulation using Magic Red staining. As shown in Fig. 4A, MK stimulation significantly reduced the activity of these caspases. This reduced caspase activity resulted in an elevation of the live population and reduced the apoptotic population, as revealed by annexin/PI staining (Fig. 4B). Furthermore, an MK-induced survival effect was also observed in CD74−/− B cells (Fig. 4A). Although the absence of CD74 results in elevated levels of apoptotic cells, MK rescued the survival of these B cells. These results suggest that MK can rescue the absence of CD74 and therefore acts downstream of the CD74-induced survival cascade. However, MK does not completely rescue the absence or activity of CD74, suggesting that other compounds might be involved in this CD74-induced survival cascade. To determine further that MK activity occurs downstream to HGF/c-Met, c-Met activity was blocked with the PHA-665752 inhibitor. Caspase activity was analyzed by Magic Red staining. As seen in Fig. 4C, MK stimulation reduced caspase activity in cells incubated either in the presence or absence of the c-Met inhibitor. Furthermore, MK was able to rescue B cells from apoptosis induced by the blocking anti-HGF Ab (Fig. 4D). Thus, MK acts downstream to the MIF/CD74 and HGF/c-Met survival cascade.

To determine whether MK has a physiological effect on B cell survival, PBS or MK were injected daily i.p. into control (C57BL6) and CD74-deficient mice. After 48 h, live versus apoptotic B cell populations were analyzed by annexin/PI staining. As demonstrated in Fig. 5A, the percent of apoptotic B populations was reduced after MK injection, resulting in elevation of the live cell population, compared with the PBS-treated cells in both C57BL/6 and CD74-deficient mice (Fig. 5A). Moreover, to determine whether the MK-induced survival cascade leads to elevation of the peripheral mature population, we next analyzed the splenic B cell populations in MK-treated mice. As demonstrated in Fig. 5B, treatment of C57BL6 and CD74-deficient mice with MK resulted in an elevation of the percent and number of mature B (CD21low CD24low) cells. The mature B cell population in mice lacking CD74 is markedly reduced (7). Our results show that the proportion of elevation in the mature population after MK stimulation in both strains is similar, suggesting that MK has a similar effect on control and CD74−/− B cells. These results suggest that

![FIGURE 5.](http://www.jimmunol.org/)
treatment with MK induces a survival cascade, which enlarges the proportion of the mature B cell population in the spleen.

Together, these results show that MK activation is a downstream event to the MIF/CD74 and HGF/c-Met induced survival cascade. MK induces a signaling cascade leading to the expression of Bcl-2, resulting in the suppression of apoptosis, cell survival, and elevation of the mature B cell population.

**RPTPζ serves as the receptor for MK in B cells**

We next wished to identify the receptor that binds MK and regulates B cell survival. RPTPζ has been recognized as one of the receptors of MK in many cell types (26); however, its expression in B cells has not been reported. To reveal whether RPTPζ serves as a receptor for MK in B cells, we initially examined its expression in these cells. Thus, B cells from control and RPTPζ knockout mice (39, 44) were isolated, and RPTPζ mRNA and protein were examined using RT-PCR and Western blot analysis, respectively. As can be seen in Fig. 6, RPTPζ mRNA was detected in the wild-type but not the RPTPζ-deficient B cells (Fig. 6A). RPTPζ protein levels were then evaluated using an Ab that recognizes the transmembrane isoform. As shown in Fig. 6B, RPTPζ protein was detected in B cells derived from control mice, whereas they were not observed in the RPTPζ-deficient cells.

To test whether MK induces its survival cascade through RPTPζ, the survival of control and RPTPζ-deficient B cells was analyzed in the presence or absence of MK. As seen in Fig. 6C, MK stimulation reduced caspase 3 and 7 activities in control B cells, whereas cells lacking RPTPζ were unresponsive to MK. Moreover, in absence of RPTPζ, neither MIF nor HGF were able to elevate cell survival, demonstrating that RPTPζ is essential for the survival cascade induced by MIF/CD74 (Fig. 6D) and HGF/c-Met (Fig. 6E). These results indicate that RPTPζ serves as the receptor for MK in B cells and is involved in the survival cascade induced by MIF/CD74 and HGF/c-Met.

To determine whether the survival cascade regulated by RPTPζ modifies the B cell repertoire, wild-type (129 Sv/Ev) and RPTPζ-deficient peripheral B cell populations were compared. FACS analysis showed a severe deficit of B cells in the spleen, the bone marrow, and the lymph nodes of RPTPζ-deficient mice compared with their numbers in those of wild-type mice. A significant reduction in the proportion and absolute numbers of mature splenic B cells (CD21low CD24high IgMlow IgDhigh) was detected (Fig. 7A, 7B, respectively). In addition, lower absolute numbers and proportion of the mature B cell population was detected in the bone marrow of RPTPζ-deficient mice relative to control mice (Fig. 7C). Moreover, in the RPTPζ knockout mice, as mostly mature B cells can be detected in the lymph nodes, the percent of mature B cells in this compartment was similar to its levels in control mice; however, the total number of mature B cells was ∼2-fold lower (Fig. 7D). Taken together, these results suggest that RPTPζ plays an important role in the survival and shaping of the mature B cell compartment. However, no significant difference in the B cell populations in the peritoneal cavity was observed (Fig. 7E), suggesting that this receptor does not play a role in survival of B cells in this compartment.

**MK regulates survival of CLL cells**

To determine whether MK regulates survival of normal human B cells, we first analyzed the expression of the MK receptor on these cells. As shown in Fig. 8A, normal human B cells express RPTPζ on their surfaces. We next followed MK secretion after MIF stimulation. Human B cells were incubated with or without MIF for 24 h, and MK secretion was then analyzed by ELISA. As demonstrated in Fig. 8B, MIF stimulation augmented MK secretion to levels in the range used in our in vitro experiments (174 ng/ml ± 56 ng/ml; n = 3).

Numerous studies have reported that serum MK levels are increased in the blood of patients with several kinds of malignancy (45–47). However, little is known about circulating levels of MK in leukemia patients. Therefore, we next evaluated the levels of MK in sera derived from control versus early and advanced CLL patients using an ELISA assay (Fig. 8C). Higher levels of MK were detected in sera derived from both early and advanced CLL patients (early CLL: mean = 285.5 ng/ml, n = 12; advanced CLL: mean = 400 ng/ml, n = 9; normal: mean = 79.5 ng/ml, n = 14), suggesting that MK can serve as a prognostic marker, even at early stages of the disease.

A similar survival pathway regulated by MIF and CD74 was shown to operate in CLL cells (12, 20). We therefore wished to determine whether MK is involved in the cascade regulating CLL survival as well. To determine whether MK is a target gene of the MIF/CD74 survival cascade in CLL cells, CLL cells from early- and advanced-stage patients were stimulated with MIF for 18 h, and MK gene expression was analyzed by qRT-PCR. As demonstrated in Fig. 8D, an elevation in MK mRNA levels was detected.
in MIF-stimulated CLL cells regardless of the disease stage. To determine whether MK induces CLL survival, CLL cells were stimulated in the presence or absence of MK, and expression of Bcl-2 was analyzed by qRT-PCR and Western blot analysis. MK elevated Bcl-2 mRNA (Fig. 8E) and protein (Fig. 8F) levels. This cascade led to a significant reduction in the level of caspase activity (Fig. 8G). Thus, in analogy to its role in normal B cells, MK mediates the survival of CLL cells.

To gain further insight into the role of RPTPζ in CLL survival, the receptor was inhibited using an anti-RPTPζ blocking Ab. To this end, CLL cells were treated with RPTPζ blocking or with an isotype-control Ab for 24 h, and caspase activity was examined by Magic Red. As can be seen in Fig. 8H, blocking of RPTPζ significantly increased the caspase activity. In addition, to confirm that MIF/CD74 induces a survival cascade that is RPTPζ dependent, CLL cells were stimulated with MIF in the presence or absence of RPTPζ blocking or isotype control Abs. The MIF-induced survival was downregulated in cells whose RPTPζ receptor was blocked (Fig. 8H). However, blocking RPTPζ did not completely abolish the MIF-induced survival cascade, suggesting that there might be additional branches in the pathway controlled by CD74 or alternatively that the Ab did not entirely block RPTPζ function. Altogether, these results show that RPTPζ is involved in mediating the antiapoptotic activity of MK and of the MIF/CD74 survival cascade in CLL cells.

Discussion
Adaptive immunity depends on the production and maintenance of a pool of mature peripheral lymphocytes throughout life. In healthy individuals, the pool of peripheral lymphocytes remains constant in size. The control of lymphoid homeostasis is the result of a very fine balance between lymphocyte production, survival, and proliferation. Survival factors have been shown to play a critical role in maintaining lymphocyte homeostasis (1). Moreover, resistance to apoptosis, leading to enhanced survival, is associated with initiation and progression of B cell malignancies such as CLL (48).

Our previous studies have demonstrated that mature B cell survival is mediated by CD74 and its natural ligand, MIF. MIF is a ubiquitous protein that has a broad tissue distribution and is found in virtually all cells (49); thus, it undergoes tonic production in the spleen as well. Engagement of the CD74/CD44 complex by MIF triggers recruitment of the tyrosine kinase receptor, c-Met, to this complex (13), resulting in induction of an antiapoptotic Syk–PI3K/Akt signaling pathway and HGF secretion, which stimulates the survival of the mature B cell population in an autocrine manner (5, 12, 13, 50). In this study, we identified two novel components of the CD74/CD44-induced B cell survival pathway, the heparin-binding cytokine, MK, and its receptor, RPTPζ.

MK is a heparin-binding cytokine that promotes growth, survival, migration, and other activities of its target cells (31). MK was shown to promote growth and suppress apoptosis through activation of Raf-1/MEK/ERK and PI3K/Akt kinase pathways in neurons, cardiomyocytes, and some malignant neuroblastosomas. The activated MAPK pathway was reported to downregulate caspase 3 activity (51, 52). In addition, MK-induced Akt phosphorylation promotes a series of antiapoptotic pathways (53). In B cells, the PI 3K/Akt pathway has a major role in determining cell fate and
resting B cell survival (42, 43). Akt is activated in a Syk-dependent pathway (54, 55). Disruption of Syk expression by genetic deletion indicates that it is essential for regulation of cell survival (56). In this study, we show that exogenous MK stimulation triggers the Syk and Akt signaling cascade, which leads to B cell survival. Thus, MK appears to be necessary for the Syk and Akt phosphorylation induced by MIF.

Several reports have previously demonstrated that MK acts as an antiapoptotic growth factor, at least in part, through the regulation of Bcl-2 expression (57, 58). Our current data show that the MK-induced signaling cascade enhances the expression of Bcl-2 and inhibits activity of caspases 3 and 7, leading to cell survival. Thus, MK appears to be necessary for the Syk and Akt phosphorylation induced by MIF.

Several receptors are known to be activated by MK (31). In this study, we report that RPTPζ is expressed in mature B cells and functions as a survival receptor in these cells. RPTPζ forms a cell surface complex with LRP (33), α4β1-integrin, and α6β1-integrin (35), which also serve as MK receptors. MK, which acts as a dimer, binds to components of the receptor including glycosaminoglycan chains and promotes formation of the receptor complex. It was previously shown in embryonic neurons that the survival-promoting signal of MK is induced by binding to a receptor complex containing RPTPζ (59). Similarly, our study demonstrated the importance of RPTPζ in reducing cell death in B cells. In addition, characterization of the peripheral B cell repertoire of RPTPζ-deficient mice revealed a significant decrease in the number of mature B cells in RPTPζ-deficient compared with control mice. These results demonstrate the essential role of RPTPζ in controlling and shaping the B cell repertoire.

MIF/CD74 (6, 60) and HGF/c-Met (61) induced pathways were shown to regulate tumor progression and survival. In addition, in CLL, we previously showed that MIF and CD74 play a pivotal role in the regulation of CLL cell survival (20, 21). MK has also been associated with tumor progression (22). Moreover, significantly higher MK expression levels were detected in B-ALL, AML-M2, and AML-M3 than those in normal controls (62).
However, expression and function of MK in leukemia cells was never characterized, although it was demonstrated that transcription of MK into the IL-3–dependent pro-B cell line Ba/F3 promotes cell cycle progression and partially inhibits apoptosis in these cells (37).

Our study demonstrates that MK and its receptor, RPTPζ, play a major role in survival of CLL cells. MK suppresses CLL apoptosis by elevating the expression of Bcl-2 and inhibiting caspase 3 and 7 activities. Moreover, blocking RPTPζ in CLL cells resulted in the induction of cell death and inhibition of the MIF/CD74-induced survival cascade. However, blocking RPTPζ did not completely abolish the MIF-induced survival cascade, suggesting that there might be additional branches in the pathway controlled by CD74. We further show that MK serum levels are elevated in CLL patients compared with their levels in normal individuals, regardless of the disease stage. These findings are in agreement with other studies in which MK was significantly elevated in serum derived from cancer patients (45, 46). Because reliable predictors of prognosis at the early stages of CLL are lacking in the clinical workup of CLL patients (63), the identification of MK as a prognostic marker may contribute to improving the clinical management of CLL patients. To conclude, our findings establish a key and novel role for MK and its receptor, the clinical management of CLL patients. To conclude, our findings of MK as a prognostic marker may contribute to improving the clinical workup of CLL patients (63), the identification of MK as a prognostic marker may contribute to improving the clinical management of CLL patients.