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*J Immunol* 2010; 185:2020-2031; Prepublished online 16 July 2010; doi: 10.4049/jimmunol.0902566

http://www.jimmunol.org/content/185/4/2020

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/07/16/jimmunol.0902566.DC1

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*The Journal of Immunology* is published twice each month by

The American Association of Immunologists, Inc.,

1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
c-Met and Its Ligand Hepatocyte Growth Factor/Scatter Factor Regulate Mature B Cell Survival in a Pathway Induced by CD74

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The signals regulating the survival of mature splenic B cells have become a major focus in recent studies of B cell immunology. Durable B cell persistence in the periphery is dependent on survival signals that are transduced by cell surface receptors. In this study, we describe a novel biological mechanism involved in mature B cell homeostasis, the hepatocyte growth factor/scatter factor (HGF)/c-Met pathway. We demonstrate that c-Met activation by HGF leads to a survival cascade, whereas its blockade results in induction of mature B cell death. Our results emphasize a unique and critical function for c-Met signaling in the previously described macrophage migration inhibitory factor/CD74-induced survival pathway. Macrophage migration inhibitory factor recruits c-Met to the CD74/CD44 complex and thereby enables the induction of a signaling cascade within the cell. This signal results in HGF secretion, which stimulates the survival of the mature B cell population in an autocrine manner. Thus, the CD74–HGF/c-Met axis defines a novel physiologic survival pathway in mature B cells, resulting in the control of the humoral immune response. The Journal of Immunology, 2010, 185: 2020–2031.

During their development, B cells encounter various checkpoints that control cell survival. Under steady-state conditions, the number and distribution of B cells are under homeostatic control maintained by a balance between survival and apoptosis.

Regulation of mature B cell survival involves multiple mechanisms. The BCR provides survival signals essential for maintaining the mature B cell pool. Deletion of the IgH gene (1) or conditional deletion of either IgH or the signal-transducing Cd79a (Igs) genes (2) leads to a loss of B cells. In addition, survival of mature naive B cells depends on signals delivered by the ligand–receptor pair B cell-activating factor (BAFF) and BAFFR (3, 4). Mice that lack BAFF expression or that are subjected to treatments designed to block the action of BAFF fail to produce or to maintain a mature B cell pool (5–7).

Recently, we described an additional mechanism that regulates B cell survival, which depends on CD74 (invariant chain, li). CD74 is a type II integral membrane protein, containing a short N-terminal cytoplasmic tail of 28 aa, followed by a single 24-aa transmembrane region and an ~150-aa luminal domain. The CD74 chain was originally thought to function mainly as an MHC class II chaperone, promoting the exit of MHC class II molecules from the endoplasmic reticulum, directing them to endocytic compartments, preventing peptide binding within the endoplasmic reticulum, and contributing to peptide editing in the MHC class II compartment (8).

In addition to its chaperone function, CD74 was shown to have a role as an accessory signaling molecule. A small proportion of CD74 is modified by the addition of chondroitin sulfate, and this form of CD74 is expressed on the surface of APCs, including monocytes and B cells. Ab blocking studies have shown that CD74 modified by the addition of chondroitin sulfate interacts with CD44 (9, 10). In addition, it was shown that macrophage migration inhibitory factor (MIF) binds to the CD74 extracellular domain on macrophages, a process that results in initiation of a signaling pathway in these cells (11).

Our studies have shown that CD74 expressed on B cells is directly involved in shaping the peripheral B cell populations by regulating mature B cell survival (12) through a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its coactivator TAFII105 (13, 14). CD74 stimulation by MIF activates the Syk and PI3K/Akt pathways, leading to NF-κB activation, enabling entry of the stimulated B cells into the S phase, an increase in DNA synthesis, cell division, and augmented expression of antiapoptotic proteins in a CD44-dependent manner. These findings confirmed that surface CD74 functions as a survival receptor (15–17).

Interestingly, the cell surface receptor, CD44, has also been implicated in the regulation of the signaling of the tyrosine kinase receptor c-Met (18–20), although the precise mechanism of this interaction is unknown.

c-Met is a unique disulfide-linked α-β heterodimeric receptor tyrosine kinase with a versatile role in regulating numerous biological functions in response to its natural ligand hepatocyte growth factor/scatter factor (HGF). HGF is a multifunctional cytokine with a domain structure and proteolytic mechanism of activation similar
to that of the serine protease plasminogen. Activation of the HGF/c-Met signaling pathway, which requires phosphorylation of various specific tyrosine residues on c-Met itself, leads to cellular responses, including increased motility, proliferation, morphogenesis, and cell survival (21–27).

Little is known about the role of HGF/c-Met in the regulation of lymphopoiesis in general and specifically that of B cells. HGF has been reported to regulate hematopoiesis in mouse fetal liver and in adult bone marrow (28, 29) and was found to have a stimulatory effect on Ig secretion in cultures of mouse spleenocytes (30). On the basis of the known interactions between c-Met and CD44, we wished to determine whether c-Met is expressed in naive B cells and whether it is involved in the regulation of their survival. In this study, we demonstrate that naive murine B cells express both c-Met and its ligand HGF, which regulate peripheral B cell survival. In addition, our study shows that c-Met participates in controlling MIF-induced signaling by forming a survival complex together with CD74 and CD44 in B cells. These findings establish a key and novel role for HGF/c-Met pair in the regulation of B cell survival, demonstrating an additional level of control of the humoral immune response.

Materials and Methods

Cells
Splenic cells were obtained from C57BL/6 CD44+/− (31) or CD74+/− (32) mice. All of the animal procedures were approved by the Animal Research Committee at the Weizmann Institute.

Cells and B cell separation
Splenic cells were obtained from the indicated mice at 6–8 wk of age, as previously described (33). B cells then were purified from each mouse strain using CD45R beads (BD Biosciences, San Jose, CA). The purity of the isolated cells (96–99%) was confirmed by FACS (using the B220+ marker) after each experiment. For isolation of AA4+ cells, purified splenocytes were stained with anti-AA4 Ab conjugated to PE, as previously described (16). Separation of cells was performed using PE-conjugated beads (BD Biosciences). The purity of the isolated cells (96–99%) was confirmed by FACS (using the IgD marker [eBioscience, San Diego, CA]) after each experiment.

For isolation of IgD+ cells, control IgD+ cells were separated from total splenocytes, as previously described (34).

Sorting of the transitional 1 (T1), transitional 2 (T2), marginal zone (MZ), and mature populations was performed using anti-CD45R/B220, anti-CD21 (CR2/CR), anti-CD24 (heat stable Ag), and anti-CD23 Abs (all from eBioscience), as previously described (35).

T1 B cells were obtained from 1-wk-old mice, as previously described (36).

MIF and HGF stimulation
Recombinant murine MIF was prepared with its native sequence and purified from an expression system, as previously described (37). Contaminating endotoxin was removed by C8 affinity chromatography prior to protein renaturation, and the experimental preparations contained 1800 EU/mg MIF. For MIF stimulation, 1 × 10^6 primary B cells were incubated in RPMI 1640 medium containing 0.1% (v/v) FCS at 37˚C for 3 h. Next, cells were resuspended in medium containing 100 ng/ml rMIF and incubated at 37˚C for various periods. For HGF stimulation, 1 × 10^6 primary B cells incubated in RPMI 1640 medium containing 1% (v/v) FCS at 37˚C. Cells were then resuspended in medium containing 10 ng/ml rHGF (R&D Systems, Minneapolis, MN) and incubated at 37˚C for the indicated periods.

CD74 stimulation
First, 1 × 10^6 primary B cells were suspended in 1 ml RPMI 1640 medium containing 10% (v/v) FCS. Next, 5 μg Ab specific for the extracellular domain of both murine and human CD74 (C-16; Santa Cruz Biotechnology, Santa Cruz, CA) or an isotype control (Santa Cruz Biotechnology) was added to the cells and incubated at 37˚C for various periods.

HGF and c-Met blocking
For HGF blocking, 1 × 10^7 B cells were incubated in 1 ml RPMI 1640 medium containing 10% (v/v) FCS at 37˚C for 8 h at 37˚C. For c-Met blocking, 1 × 10^7 B cells were incubated in 1 ml RPMI 1640 medium containing 0.1% (v/v) FCS in the presence of 3 μg/ml anti-murine HGF Ab (R&D Systems) and 3 μg/ml anti-isotype control Ab for 8 h at 37˚C. For c-Met blocking, 1 × 10^7 B cells were incubated in 1 ml RPMI 1640 medium containing 0.1% (v/v) FCS in the presence of 0.3 ng/ml PHA-665752, a c-Met inhibitor (a gift from Pfizer, New York, NY), at 37˚C.

RNA isolation and reverse transcription
Total RNA was isolated from cells using the TRIzol Reagent kit (MRC, Cincinnati, OH). Reverse transcription was carried out using Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA). Primers that were used included: c-Met, 5′-GACCTCGACCATCCTGATTG-3′, 3′-GGTTGAACCTCTGGTTGC-5′; HGF, 5′-AGCGCTTCCCTCCCTTCTTT-3′; TCAACAGAATCCAGCAAGA5′-3′; Bcl-2, 5′-CAGCGCATGTTGGC-3′; 3′-CTGGCATCTTCCCTTCTCC-5′; cyclin E, 5′-GAAAATCGACCCACCCAGACG-3′, 3′-GAAATGATACACAGGCAACGAGCC-5′; hypo- xanthine ribosyltransferase (HPRT), 5′-GAGGTTAGAGCTGCTATGGCT-3′, 3′-GGTTGATACCGGACGATTTTGG-5′.

Real-time RT-PCR analysis
Levels of mRNA of actin, c-Met, and Bcl-2 were analyzed by quantitative real-time RT-PCR using a LightCycler instrument (Roche Diagnostic Systems, Burgess Hill, West Sussex, U.K.). Total RNA was isolated from cells using the TRI Reagent kit. Reverse transcription was carried out using Superscript II reverse transcriptase. The reaction volume (10 ml) contained 3 mM MgCl2, LightCycler HotStart DNA SYBR Green I mix (Roche Diagnostics), specific primer pairs, and 2.5 μg cDNA. Conditions for PCR were as follows: 10 min at 95˚C followed by 40–50 cycles of 15 s at 95˚C, 15 s at 60˚C, and 15 s at 72˚C. PCR was performed in duplicates, as previously described (38). Primer sequences were as follows: β-actin, as previously described (39); c-Met, 5′-GTGCCCAAGCTCAGCT-3′; 5′-CTTCGATACGGCCTCT-3′; Bcl-2, 5′-GCTACGCTGCTGACT-3′, 3′-GCCGGTACGAGTACTC-3′.

β-Actin levels were used to normalize samples for calculation of the relative expression levels of all of the genes (c-Met and Bcl-2). The results were evaluated for significance using the t test, and the p value was <0.05.

ELISA
B cells from C57BL/6 mice were incubated for 8 h in the presence or absence of MIF (100 ng/ml). Cell supernatants were collected, and the HGF levels were determined by ELISA kit (R&D Systems), according to the manufacturer’s instructions.

HGF intracellular staining
Total splenocytes (10^7 cells per milliliter) from control mice were used directly or stimulated with anti-CD74 (C-16; Santa Cruz Biotechnology) or isotype control Ab (Santa Cruz Biotechnology) for 5 h. HGF intracellular staining was performed using the Cell Fixation/Permeabilization Kit for Intracellular Cytokine Analysis according to the manufacturer’s instructions (BD Cytofix/Cytoperm Kit; BD Biosciences). The cells were pre-stained with anti-CD45R/B220 and anti-IgD Abs (both from eBioscience). Then, the permeabilized cells were stained with goat anti-mouse HGF Ab (R&D Systems) followed by donkey anti-goat Ab (Jackson Immunoresearch Laboratories, West Grove, PA).

Preparation of cell extracts
Stimulated cells were lysed in buffer containing: 25 mM Tris (pH 7.4), 2 mM vanadate, 75 mM β-glycophosphate (pH 7.2), 2 mM EDTA, 2 mM EGTA, 10 mM NaPi, and 0.5% Nonidet P-40 in the presence of the following protease inhibitors: 10 μg/ml leupentin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml chymostatin (Roche, Basel, Switzerland), 1 mM PMSF (Sigma-Aldrich, St. Louis, MO), and 20 mM N-ethylmaleimide (Sigma-Aldrich).

Immunoprecipitation and Western blot
Protein G-Sepharose beads (Pharic, Pisicaway, NJ) were conjugated to c-Met mAb (B-2, SC-8057; Santa Cruz Biotechnology), CD44 (BD Biosciences), or TyrP (Santa Cruz Biotechnology) mAb for 2 h at 4˚C, followed by three washes in PBS. Beads were added to the cell lysates, and c-Met, CD44, or TyrP proteins were immunoprecipitated 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. The protein bands were transferred onto a nitrocellulose membrane and probed with anti-CD74 (1N; anti-c-Met (AFS27; R&D Systems), and anti-Syk (L.R; Santa Cruz Biotechnology) Abs, respectively, followed by HRP-conjugated anti-rabbit IgG or anti-goat IgG (Jackson Immunoresearch Laboratories).
Blocking peptide

Protein G-Sepharose beads (Pharmacia) were incubated in the presence of c-Met mAb (B-2, SC-8057; Santa Cruz Biotechnology) and the c-Met blocking peptide (B-2, SC-8057P; Santa Cruz Biotechnology), which recognizes the c-Met cytoplasmic domain of murine origin. Immunoprecipitation and Western blotting were then performed, as described above.

Western blot analysis

To detect changes in protein phosphorylation, lysates or immunoprecipitates were separated by 12% (w/v) SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti–β-actin (C2; Santa Cruz Biotechnology) or anti–c-Met (AF527; R&D Systems) followed by HRP-conjugated anti-mouse or anti-goat (Jackson ImmunoResearch Laboratories), respectively. The membranes then were stripped and reprobed with anti-tubulin Ab (Sigma-Aldrich), followed by peroxidase-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories).

HGF injections

C57BL/6 mice were i.p. injected daily with 4 μg HGF or PBS for 2 d. Spleens were collected, and splenocytes were analyzed for their B cell populations and survival.

Injections with c-Met inhibitor

C57BL/6 mice were i.p. injected daily with 0.15 mg PHA-665752, a c-Met blocker (a kind gift from Pfizer), or DMSO twice a day for 5 d. Spleens were collected, and splenocytes were analyzed for their B cell populations and survival.

Propidium iodide staining

Spleens were collected from HGF- or PBS-injected mice as described. Purified splenic B cells were cultured in six-well plates with 1 × 10^7 cells per well in RPMI 1640 medium supplemented with 1% FCS, 2 mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin, with or without HGF (10 ng/ml) for 18 h. Cells were collected by centrifugation, washed, and fixed in 70% cold ethanol and incubated in the presence of RNase (25 μg/ml) (Sigma-Aldrich). Propidium iodide (PI; 25 μg/ml) (Sigma-Aldrich) was added for 20 min at room temperature. PI staining was analyzed by FACS. Doublet discrimination settings were used to eliminate cell clusters from consideration.

Cell death detection

Magic Red apoptosis detection kit. Cells were incubated with Magic Red (Immunochrometry Technology, Bloomington, MN), according to the manufacturer’s instructions, at 37°C for 1 h. Then, Magic Red staining was quantified by FACS analysis.

Fluorescent-labeled inhibitor of caspases apoptosis detection kit. Cells were incubated with fluorescent-labeled inhibitor of caspases (FLICA; Immunochrometry Technology), according to the manufacturer’s instructions, at 37°C for 1 h. Cells were then washed three times with FLICA washing buffer. FLICA staining was measured by FACS analysis. The positive population was identified by comparison of the staining to that of the negative population.

Annexin/PI staining

Spleens were collected from HGF- or PBS-injected mice. Cells were washed and stained with annexin (BD Biosciences) and PI (Bender Med-Systems, Vienna, Austria) for 15 min at room temperature. Annexin and PI staining were analyzed by FACS. The positive population was identified by comparison of the staining to that of the negative stained population.

Immunofluorescence and flow cytometry

c-Met cell surface expression was analyzed using directly conjugated anti-mouse c-Met (HGF eBioclone 7; eBioscience) (40, 41) or goat anti-mouse HGF (AF527; R&D Systems) Ab, followed by HRP-conjugated anti-goat Ab (Jackson ImmunoResearch Laboratories). CD74 cell surface staining was analyzed using goat anti-mouse CD74 (C-16; Santa Cruz Biotechnology), followed by HRP-conjugated anti-goat Ab (Jackson ImmunoResearch Laboratories).

Characterization of B cells

Purified B cells from control and CD74−/− mice that were incubated for 18 h in the presence or absence of HGF (10 ng/ml) and freshly isolated splenocytes were stained for RA3-6B2 anti-CD45R/B220, anti-IgM, anti-IgD, anti-AA4, anti-CD21 (CR2/CR), anti-CD24 (heat stable Ag), and anti-CD23 (all from eBioscience) and analyzed by FACS.

FIGURE 1. CD74/c-Met forms a cell surface complex with c-Met in B cells. A, T1, T2, MZ, and mature B cells were purified as described in Materials and Methods. Total RNA was isolated, reverse transcription using primers for c-Met and HPRT was performed as described in Materials and Methods, and fold activation was calculated. The intensity of the c-Met band for each population was normalized by dividing the measured intensity of the HPRT band from the same cell population. The activation fold ratio in the mature population was normalized to 1, and the ratio for the other populations was calculated as the intensity of the each sample relative to 1. B–D, Splenocytes from C57BL/6 mice were stained with (B) anti-B220 and anti-c-Met (eBioscience) or isotype control Abs, (C) anti-B220, anti-IgD, and anti–c-Met (eBioscience) Abs; or (D) anti-B220, anti-CD21, anti-CD24, and anti–c-Met (eBioscience) Abs. Histograms present cell surface expression of c-Met on B220+ cells (B), IgD+B220+ cells (C), and CD21hi, CD24hi B220+ cells (D). E, IgD+B220+ B cells or B220+ B cells were purified as described in Materials and Methods. Cells were lysed, and the level of c-Met was analyzed by Western blot analysis. F–H, Immunoprecipitations: control (F); CD44+M (G); or CD74−/− (H). B220+ B cells were lysed. After anti–c-Met immunoprecipitation, proteins were separated by SDS-PAGE and transferred onto nitrocellulose. CD74 was detected by Western blot analysis. The results presented are representative of at least three different experiments. I, Control B220+ B cells were lysed. After anti-CD44 or isotype Ab immunoprecipitation, proteins were separated by SDS-PAGE and transferred onto nitrocellulose. c-Met was detected by Western blot analysis.
Statistical analysis

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

Results

CD74/CD44 forms a cell surface complex with c-Met in B cells

We first wished to determine whether CD74/CD44 might form a cell surface complex with c-Met in B cells; to this end, we analyzed...
c-Met expression in B cells. T1, T2, MZ, and mature B cell populations were sorted using cell surface markers specific for each population (35), and c-Met message was followed by RT-PCR. As seen in Fig. 1A, all of the splenic B cell populations transcribed c-Met. To confirm that B cells express c-Met protein as well as mRNA, c-Met cell surface expression levels were analyzed in control and naive mature B cell populations, identified using various maturation markers. B cells, including the mature B cell population, expressed c-Met on their cell surfaces (Fig. 1B–D, Supplemental Fig. 1A, 1B). c-Met protein expression was also detected by Western blot analysis in the mature (B220<sup>+</sup>IgD+) population (Fig. 1F).

To identify complex formation between c-Met and CD74/CD44, control B cells were incubated in the presence or absence of HGF (10 ng/ml) for 18 h and subjected to PI staining. The first gate includes cells in the G0/G1 phase, and the second one includes cells in the S/G2/M phases. The graph shows an average of four independent experiments.

FIGURE 4. In vitro treatment with HGF induces survival and proliferation cascades in B cells. A, B220<sup>+</sup> B cells derived from control mice were incubated in the presence or absence of HGF (10 ng/ml) for various time periods. Immediately after stimulation, cell pellets were washed and fast-frozen in liquid nitrogen. The cells then were lysed, and an aliquot was reserved for total Syk analysis. Phosphorylated proteins from the remaining lysate were immuno-precipitated with an anti-Tyr(P) Ab. Immunoprecipitates and total lysate proteins were separated by 10% (w/v) SDS-PAGE and blotted with an anti-Syk Ab. The results presented are representative of at least three different experiments.

B, Control B cells, c-Met was specifically pulled down by anti-CD44 Ab. Immunoprecipitation of c-Met (Supplemental Fig. 1C) specifically coprecipitated CD74, mainly the [31P] isoform, showing that CD74 forms a complex with c-Met in B cells (Fig. 1F). This interaction was not observed in CD44- or CD44-deficient B cells (Fig. 1G, 1H), suggesting that CD44 is essential for the formation of the CD74/CD44/c-Met complex. To further confirm that c-Met forms a cell surface complex with CD44, CD44 was immunoprecipitated, and the coimmunoprecipitation of c-Met was confirmed by Western blot analysis. As shown in Fig. 1I, c-Met was specifically pulled down by anti-CD44 Ab, further indicating that c-Met engages with CD44 in B cells.

c-Met and HGF are MIF/CD74 target genes

To determine whether the survival cascade induced by CD74 is c-Met-dependent, we first investigated whether c-Met expression is modulated by CD74. B cells were stimulated with MIF for 8 h, and c-Met gene expression was analyzed by RT-PCR. As shown in Fig. 2A and 2B, c-Met mRNA levels were elevated after CD74...
activation. This elevation in c-Met mRNA levels was specific to MIF stimulation and did not occur in CD74- or CD44-deficient B cells (Fig. 2C). To follow c-Met protein levels in CD74-stimulated cells, c-Met protein and cell surface expression were analyzed after MIF stimulation. c-Met protein levels were upregulated after MIF stimulation, as detected by Western blot analysis (Fig. 2D, Supplemental Fig. 2D). Similarly, a specific elevation in c-Met cell surface expression after MIF stimulation was observed in control B cells using different c-Met Abs (Fig. 2E, Supplemental Fig. 2A–C), whereas there was no change in its levels in CD74-deficient B cells (Fig. 2E).

We next investigated whether HGF expression is modulated by CD74; to this end, we first measured HGF mRNA expression in control B cells. The T1 and mature B cell populations were sorted as described in Materials and Methods, and HGF mRNA was followed by RT-PCR. As shown in Fig. 3A, HGF expression was detected in both T1 and mature subpopulations. HGF expression in total B cells (B220+) and mature cells (B220+IgD+) was also confirmed by intracellular staining (Fig. 3B). We next examined whether HGF mRNA expression is modulated by MIF stimulation. As shown in Fig. 3C, a specific increase in HGF mRNA levels was detected in control B cells after CD74 stimulation, whereas no change was detected in CD74-deficient B cells. Moreover, CD74 stimulation elevated HGF intracellular expression (Fig. 3D) and secretion into the conditioned medium, as analyzed by ELISA assay (Fig. 3E, 3F). Together, these results demonstrate that stimulation of the CD74/CD44 complex expressed on B cells augments both c-Met cell surface expression and HGF secretion.

**HGF induces survival and proliferation cascades in B cells resulting in regulation of the peripheral B cell subsets**

Activation of the HGF/c-Met signaling pathway leads to cellular responses, including cell survival (22, 24, 27, 42, 43). To reveal whether HGF induces a survival cascade in B cells, we first followed the HGF-induced signaling cascade. After HGF stimulation, cells were lysed, and phosphorylated proteins were analyzed by Western blot analysis.

The BCR transduces Ag binding into alterations in the activity of intracellular signaling pathways through its ability to recruit and activate the cytoplasmic protein-tyrosine kinase Syk. The recruitment of Syk to the receptor, its activation, and its subsequent interactions with downstream effectors regulate B cell survival (44). In addition, we have recently shown that MIF induces Syk phosphorylation, also resulting in B cell survival (15, 16). Therefore, Syk phosphorylation after HGF stimulation was analyzed. B cells derived from control mice were incubated in the presence or absence of HGF for 0–5 min. The cells were then lysed, and Syk phosphorylation was analyzed. As can be seen in Fig. 4A, HGF stimulation augmented Syk phosphorylation.

Next, the role of HGF in B cell survival was examined. B cells were incubated in the presence or absence of HGF, and the expression of the antiapoptotic protein Bcl-2 was analyzed. As shown...
in Fig. 4B–E, HGF stimulation upregulated Bcl-2 mRNA (Fig. 4B–D) and protein (Fig. 4E) levels. To further determine whether HGF regulates B cell survival, the B cell apoptotic populations after HGF stimulation were analyzed by annexin/PI staining. As shown in Fig. 4F, HGF reduced B cell apoptosis, resulting in an elevation of the live population. Because B cells survive poorly in vitro and after incubation for 24 h a large proportion of the cells are already dead, we followed cell survival over a shorter time period. Therefore, cells were incubated in the presence or absence of HGF for 8 h, and their caspase 3 and 7 activity was analyzed as a marker of apoptosis. As shown in Fig. 4G, a significant reduction in the apoptotic population was detected in control B cells stimulated with HGF.

The possible regulation of B cell proliferation by HGF in vitro was studied by analyzing cell entry into the S phase. Cell cycle progression is regulated by cyclin-dependent kinases. We previously showed that CD74 activation induces expression of cyclin E (15, 16), which regulates cell entry into the S phase. To determine whether HGF also regulates cell entry to the S phase, cyclin E mRNA levels were followed. As demonstrated in Fig. 4H, HGF elevated the intracellular level of cyclin E mRNA. To further evaluate cell cycle entry and DNA synthesis after HGF stimulation, PI staining was performed. As shown in Fig. 4I, HGF stimulation induced the entry of B cells into the S phase. The HGF-induced survival pathway may control peripheral B cell subsets; B cells from control mice were therefore incubated with or without HGF for 18 h. Then, the different B cell populations were analyzed for the expression of various maturation markers. As shown in Fig. 5A and 5B, accumulation of the mature B cell subpopulation (IgD$^{high}$AA4$^+$) was observed after HGF stimulation, which resulted from elevated survival of the mature (AA4$^+$, Fig. 5C2, 5D2; CD21$^{low}$CD24$^{low}$, Fig. 5D1) population, whereas there was no change in the cell death of the immature (AA4$^+$, Fig. 5C3, 5D3) population. Thus, HGF preferentially promotes the viability of mature B cells. Next, to investigate whether HGF controls differentiation of the transitional B cell populations, purified T1 cells derived from a 1-wk-old mouse (Fig. 5E1; AA4$^+$, Fig. 5E2) B cells were incubated with or without HGF for 18 h and were analyzed for the expression of maturation markers. No change in the transitional and mature B populations was observed in HGF-stimulated cells. These results suggest that HGF regulates mature B cell survival and is not involved in the differentiation of transitional B cells into mature cells.

To follow the in vivo effect of HGF on B cells, we injected HGF (4 μg) into mice for 2 d and analyzed the apoptotic populations by annexin/PI staining of B cells recovered from the spleens. As demonstrated in Fig. 6A, a high proportion of live B splenocytes was detected in both HGF- and PBS-injected mice. However, HGF significantly reduced the apoptosis of B cells. To further evaluate cell division, cell cycle entry, and DNA synthesis after HGF injection, PI staining was performed. As shown in Fig. 6B, the

**FIGURE 6.** The HGF-induced survival pathway regulates peripheral B cell subsets in vivo. Control mice were injected with HGF (4 μg) or PBS for 2 d. A, Cell survival was analyzed by annexin V and PI staining of intact B220$^+$ cells. B, Cell cycle entry in B220$^+$ cells was analyzed by PI staining after cell fixation. The first gate includes cells in the G0/G1 phase, and the second one includes cells in the S/G2/M phases. The results presented are representative of at least three separate experiments. C–E, Control mice were injected with HGF (4 μg) or PBS for 2 d, and the B cell subpopulations then were analyzed for IgD and IgM (C); AA4 (D); and CD21, CD24, and CD23 (E) expression on B220$^+$ cells. The graph shows an average of four independent experiments. F and G, Control mice were injected with the c-Met inhibitor PHA-665752 (0.15 mg) twice per day for 5 d. B cell survival was analyzed by annexin V and PI staining of intact cells (F). B cell subpopulations were analyzed for IgD and AA4 expression on B220$^+$ cells (G). The results presented are representative of four separate experiments.
To determine whether the c-Met-induced survival cascade is a CD74 manner and suppression of apoptosis, which enlarge the mature B cell together, these results show that activation of c-Met induces cell division. As shown in Fig. 8C, Bcl-2 transcription levels were analyzed by means of RT-PCR. The results presented are representative of at least five different experiments. B, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Cells were lysed, and levels of Bcl-2 and tubulin were analyzed by Western blot analysis. The results presented are representative of at least three separate experiments. C, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Cell death was measured by Magic Red apoptosis detection kit. The graph shows an average of the results obtained from four independent experiments. D, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Nontreated cells were analyzed using maturation markers for C57BL/6 mice were injected with PHA-665752, a specific c-Met inhibitor. PHA-665752 is a selective, small-molecule, active-site inhibitor of the catalytic activity of c-Met kinase (Ki = 4 nM) that competes with its ATP binding. As shown in Fig. 6F and 6G, blocking c-Met in vivo induced apoptosis of B cells (Fig. 6F), resulting in the reduction of the mature population in the spleen (Fig. 6G). Together, these results show that activation of c-Met induces cell division and suppression of apoptosis, which enlarge the mature B cell compartment, whereas its blockade results in mature B cell death.

**CD74 induces B cell survival in an HGF/c-Met-dependent manner**

To determine whether the c-Met-induced survival cascade is a CD74 downstream event, we first tested whether exogenous HGF could bypass reduced survival in CD74−/− cells. CD74−/− B cells were treated in the presence or absence of HGF, and Bcl-2 mRNA and protein levels were evaluated. As shown in Fig. 7, HGF stimulation upregulated Bcl-2 mRNA (Fig. 7A) and protein (Fig. 7B) levels in cells lacking CD74. Next, CD74−/− B cells were incubated in the presence or absence of HGF for 18 h, and cell death was analyzed. As shown in Fig. 7C, a reduced apoptotic population was observed in HGF-stimulated cells. To directly determine whether HGF can shape B peripheral cell subsets by regulating the survival of the mature population, CD74−/− B cells were incubated in the presence or absence of HGF, and the various B cell populations were observed in mice treated with HGF or with c-Met inhibitor. C57BL/6 mice were injected with PHA-665752, a specific c-Met inhibitor, PHA-665752 is a selective, small-molecule, active-site inhibitor of the catalytic activity of c-Met kinase (Ki = 4 nM) that competes with its ATP binding. As shown in Fig. 6F and 6G, blocking c-Met in vivo induced apoptosis of B cells (Fig. 6F), resulting in the reduction of the mature population in the spleen (Fig. 6G). Together, these results show that activation of c-Met induces cell division and suppression of apoptosis, which enlarge the mature B cell compartment, whereas its blockade results in mature B cell death.

**FIGURE 7.** HGF transmits a survival signal in CD74-deficient cells, and stimulation with exogenous HGF restores the mature population missing in cells lacking CD74. A, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 8 h. Total RNA was isolated, and reverse transcription using primers for Bcl-2 or HPRT was carried out. The results presented are representative of at least five different experiments. B, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Cells were lysed, and levels of Bcl-2 and tubulin were analyzed by Western blot analysis. The results presented are representative of at least three separate experiments. C, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Cell death was measured by Magic Red apoptosis detection kit. The graph shows an average of the results obtained from four independent experiments. D, B220+ B cells derived from CD74−/− mice were incubated in the presence or absence of HGF (10 ng/ml) for 18 h. Cell subpopulations were analyzed by FACs. The graph shows an average of results obtained from four independent experiments.

Finally, we tested whether the formation of the CD74/CD44/c-Met cell surface complex is MIF-dependent. As shown in Fig. 8A, stimulation of B cells with MIF induced the coprecipitation of CD74 with c-Met, showing that MIF regulates the formation of the CD74/CD44/c-Met cell surface complex. Next, B cells were incubated in the presence or absence of the c-Met inhibitor PHA-665752 (0.3 ng/ml) for 3 h. After incubation with PHA-665752, the cells were stimulated with MIF for 0–5 min. Then, the cells were lysed, and Syk phosphorylation was analyzed. As shown in Fig. 8B, the c-Met inhibitor abolished MIF-induced Syk phosphorylation. Thus, c-Met activation is required for the MIF/CD74 signaling cascade.

We then analyzed the CD74-induced survival cascade in the presence of MIF and the c-Met inhibitor PHA-665752, as above. Bcl-2 transcription levels were analyzed by means of RT-PCR. As shown in Fig. 8C and 8D, addition of the c-Met inhibitor significantly downregulated the MIF-induced Bcl-2 mRNA levels. In addition, as shown in Fig. 8E, the MIF-induced support of cell survival was abolished in PHA-665752-treated cells. Finally, cells were stimulated with MIF in the presence or absence of an anti-HGF blocking Ab or an isotype control Ab, and Bcl-2 mRNA and protein levels were analyzed. As shown in Fig. 8F and 8G, although stimulation with MIF augmented Bcl-2 mRNA and protein levels, blocking of HGF significantly reduced this elevation. Downregulation of Bcl-2 transcription and expression was specific and did not occur in the cells incubated in the presence of an isotype control Ab. In addition, blocking of the secreted HGF by the anti-HGF blocking Ab abolished the MIF-induced elevation of survival. Thus, the MIF-dependent survival of mature cells is regulated by secreted HGF in an autocrine manner.

Together, these results show that blocking the HGF/c-Met pathway results in induction of cell death and inhibition of the MIF/CD74-induced survival cascade. Thus, HGF/c-Met axis functions as a CD74 downstream pathway, regulating B cell survival in an autocrine manner.

**Discussion**

In B cells, as in every other tissue, the balance between cell survival and apoptosis is essential for homeostasis. Adaptive immunity depends on the production and maintenance of a pool of mature peripheral lymphocytes throughout life. Most of these cells circu-
late in the periphery in a quiescent state, without actively contributing to a given acute immune response. This vast number of resting cells must be maintained to preserve a diverse B cell repertoire. Long-term B cell persistence in the periphery is dependent on survival signals that are transduced by cell surface receptors. Conversely, resistance to apoptosis, leading to enhanced survival, is associated with initiation and progression of B cell malignancies.

It was previously shown that the regulation of B cell homeostasis depends on tonic and induced BCR signaling and on receptors sensitive to trophic factors, such as BAFFR (BR3), during development and maintenance (1, 3, 4). Our previous studies demonstrated that survival of mature B cells is controlled by MIF. MIF is a ubiquitous protein that has a broad tissue distribution and is found in virtually all cells (46); thus, it undergoes tonic production in the spleen as well. Engagement of the CD74/CD44 complex by MIF triggers the antiapoptotic Syk–PI3K/Akt signaling pathway and thus promotes B cell survival (15, 16, 47). In this study, we identified two novel components of the CD74/CD44 B cell survival complex, the c-Met receptor and its ligand HGF.

**FIGURE 8.** c-Met regulates the CD74-induced survival cascade. A, B220+ B cells derived from control mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. Immediately after stimulation, cells were washed and fast-frozen in liquid nitrogen. The cells then were lysed, and an aliquot was reserved for total Syk analysis. Phosphorylated proteins from the remaining lysate were immunoprecipitated with an anti-Tyr(P) Ab. Immunoprecipitates and total lysate proteins were separated by 10% (w/v) SDS-PAGE and blotted with an anti-Syk Ab. B, B220+ B cells derived from control mice were incubated in the presence or absence of MIF (100 ng/ml) or the c-Met inhibitor PHA-665752 (0.3 ng/ml) for 8 h. Total RNA was isolated, and reverse transcription using primers for Bcl-2 or HPRT was carried out. The results presented are representative of at least three different experiments. C, B220+ B cells derived from control mice were incubated in the presence or absence of MIF (100 ng/ml) for 8 h. Total RNA was isolated, and reverse transcription using primers for Bcl-2 or HPRT was carried out. The results presented are representative of at least three different experiments. D, Quantitative real-time PCR was performed using primers for Bcl-2 and β-actin as described in Materials and Methods. β-Actin levels were used to normalize samples for calculation of the relative expression levels of c-Met. Results are expressed as fold change in Bcl-2 expression in stimulated cells compared with that in unstimulated cells, which was defined as 1. Results shown are a summary of three separate experiments. E, B220+ B cells derived from control mice were incubated in the presence or absence of MIF (100 ng/ml) or the c-Met inhibitor PHA-665752 (0.3 ng/ml) for 5 h. Cell death was analyzed by Magic Red and FLICA apoptosis detection kits. Graphs summarize results of three different experiments (E). After 12 h, cells were lysed, and levels of Bcl-2 and tubulin (loading control) were analyzed by Western blot analysis. The results presented are representative of at least three different experiments (E). After 12 h, cell death was analyzed by Magic Red apoptosis detection kits. The graph summarizes results of three different experiments (F).
subjected to various apoptotic inducers (48). HGF is a paracrine factor that is produced by stromal and mesenchymal cells (21, 49). We found that HGF can be produced by B cells, indicating a possible autocrine mechanism of c-Met activation. Although paracrine HGF appears to be sufficient for survival of CD74−/− B cells, we believe that because in CD74−/− mice B cell survival is nevertheless impaired, the autocrine pathway induced by MIF/CD74 plays an essential role in upregulating HGF secretion inducing optimal B cell survival.

Our results show that HGF does not affect differentiation of transitional cells in the spleen but rather induces a signaling cascade involving Syk, leading to B cell entry into the S phase and to cell survival of mature B cells. In addition, our studies show that the HGF/c-Met–induced survival cascade is regulated by the CD74/CD44 cell surface complex, because exogenous HGF can bypass the absent survival signal and rescue the mature population missing in cells lacking CD74. Moreover, blocking HGF or c-Met activity abolishes MIF-induced Syk phosphorylation and Bcl-2 elevation, thereby inhibiting cell survival.

The precise mechanism by which MIF activates c-Met is still unclear. It is possible that MIF binding to the CD74/CD44 complex can induce c-Met activation in an HGF-independent manner, as was shown in tumor cells (50, 51) or after cellular adherence (52). However, because HGF was shown in our studies to be sufficient to support survival of mature B cells and its blocking inhibits the MIF-induced survival pathway, we believe that HGF is involved in the MIF-induced survival cascade. We suggest that MIF activation induces transient CD74/CD44/c-Met complex formation. This can lead to induction of a signaling cascade leading to Syk phosphorylation and augmented transcription of genes, including the survival genes and c-Met and HGF. Subsequently, c-Met cell surface expression becomes elevated together with the augmented levels of HGF.

There is limited information regarding the expression and function of c-Met and HGF in naive mature B cells. Previous studies showed that human tonsillar and human peripheral B cells do not express c-Met (53–55) and HGF (55), whereas germinal center activated cells and plasma cells exhibit c-Met expression. These results suggest that B cell activation significantly elevates c-Met expression, whereas its expression in resting mouse and human splenic B cells might be lower. Our study shows that naive murine splenic B cells express c-Met and its ligand HGF. Thus, upon activation, it is likely that c-Met expression is significantly upregulated. Further studies will be required to compare c-Met expression in activated B cells versus that in naive murine or human cells.

One of the signaling proteins that plays a crucial role in B cell development is the Syk protein-tyrosine kinase. Syk belongs to the Syk/ZAP70 family of protein-tyrosine kinases and participates in B cell fate decisions and Ag processing. Recent findings indicate that expression of Syk in nonhematopoietic cells is involved in a wide variety of cellular functions and in the pathogenesis of malignant tumors. Syk was previously shown to be required for the activation of Akt in a PI3K-dependent manner. Syk is a critical component of this signaling machinery. Studies in knockout mice and cell lines indicate that Syk is essential for most of the biochemical responses to BCR engagement, including regulation of cell survival (44). Previously, we showed that MIF initiates a signaling cascade in mature B cells that involves Syk and Akt phosphorylation and is dependent on PI3K (16). In this study, we demonstrate that in B cells HGF stimulation leads to Syk phosphorylation. Blocking c-Met activity abolishes MIF-induced Syk phosphorylation. Therefore, c-Met appears to be necessary for the signaling mechanism induced by MIF in a Syk-dependent manner.

Our model suggests that both c-Met and HGF are CD74/CD44 target genes. After MIF stimulation, c-Met engages with CD74 and CD44 on the cell membrane and, together with HGF, triggers an additional signaling pathway, which is necessary to initiate the MIF-induced survival signaling cascade. It is interesting to note that both MIF/CD74 and HGF/c-Met pathways have been implicated in tumor progression. It has been reported that MIF is overexpressed in solid tumors (56, 57) and that its expression is associated with the growth of malignant cells (58). In addition, anti-MIF Ig therapy has been shown to suppress tumor growth (59). Many studies have demonstrated the overexpression of CD74 in various cancers (60–65), and CD74 has been suggested to serve as a prognostic factor in several tumor types, with higher relative expression of CD74 behaving as a marker of tumor progression (66). Moreover, a humanized anti-CD74 mAb (hLl1) was shown to have therapeutic activity in multiple myeloma, perhaps due to the high level of expression of CD74 in this plasma cell malignancy (67). Finally, we recently showed that activation of cell surface CD74, expressed at high levels from an early stage of B-chronic lymphocytic leukemia by MIF initiates a signaling cascade that contributes to tumor progression (68). Uncontrolled activation of c-Met is oncogenic and has been implicated in the growth, invasion, and metastasis of a variety of tumors in both mice and humans (69). It has been shown that receptor overexpression is by far the most frequent alteration of c-Met in human tumors (70). c-Met overexpression has been described in a variety of human cancers, including B cell malignancies, such as multiple myeloma (MM) (42, 71). Furthermore, in MM, Hodgkin lymphoma, and diffuse large B cell lymphoma, elevated serum HGF levels correlate with unfavorable prognosis (72, 73). Moreover, it was shown that HGF induces a potent proliferative and antiapoptotic response in MM cell lines and primary MMs (42, 43). Thus, CD74 and c-Met might operate together in supporting tumor cell survival.

These studies establish the function of the CD74/CD44/c-Met complex in delivering signals important for B cell survival and may provide a basis for therapy of B cell malignancies with enhanced specificity.

Acknowledgments

We thank Pfizer for accepting the proposal for this study and for providing the PHA-665752 (c-Met inhibitor) reagent. We also thank Prof. Tsve Lapidot and members of the Shachar laboratory team for helpful discussions and review of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

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