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Hemokinin-1 Activates the MAPK Pathway and Enhances B Cell Proliferation and Antibody Production

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Hemokinin 1 (HK-1) is a substance P-like tachykinin peptide predominantly expressed in non-neuronal tissues. In addition to a prominent function in lymphoid development, recent studies indicate a potential role for HK-1 in immunoregulation. The current study was focused on its action on mature B cells. Despite the negligible effect on its own, HK-1 exhibited a profound influence on B cell activation elicited by several classical signals, including LPS stimulation, BCR cross-linking, and CD40 ligation. Cells therefore showed enhanced proliferation, survival, and CD80/86 expression, and produced more IgM with a higher frequency of Ab-forming cells. Biochemical analysis revealed that HK-1 alone was sufficient to induce the activation of MAPKs and the expression of Blimp-1 and Xbp-1 in B cells. Nevertheless, costimulation with a known B cell activator resulted in much enhanced phosphorylation of MAPKs and transcriptional activation of Blimp-1 and Xbp-1. Overall, these data support that HK-1 provides an important costimulatory signal for B cell activation, possibly through synergistic activation of the MAPK pathway and induction of transcription factors critical for plasma cell differentiation. The Journal of Immunology, 2010, 184: 3590–3597.

Antigen-induced B cell activation and differentiation is an ordered cascade of cellular events (1–3). After Ag stimulation, naive B cells differentiate along either the extrafollicular or the germinal center (GC) pathway. The extrafollicular pathway gives rise to short-lived plasma cells which secrete germ-line-encoded Abs. The GC reaction is characterized by massive and rapid clonal expansion, somatic hypermutation and affinity-based selection, and class switching, leading to the production of long-lived plasma cells and memory B cells. GC-derived plasma cells home to the bone marrow where they continue to produce a large quantity of high-affinity Abs. Memory B cells persist after Ag stimulation, and mount rapid responses to Ag rechallenge. The Ag-driven B cell response is the consequence of the concerted action of a panoply of transcription factors, most notably Bcl-6, Blimp-1, Pax-5, and Xbp-1. Bcl-6 is absolutely required for the GC formation (4), whereas Blimp-1 is a master regulator of plasma cell differentiation (5). Pax-5, in contrast, is critical for the maintenance of B cell identity. The direct repression of Pax-5 by Blimp-1 releases its control over Xbp-1 (6), which in turn regulates the expression of a wide spectrum of genes important for professional secretory cells (7).

Effective Ab response depends on the integration of multiple signals. Although engagement of BCR by specific Ags initiates the cascade, non-Ag–specific stimuli have profound influence on the quantity and quality of the response (2, 3). TH cells secrete a variety of cytokines. Among many other activities, they are particularly important for class switching in vivo. IL-4, for example, drives the switch to IgG1 and IgE (8), whereas IFN-γ and TGF-β are implicated in the induction of IgG2a and IgA, respectively (8, 9). T cell help in B cell response is further manifested by the signaling capacity of a diverse array of cell surface molecules (10). Most prominent is the interaction between CD40 on B cells and CD40L on activated T cells, which is required for initiation of the GC reaction as well as class switching to non-IgM isotypes in the extrafollicular pathway (11). In addition to Th-derived signals, other factors in the microenvironment contribute to B cell activation and differentiation. BAFF, which is primarily produced by myeloid cells, not only have a substantial influence on the generation and maintenance of preimmune B cell pool (12, 13), but are also implicated in the regulation of B cell immune response (14, 15). More intriguingly, a recent study by Medzhitov and colleagues suggested that generation of T-dependent Ag-specific Ab responses requires TLR signal, such as the one elicited by LPS, in B cells (16). It is anticipated that optimal humoral response may involve additional factors.

Tachykinin defines a large group of 10–12 aa neuropeptides characterized by the presence of a common C-terminal motif, Phe-X-Gly-Leu-Met-NH2 (17–19). Substance P (SP) represents the best known member of this family. In mammals, tachykinins are implicated in a wide variety of biological actions, such as smooth muscle contraction, vasodilation, pain transmission, neurogenic inflammation, and immune regulation. Most of these activities are mediated by one of the three known G-protein coupled neurokinin receptors, NK-1, NK-2, or NK-3 (18). Hemokinin-1 (HK-1) is the most recent addition to the tachykinin family (20). Structurally, it most closely resembles SP in that both have multiple cationic residues at the N terminus and an aromatic amino acid occupying the variable X position of the tachykinin motif. Moreover, HK-1 demonstrates similar binding affinity and preference for the SP receptor, NK-1 (21–26). However, there are remarkable differences in tissue distribution between HK-1 and SP. Although SP expression is largely restricted to neuronal tissues, HK-1 shows a much broader distribution in peripheral tissues (21, 24, 27, 28). The distinct expression pattern but similar receptor selectivity leads to the speculation that HK-1 may represent the naturally occurring peripheral SP-like agonist (29).

The physiological function of HK-1 remains to be determined. Data currently available argue for an active role in lymphoid development. HK-1 promoted the proliferation and survival of lymphoid
precursors in vitro, and blocking its action with the NK-1 antagonist impaired lymphoid development in vivo (20, 30). Echoing these results, Wang and Clarke reported that HK-1 was important for the generation of the pre-BII compartment in a BCR transgenic mouse model. More interestingly, they found that the HK-1 transcription level was directly correlated with the surface density of the pre-BCR, suggesting a link between the pre-BCR signal and HK-1 signal (31). In contrast to the relatively abundant data on developing lymphocytes, the impact of HK-1 on mature lymphocytes is completely elusive. Prompted by the reviving interest in the immune regulatory function of tachykinins in recent years, the current study was undertaken to explore the potential influence of HK-1 on the activation of mature B cells, including their proliferation, survival, and differentiation to plasma cells. Furthermore, efforts were made to determine how HK-1 signals in B cells and how it is integrated with classical B cell activation signals.

Materials and Methods

Mice

C57BL/6, C3H/He and C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained in the animal breeding facility at Peking University Health Science Center under specific pathogen-free conditions. The experimental procedures on use and care of animals had been approved by the ethics committee of Peking University Health Science Center. All animals were used at the age of 6–8 wk.

B cell isolation and culture

Splenic B cells were isolated using anti-B220 coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instruction. The purity was routinely >95% as indicated by flow cytometric analysis. Memory B cells, defined as B220+IgM+IgD-, were isolated from the spleen by fluorescence activated cell sorting (FACSaria, BD Biosciences, San Jose, CA) with a purity >98%. Purified B cells were cultured in Opti-MEM (Invitrogen, San Diego, CA) supplemented with 10% FCS and gentamycin (200 U/ml). LPS (Sigma-Aldrich, St. Louis, MO), goat anti-mouse IgM (ab1523; fragment Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-mouse polyvalent IgGs (whole molecule) (Sigma-Aldrich), anti-CD40 (BD Pharmingen, San Diego, CA), HK-1 (Chinese peptide, Hangzhou, China), SP (Sigma-Aldrich), or NK-1 receptor antagonist L-732,138 (Sigma-Aldrich) was added to the culture at concentrations as specified in the Results.

Analysis of cell proliferation

Cell proliferation was analyzed by [3H]thymidine incorporation or CFSE dilution. For [3H]thymidine incorporation, B cells were cultured in 96-well plates at 2 × 10^5 cells/well under various conditions for 72 h, and [3H] thymidine (0.5 μCi/well) was included in the culture in the last 12 h. Cells were then harvested, and thymidine incorporation was measured in a β-scintillation counter (Beckman, Fullerton, CA). For CFSE dilution assay, splenic B cells were incubated with 5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C, and then washed with medium containing 10% FCS. CFSE-labeled cells were cultured under various conditions and harvested on day 3. Data were acquired on FACSCalibur (BD Biosciences) and analyzed using the Modfit software (Verity Software House, Topsham, ME).

Flow cytometric analysis

Cells were stained with B220-APC (RA3-6B2), CD80-FITC (16-10A1), CD86-PE (GL1), or CD138-PE (281-2). Appropriate isotype-matched Abs were included for compensation adjustment. All fluorochrome-labeled Abs were purchased from BD Pharmingen. Data acquisition and analysis was performed on FACSCalibur using the CellQuest software (BD Biosciences). Dead cells were excluded on the basis of low forward-light scatter (FSC) and propidium staining.

ELISA

 Supernatant were collected from d 7 cultures, and the Ig levels were determined using ELISA. Briefly, 96-well high-binding-capacity plates (NUNC) were coated with anti-IgA (C10-3), anti-IgG1 (A85-3), anti-IgG2a (R11-89), anti-IgG2b (R9-91), anti-IgG3 (R2-38), or anti-IgM (II/41) Abs (500 ng in 100 μl) (BD Pharmingen) at 4°C overnight. After blocking with PBS containing 1% BSA, the culture supernatant (100 μl) was added to the plate and allowed to incubate for 3 h at room temperature. The bound Abs were then detected with alkaline phosphatase-conjugated goat anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) (Sigma-Aldrich) using p-nitrophenyl phosphate disodium hexahydrate (Sigma-Aldrich) as substrate. OD was read at 405 nm and converted into absolute amount using a standard curve obtained with IgGs of known concentrations.

ELISPOT assay

ELISPOT assay was performed to determine the frequency of Ab-secreting cells. B cells were first exposed to various stimulators for 48 h, and then harvested and replated at a density of 2.5 × 10^6 cells/well into a flat-bottomed 96-well nitrocellulose plate (Millipore, Bedford, MA), which was precoated with goat anti-mouse IgG/A/M (H&L) Abs (AbD Serotec, Oxford, U.K.) and blocked with Opti-MEM medium containing 10% FCS. The cells were cultured with the original stimulator(s) for another 24 h before being washed off. After incubation with biotin-conjugated anti-IgG/A/M (H&L) Abs (AbD Serotec) for 2 h at 37°C, the plates were developed with streptavidin–alkaline phosphatase (Mabtech, Nacka, Sweden) using 5-bromo-4-chloro-3-indoly1 phosphatetetrazolium as substrate. After the final wash, the dark-violet spots on the plate membrane were counted under the microscope. The averages of the spots in duplicate wells were calculated and expressed as the number of Ab-secreting B cells.

Detection of mRNA expression by RT-PCR

RNA was prepared from mouse brain tissues, freshly isolated splenic B cells, or B cells cultured under various conditions for 4 d using TRizol reagent (Invitrogen). Gene expression was determined by RT-PCR. The primer pairs used for PCR were as follows: 5’-GCC AAC CAC GAA CTT CTG GTG T-3’ and 5’-TGTT ATG ATA AAC CAC CGG AAG GT3’-TG for Blimp-1; 5’-CAG CTA CAG ACC TCT G-3’ and 5’-TAT TGC ACC ATT GTG GTG G-3’ for Bcl-6; 5’-AAA CAG AGT AGC AGC GCA GCA GAC TGG-3’ and 5’-TCC TTC TGG GTG GTA CAT CTT GAG GAG-3’ for Xbp-1; 5’-GTC CCA GCT TCC AGT AGC AGG-3’ and 5’-CAT GGC TGA ATA CTC TCT GGT-3’ for Pax3; and 5’-TCTG GCA GCA CCA CCT CTT CA-3’ and 5’-TCG TAC TCC TGC TGG CTG ATC-3’ for β-actin. The amplification was carried out for a total of 35 cycles for Blimp-1, Bcl-6, Xbp-1, and Pax5 or 25 cycles for β-actin.

Western blot analysis

Analysis of ERK, p38, and JNK phosphorylation was performed as described (32). Briefly, the purified splenic B cells at a density of 1 × 10^6 cells/ml were stimulated at 37°C. At various time points, cells were harvested and lysed in 20 mM Tris (pH 8.0), 137 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM EDTA, 1 mM Na3VO4, 1 mM tetrasodium pyrophosphate, and 10 mM NaF. The lysate was clarified by centrifugation, and the protein concentrations were determined by the Bradford assay. Samples at 40 μg protein/lane were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with anti–Phospho-p44/p42 MAPK, anti–Phospho-p38, or anti–Phospho-JNK Abs (Cell Signaling Technology, Danvers, MA). The Ab bound to the membrane was detected using IRDye 800CW conjugated goat anti-rabbit IgG (LI-COR Biosciences, Berlin, Germany). After stripping, blots were reprobed with Abs against total p44/42 MAPK, p38 or JNK (Cell Signaling Technology) to ensure equal loading.

Calcium flux

B cells were added into poly-l-lysine–coated glass bottom microwell dishes designed for confocal microscopic examination (MatTek, Ashland, MA) at a density of 1 × 10^6 cells/ml, and allowed to attach for 12 h. The cells were then loaded with Fluor-3 acetoxyethyl ester (Biotium, Hayward, CA) at 37°C for 60 min. Intracellular Ca2+ concentration was measured by monitoring the fluorescence of individual cells at 525 nm using a laser scanning confocal microscope (DM IRB, Leica Microsystems, Deerfield, IL). Baseline fluorescence were assessed for 30 s before adding anti-IgM (200 ng/ml), HK-1 (0.5 μM), or anti-IgM plus HK-1. Image recording continued for additional 10 min after stimulation. Ca2+ concentrations were expressed as the average fluorescence intensity of 15–20 cells randomly picked up from at least two fields.

Statistical analysis

Unpaired Student t test was performed using GraphPad Prism software (GraphPad, La Jolla, CA). Differences were considered significant when p values were <0.05. All data are representative of at least three independent experiments.
Results

HK-1 promotes the proliferation and survival of activated B cells

To investigate the potential effect of HK-1 on peripheral B cells, splenic B220<sup>+</sup> cells were isolated using anti-B220–coated microbeads. The cells were then put into culture with or without HK-1, and their proliferation was measured by [H]thymidine incorporation. At all concentration tested from 10 nM to 1 μM, HK-1 had a noticeable effect on cell proliferation on its own (Fig. 1A and data not shown).

We next examined whether HK-1 modulated B cell activation induced by known stimulators. Anti-IgM, anti-CD40, and LPS were chosen as representatives. Titration was first performed to determine the suboptimal concentration showing a minimal proliferative effect, which was 200 ng/ml for anti-IgM, 10 ng/ml for anti-CD40, and 100 ng/ml for LPS. As shown in Fig. 1A, addition of HK-1 resulted in a distinctive increase in the proliferation induced by these classical B cell stimulators at suboptimal concentrations. A similar but less robust effect was also observed for SP in LPS-induced proliferation. Notably, the HK-1-mediated enhancement was blocked by an NK-1 antagonist, L-732,138 (Fig. 1B). Moreover, no synergistic effect was observed between LPS and HK-1 with B cell from the TLR4-defective C3H/HeJ mouse (Fig. 1C). To extend this observation to memory B cells, B220<sup>+</sup>IgM<sup>hi</sup>IgD<sup>+</sup> splenic B cells were isolated, and then stimulated with anti-polyvalent Ig, anti-CD40, or LPS in the presence or absence of HK-1. Notably, HK-1 demonstrated an even more potent effect on this subpopulation (Fig. 1D).

The increased thymidine incorporation might result from accelerated cell cycle progression, improved cell survival, or both. To distinguish these possibilities, we monitored cell division using CFSE dilution assay. Approximately 25% of cells underwent one or more generations of cell division with suboptimal concentrations of LPS, anti-IgM, or anti-CD40 with or without the addition of HK-1. As shown in Fig. 2A, LPS-induced proliferation of B cells preactivated by conventional stimuli to enhance their survival. Taken together, these results suggest that both enhanced cell cycle progression and cell survival may contribute to the increased thymidine incorporation.

HK-1 enhances costimulatory molecule expression in activated B cells

B cell activation is often accompanied by increased expression of costimulatory molecules CD80 and CD86. The surface expression of CD80 and CD86 was measured 72 h after stimulation with suboptimal concentrations of LPS, anti-IgM, or anti-CD40 with or without the addition of HK-1. As shown in Fig. 3A, LPS and anti-IgM–induced expression of CD80 and CD86 was further elevated by the addition of HK-1, whereas HK-1 seemed to preferentially affect CD86 expression in anti-CD40–stimulated cells. Importantly, such an effect was largely diminished in the presence of L-732,138 (Fig 3B), indicating the involvement of NK-1 receptor.

HK-1 increases IgM secretion by activated B cells

HK-1 was further evaluated for its impact on Ab secretion by activated B cells. Seven days after stimulation with suboptimal concentrations of LPS, anti-IgM, or anti-CD40, Ig in the culture supernatant was quantified by ELISA. Probably because of the weak strength of the stimuli, IgA and IgG were barely detectable in the culture, and showed no difference under the various conditions (data not shown). IgM secretion, in contrast, was readily detected, albeit at a level much lower than that with full stimulation. The presence of HK-1 markedly increased IgM production by LPS- or anti-CD40–stimulated B cells. A slight but statistically insignificant increase was also observed after costimulation with anti-IgM and HK-1 (Fig. 4A). SP mimicked the effect of HK-1, whereas L-732,138 suppressed the effect mediated by HK-1 (Fig. 4B).

The increased levels of IgM in the culture could be either due to the increased frequency of Ab-producing cells or the enhanced IgM

**FIGURE 1.** The synergistic effect of HK-1 on B cell proliferation. A, B220<sup>+</sup> splenic B cells were stimulated with suboptimal concentration of LPS (100 ng/ml), anti-IgM (200 ng/ml), or anti-CD40 (10 ng/ml) in the presence (0.5 μM) or absence of HK-1 or SP for 72 h. Cell proliferation was determined by thymidine incorporation. B, LPS-induced cell proliferation in the presence of HK-1 with or without the NK-1 antagonist L-732,138 (0.05 μM). C, LPS-induced proliferation of B cells from C3H/He or C3H/HeJ mice in the presence or absence of HK-1. D, The proliferation of B220<sup>+</sup>IgM<sup>hi</sup>IgD<sup>+</sup> memory B cells in response to LPS, anti-CD40, or antipolyvalent IgS with or without the addition of HK-1. Data from at least three independent experiments are presented as means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
production by individual cells. ELISpot assay was therefore performed to determine the number of Ab-secreting cells in the culture. Given the high background commonly associated with long-term culture in the nitrocellulose plates, B cells were first grown in regular 96-well plates for 48 h before being transferred into nitrocellulose plates for continued culture for another 24 h. In comparison with cultures stimulated by LPS or anti-CD40 alone, addition of HK-1 or SP resulted in a 2- to 3-fold increase in the number of spots (Fig. 4C). Again, this effect could be blocked by L-732,138 (Fig. 4D). Intriguingly, the spot number was also found to be markedly increased in cultures stimulated with anti-IgM and HK-1 even though this combination showed no significant effect on the secretion of total IgM (Fig. 4A,4C). The reason for this discrepancy is not clear.

HK-1 enhances LPS-induced plasma cell differentiation

B cell activation by LPS has been widely adopted as an in vitro model to study plasma cell differentiation. A small number of B220+ CD138+ cells were generated after suboptimal stimulation with LPS. Addition of HK-1 to the culture led to a 3-fold increase of this population (Fig. 5A), supporting a potential role of HK-1 in the terminal differentiation of activated B cells into Ab-secreting plasma cells.

It is well established that plasma cell differentiation depends on the coordinated expression of several transcription factors, namely, the induction of Blimp-1 and Xbp-1, and the suppression of Bcl-6 and Pax-5 (33). We examined mRNA expression of these transcription factors in response to HK-1 and/or LPS. RT-PCR demonstrated that HK-1 alone led to a significant induction of Blimp-1 and Xbp-1. When HK-1 and LPS were applied together, a much stronger expression was achieved (Fig. 5B). The induction of Blimp-1 and Xbp-1 by HK-1 alone is of particular interest in view of the apparent lack of Ig secretion under similar conditions (Fig. 4A). It may well be possible that the full maturation and functioning of plasma cells requires more than the activation of Blimp-1 and Xbp-1. The expression pattern of Bcl-6 and Pax-5 was more intriguing. Resting B cells lost the expression of both factors after 4 d culture in vitro. However, a low level of Pax-5 expression was maintained in the presence of HK-1 and/or LPS (Fig. 5B). This seemed to be contradictory to the expected downregulation of Pax-5 in plasma cells. We speculate that it may be due to the heterogenous nature of the culture. After all, plasma cells only accounted for a small fraction of the culture as indicated by CD138 staining (Fig. 5A).

HK-1 activates the MAPK signaling pathway in B cells

Next, we sought to determine how HK-1 signals to B cells and how the HK-1–mediated signal is integrated with other B cell activation signals. One of the earliest events after engagement of tachykinin receptors is the hydrolysis of phosphoinositides and Ca2+ mobilization (18). HK-1 was also found to be able to induce an increase of intracellular [Ca2+] in B cells. But in contrast to the rapid and robust Ca2+ response observed in many NK-1–expressing cells (21–24,
the HK-1–induced Ca^{2+} influx in B cells was slow and modest. We further examined the synergism between HK-1 and anti-IgM in the induction of Ca^{2+} influx. As shown in Fig. 6A, anti-IgM induced a typical spike of intracellular [Ca^{2+}], which rapidly retreated. In the presence of both anti-IgM and HK-1, however, an elevated level of intracellular [Ca^{2+}] was maintained for a much protracted period.

**FIGURE 3.** The impact of HK-1 on CD80 and CD86 expression. A, B220+ splenic B cells were stimulated with suboptimal concentration of LPS (100 ng/ml), anti-IgM (200 ng/ml), or anti-CD40 (10 ng/ml) in the presence (0.5 μM) or absence of HK-1 for 72 h. CD80 and CD86 expression was determined by flow cytometry. B, Blockade of HK-1 effect on LPS-induced CD80 and CD86 expression by the NK-1 antagonist L-732,138 (0.05 μM). The experiments were repeated 3–5 times with similar results. The histogram from one representative experiment is shown.

**FIGURE 4.** Modulation of IgM secretion by HK-1. A and B, B220+ splenic B cells were cultured for 7 d in media containing LPS (100 ng/ml), anti-IgM (200 ng/ml), anti-CD40 (10 ng/ml), HK-1 (0.5 μM), SP (0.5 μM), L-732,138 (0.05 μM), or combinations of them. IgM secretion was measured by ELISA. C and D, Cells were first cultured for 48 h under conditions as specified previously, and then replated at a density of 2.5 × 10^4 cells/well. The frequency of Ab-forming cells was determined by ELISPOT assay. Data from at least three independent experiments are present as means ± SD. *p < 0.05; **p < 0.01.
The signaling cascade downstream of Ca²⁺ influx is overall poorly defined for tachykinins. In consideration of the importance of the MAPK pathway in B cell activation (35) and the potential link between the Ca²⁺ and MAPK pathways (36, 37), our subsequent analysis of HK-1 signaling in B cells was focused on the activation of MAP kinases. As shown in Fig. 6B, HK-1 and LPS each induced significant phosphorylation of ERK and JNK, and in combination they induced even higher levels of phosphorylated ERK and JNK. The synergism was blocked by adding the NK-1 antagonist L-732,138, whereas SP could substitute HK-1 for such an effect. With respect to p38, there was a relatively high basal level of phosphorylation. Stimulation with either HK-1 or LPS alone caused no obvious change, but increased phosphorylation did occur with HK-1 and LPS (Fig. 6B).

The synergistic effect between HK-1 and LPS was more clearly illustrated by analyzing the kinetics of ERK activation. Phosphorylation induced by either HK-1 or LPS reached its highest level around 30 min after stimulation, and gradually decreased afterward. In the presence of both stimuli, ERK phosphorylation occurred more rapidly, reaching the highest level within 5–10 min. More strikingly, this high level of phosphorylation was maintained over the next 48 h (Fig. 6C). Therefore, the response induced by costimulation with HK-1 and LPS was not only more rapid and robust but also more sustainable.

**Discussion**

Tachykinins are traditionally viewed as neuropeptides with well-defined functions as neurotransmitters. But evidence is accumulating that they may also play an important role in the regulation of immune responses. SP and its receptor NK-1 are detected in various types of immune cells, and their expression is modulated by a number of inflammatory stimuli. More importantly, the NK-1–mediated signal affects multiple aspects of immune cell function (38–41). As the first tachykinin peptide primarily expressed in non-neuronal tissues, the immunoregulatory function of HK-1 is of considerable interest. In T cells, HK-1 was found to enhance IFN-γ production after Ag stimulation with equal potency to SP (42). More recently, Janelsins and colleagues demonstrated that both HK-1 and SP rescued bone marrow-derived dendritic cells (DCs) from apoptosis induced by the deprivation of growth factors, probably by activating the PI3K-Akt pathway. Furthermore, they showed that NK-1–signaled DCs exhibited increased longevity in vivo, leading to enhanced and prolonged effector cellular immunity (43).

The current study provides evidence that HK-1, by acting as a costimulatory factor, has profound influence on B cell function. Although it showed no obvious effect on its own, HK-1–enhanced B cell function was more rapid and robust than that induced by LPS alone.
cell activation induced by suboptimal concentrations of classical stimuli, such as LPS, anti-IgM or anti-CD40, as demonstrated by the accelerated cell proliferation, improved cell survival, upregulated expression of costimulatory molecules, and increased secretion of IgM Abs. The synergistic effect seems to be specific as it could be effectively blocked by an NK-1–specific antagonist. Consistent with this notion, we were able to detect NK-1 but not NK-2 or NK-3 mRNA in B cells (data not shown). Like in many other assays (21, 23, 25, 26, 42, 43), HK-1 activity on B cells could be fully reproduced with SP, which is in accord with several earlier reports documenting enhanced Ig secretion in the presence of SP (44). Given the apparent functional overlap, an important issue is the tissue distribution of the two peptides. Duffy et al. directly compared the expression levels of HK-1 and SP in a wide range of mouse tissues using quantitative RT-PCR. Although HK-1 mRNA was found in the majority of tissues examined, SP had a more restricted distribution (highly present in the brain, but absent from the spleen) (21). As much as immune cells were concerned, constitutive HK-1 expression was readily detected in DCs and macrophages in comparison with a much lower level of SP mRNA in the same unstimulated cells (45). This differential expression pattern makes HK-1 a more likely mediator for the regulation of Ab responses in vivo.

We further explored the biochemical basis for the synergistic effect of HK-1 in B cell activation using costimulation with LPS and HK-1 as an example. LPS and HK-1 each induced the activation of ERK and JNK. When applied together, much enhanced phosphorylation of ERK and JNK was observed. Kinetic analysis of ERK activation indicated that the costimulation not only resulted in increased ERK phosphorylation, but also led to a much more rapid and sustained response. As to the other member of the MAPK family p38, neither LPS nor HK-1 affected the basal level of phosphorylation. Nevertheless, their coadministration caused a slightly increased phosphorylation. We also examined the cooperation between HK-1 stimulation and BCR or CD40 ligation in the activation of MAPKs. Similar results were obtained (data not shown). Therefore, the MAPK pathway may serve as an integration point for the HK-1–mediated signal and other B cell activation signals. Given the pivotal role of the MAPK pathway in the B cell response (35), the enhanced phosphorylation of ERK, JNK, and p38 in the presence of HK-1 should be at least partly responsible for the synergism in cell activation.

B cell activation ultimately leads to the generation of Ab-secreting plasma cells. Several transcription factors are critically involved in the plasmacytic differentiation (33). Centrally positioned in this process is the developmental feedback loop formed between Bcl-6 and Blimp-1. In GC B cells, Bcl-6 expression inhibits Blimp-1 expression and plasmacytic differentiation. Once Bcl-6 is downregulated and Blimp-1 becomes activated, post-GC cells undergo irreversible plasmacytic differentiation by extinguishing gene expression important for proliferation, GC reaction, and initiating expression of important plasma cell genes such as Xbp-1. It remains to be determined how Bcl-6 is initially downregulated in a GC B cell. But several studies indicate the implication of MAPK–mediated phosphorylation and proteosomal degradation of Bcl-6 (46, 47). In this context, it is interesting to note that HK-1 provides an independent signal for the activation of the MAPK pathway and the induction of Blimp-1 and Xbp-1. Unfortunately, the in vitro assay used in this study did not allow us to test the direct effect of HK-1 on Bcl-6 expression.

The signal pathway for HK-1 is largely unresolved. Pharmacological studies suggest that HK-1 and SP may share the same receptor NK-1 (21–26). The B cell response to HK-1 may also be mediated by NK-1 as it could be effectively blocked with an NK-1–specific antagonist. Like in many other HK-1–responsive cells (21–24, 34), HK-1 stimulation of B cells induced Ca²⁺ influx. But the response appears to be slow and modest in B cells. Other than Ca²⁺ mobilization, little is known about HK-1 signaling. Several studies have demonstrated that SP or its analog induced ERK phosphorylation, protein kinase C translocation, or the activation of the PI3K-Akt pathway in different cell types (43, 48). In this study, we showed that HK-1 was a potent activator of the MAPKs in B cells. More intriguingly, signals from HK-1 and other B cell stimuli seem to converge at the level of MAPK phosphorylation.

In summary, the current study has demonstrated that HK-1 acts as a costimulatory factor in B cell activation. In collaboration with other B cell activators, HK-1 promotes B cell proliferation and Ab production, possibly through the synergistic activation of the MAPK pathway. In addition, HK-1 provides an independent signal for the initiation of plasmacytic differentiation by inducing Blimp-1 and Xbp-1 expression. The studies by us and others on different types of immune cells all point to a substantial and overlapping role for HK-1 and SP in immune regulation. It warrants further exploration which peptide actually mediates a specific function in vivo.

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Disclosures
The authors have no financial conflicts of interest.

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