

Insulin and insulin-like growth factor-1 promote mast cell survival via activation of the phosphatidylinositol-3-kinase pathway

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Objective. Mast cells (MCs) play central roles for the onset and development of immediate-type and inflammatory allergic reactions. Since the inverse relationship between atopic disorders and diabetes mellitus has been observed in animals and humans, we investigated the effects of insulin (Ins) on MC signaling and biological function.

Methods. In bone marrow-derived MCs (BMMCs) from wild-type as well as SHIP-deficient mice Ins as well as insulin-like growth factor-1 (IGF-I)-triggered intracellular signaling events and MC effector functions were studied.

Results. We found that the addition of either Ins or IGF-1 to BMMCs triggers the phosphorylation of protein kinase B (PKB) and p38 kinase but not extracellular signal-regulated kinase (Erk). We also found that Ins/IGF-1 stimulates the tyrosine phosphorylation of SHIP1 and, in keeping with this, Ins/IGF-1-induced PKB phosphorylation is higher in SHIP1^{-/-} BMMCs and is inhibited in SHIP1^{+/+} as well as SHIP1^{-/-} BMMCs with inhibitors of phosphatidylinositol-3-kinase (PI3K). Ins/IGF-1, like antigen (Ag), also stimulates the Rac-dependent activation of PAK as well as the production of hydrogen peroxide (H₂O₂). To elucidate the role of Ins and IGF-1 in MC biology, we studied their effects on Ag-mediated degranulation and MC survival. Although both only slightly enhanced Ag-mediated degranulation, they significantly promoted MC survival in the absence of IL-3 in a PI3K-dependent manner.

Conclusion. The promotion of BMMC survival by induction of Ins/IGF-1 signaling may, in part, be responsible for the inverse correlation observed between atopic disorders and diabetes mellitus. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Antigen (Ag)-mediated activation of mast cells (MCs) via engagement of high-affinity IgE receptors (FcεR1) constitutes an important initial event in the development of type I hypersensitivity reactions [1]. The FcεR1 on MCs is comprised of an α-, a β-, and two disulfide-bridged γ-subunits [2]. Whereas the α-subunit binds to the IgE molecule [3], the β- and γ-subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular domains and mediate the signal transduction of this receptor [2].

Important IgE-induced signaling pathways that lead to the release of preformed pro-inflammatory mediators are,

among others, the PLC-γ pathway, which leads to the release of calcium from intracellular stores, and the phosphatidylinositol-3-kinase (PI3K) pathway, which enhances extracellular calcium entry and PKC activation [2]. The importance of the PI3K pathway was demonstrated in particular by analyzing bone marrow-derived MCs (BMMCs) from mice deficient for p110δ^{PI3K} or the SH2-containing inositol-5'-phosphatase, SHIP1, the major negative regulator of PI3K in BMMCs. Whereas p110δ^{PI3K} BMMCs were greatly impaired in Ag-induced degranulation [4], SHIP1^{-/-} BMMCs were found to be much more prone to Ag-mediated degranulation than wild-type BMMCs [5,6] and even degranulated under conditions where normal MCs did not, i.e., following stimulation with Steel Factor (SF) or IgE alone [5,7]. These studies established SHIP1 as a crucial gatekeeper of MC degranulation.

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Under physiological conditions, every cell is surrounded by a milieu of various different soluble (e.g., cytokines, chemokines, and growth factors) as well as cell-bound factors (e.g., adhesion molecules and membrane-anchored growth factors). These factors can modulate the response of a cell to any given stimulus and collectively, the response of a cell will be determined by the net effect of all integrated signals. Thus, with respect to the control of MC degranulation, it is important to study soluble factors already known to bind to MCs for their ability to influence FcεR1-triggered signal transduction. For instance, it is known that SF is not able to cause MC degranulation by itself, but acts in a synergistic fashion with Ag [8,9]. This appears, at least in part, to be due to the ability of SF to augment Ag-induced PI3K activation [8] and NTAL phosphorylation in MCs [9].

In addition to factors already documented to bind to receptors on MCs, it is important to identify new ligand/receptor systems and study their effects on MC activation. Since mast cells reside in different tissues, they encounter many different stimuli, which might change in quality and quantity under pathological conditions. Of particular interest, as learned from the SF example, are receptors known to activate the PI3K pathway. Receptors of the insulin (Ins) receptor (IR) family are known to strongly activate the PI3K pathway by means of tyrosine phosphorylation of the Ins receptor substrate proteins, IRS1 and 2 [10]. Interestingly, data from recent and previous epidemiological studies in children with type I diabetes mellitus indicate an inverse relationship between atopic disorders (asthma, eczema, and hay fever) and diabetes mellitus [11–13]. In keeping with this, diabetic rats have been reported to exhibit markedly reduced inflammatory responses to allergen challenge in the airways and the pleural space, which can be reversed by treatment of the animals with Ins [14,15]. Furthermore, a 50% reduction in the number of degranulated MCs and in histamine release has been observed in bronchial segments isolated from diabetic rats [16]. These data prompted us to analyze the signaling response of BMMCs to Ins and its effects on MC biology.

In this report we present evidence that BMMCs can be activated by both Ins and Ins-like growth factor-1 (IGF-1). However, the activation pattern known to occur in muscle, fat, and liver cells is not observed in BMMCs. Specifically, while Ins stimulates a robust activation of the PI3K pathway in BMMCs in a SHIP1-controlled manner, it does not activate the extracellular signal-regulated kinase (Erk) pathway. We also show that both Ins and IGF-1 promote BMMC survival in the absence of interleukin (IL)-3, and this may, in part, be responsible for the inverse correlation observed between atopic disorders and diabetes mellitus.

Materials and methods

Cell culture

Bone marrow cells (1×10^6 /mL) from 6- to 8-week-old male mice (129/Sv) were cultured (37°C, 5% CO₂) in a single cell suspension

in RPMI 1640 medium containing 20% fetal calf serum (FCS), 1% X63Ag8-653-conditioned medium as a source of IL-3 [17], 2 mM L-glutamine, 1×10^{-5} M 2-mercaptoethanol, 50 units/mL penicillin, and 50 mg/mL streptomycin. At weekly intervals, the nonadherent cells were reseeded at 1×10^6 cells/mL in fresh medium. By 4 to 6 weeks in culture, more than 99% of the cells were c-kit- and FcεR1-positive as assessed by phycoerythrin-labeled anti-c-kit antibodies (Pharmingen, Mississauga, Canada) and FITC-labeled IgE (anti-erythropoietin 26), respectively. SHIP1^{+/+} and SHIP1^{-/-} BMMCs were differentiated in vitro using the same protocol but starting from bone marrow cells of 6- to 8-week-old SHIP1^{+/+} and SHIP1^{-/-} littermates (129/Sv × C57BL/6).

Reagents

Monoclonal anti-phosphotyrosine (4G10), polyclonal anti-p85 (#06-195), and monoclonal anti-Erk2 antibodies were purchased from Upstate/Biomol (Hamburg, Germany). Monoclonal anti-SHIP1 (P1C1), polyclonal anti-P-PLC-γ1 (Y783) (sc-12943-R), and polyclonal anti-InsRβ (C-19) antibodies were obtained from Santa Cruz (Heidelberg, Germany). Polyclonal anti-P-p38 (T180/Y182), anti-P-PKB (S473), anti-P-PAK1 (S199/204)/PAK2 (S192/197), anti-P-FOXO1 (a.k.a. anti-P-FKHR) (S256), and anti-IκBα antibodies were purchased from Cell Signaling Technology (Frankfurt a. M., Germany). Monoclonal anti-P-Erk antibodies (T202/Y204) (12D4) were purchased from Nanotools (Teningen, Germany) and polyclonal anti-Shc antibodies from BD Transduction Laboratories (Heidelberg, Germany). Monoclonal anti-FcεR1β antibody was a kind gift from Dr. R. Siraganian. DNP-HSA containing 30 to 40 moles DNP per mole albumin, monoclonal IgE with specificity for DNP (SPE-7), and Ins were purchased from SIGMA (Deisenhofen, Germany). LY294002 and wortmannin were obtained from Calbiochem (Schwalbach, Germany). Recombinant human IGF-1 was purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany) and H₂O₂ from FLUKA (Buchs, Switzerland). 2',7'-dichlorofluorescein diacetate (H₂DCFDA) for H₂O₂ measurements was obtained from Molecular Probes (Leiden, The Netherlands). Bacterial toxins (lethal toxin (LT) from *Clostridium sordellii* and toxin B (TB1470) from *Clostridium difficile*) were tested for effective concentrations according to Djouder et al. [18].

Degranulation assay

For degranulation studies, cells were preloaded with 0.2 μg/mL IgE anti-DNP overnight. The cells were then washed and resuspended in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1 % bovine serum albumin (BSA) in 10 mM Hepes, pH 7.4). The cells were adapted to 37°C for 20 minutes and treated for 30 minutes at 37°C as mentioned. The degree of degranulation was determined by measuring release of β-hexosaminidase [7].

Antigen stimulation, immunoprecipitation, membrane preparation, and Western blotting

IgE-preloaded cells (0.2 μg/mL IgE; overnight) were washed twice in phosphate-buffered saline (PBS) and resuspended (2×10^7 /mL) in Tyrode's buffer. Cells were adapted to 37°C for 30 minutes and stimulated with the indicated concentrations of respective stimuli. After stimulation, cells were pelleted and solubilized with 0.5% NP-40 + 0.5% deoxycholate in 4°C phosphorylation solubilization buffer [19]. After normalizing for protein content, the postnuclear supernatants were either directly separated by SDS-PAGE and

subjected to Western blot analysis as described previously [19] or they were subjected to immunoprecipitation with three subsequent washing steps using solubilization buffer (PSB) containing 0.1% NP-40. The precipitates were separated by SDS-PAGE and analyzed by Western blotting. Subcellular fractionation was performed as described recently [20].

Survival assay

BMMCs were washed twice with PBS and resuspended in growth medium without FCS and IL-3, but containing 0.1 % BSA. These cells were cultured for 72 hours either without survival factor (negative control), with 30 ng/mL IL-3 (positive control), with 1 μ M Ins, or with 0.013 μ M IGF-1. After this period, cells were washed once with PBS and subjected to an annexin V assay (rh Annexin V/FITC kit; Bender Med-Systems, Vienna, Austria) according to the manufacturer's protocol.

Calcium measurements

IgE-preloaded BMMCs were washed with RPMI; resuspended at 5×10^6 cells/mL in RPMI containing 1% FCS, 30 μ M Indo-1 AM (Molecular Probes), and 0.045% pluronic F-127 (Molecular Probes); and incubated for 45 minutes at 37°C. Cells were then pelleted, resuspended in RPMI containing 1% FCS, and analyzed in a LSR II (Becton-Dickinson, Franklin Lakes, NJ, USA) after the indicated stimulation procedures. The FACS profiles were converted to line graph data using the FlowJo application.

Flow cytometric analysis of H_2O_2 production

IgE-sensitized BMMCs were washed twice with PBS, resuspended in 500 μ L PBS, and stained with H_2DCFDA (final concentration: 10 μ M) for 30 minutes at 37°C in the dark. For studies involving signaling inhibitors, cells were incubated with a premix of H_2DCFDA and the respective inhibitor for 30 minutes. Incubation in the dark was continued after addition of the stimuli (Ins, IGF-1, and antigen) for 5 minutes and flow cytometric analysis of cell samples was carried out subsequently using a FACScan cell analyzer (Becton-Dickinson, San Jose, CA, USA). Fluorescence intensity data (channel FL1) were collected from 25,000 cells per measurement.

Statistical analysis

All values in the figures (concerning bar graphs) are expressed as mean of SD of n observations (with n indicated in the respective figure legends). The relevant data sets were compared by unpaired two-tailed Student's t -test using Graph Pad Prism3 evaluation software (Graph Pad, San Diego, CA, USA). p values less than *0.05, **0.005, and ***0.0005 were considered statistically significant. Significance of data obtained by FACS analysis was calculated from the geo-means of at least three individual experiments. Densitometric analysis of Western blots was performed using publicly available ImageJ software [21].

Results

Insulin stimulates PKB but not Erk phosphorylation in BMMCs

Since there have been several reports showing an inverse relationship between atopic diseases, such as asthma, and diabetes mellitus (i.e., low plasma Ins levels) [11,12], we were interested in determining whether MCs were respon-

sive to Ins. Since Ins is a well-known activator of PI3K (important for IgE+Ag-mediated MC degranulation [5]) and Erk (playing a role in IgE+Ag-mediated cytokine secretion from MCs [22]) in typical Ins-responsive cells, we stimulated BMMCs with Ins for various times and carried out Western analysis of total cell lysates using phospho-specific antibodies. We found that Ins stimulated a rapid and sustained (up to 60 min) phosphorylation of PKB at S473, indicating activation of the PI3K pathway (Fig. 1A). This was

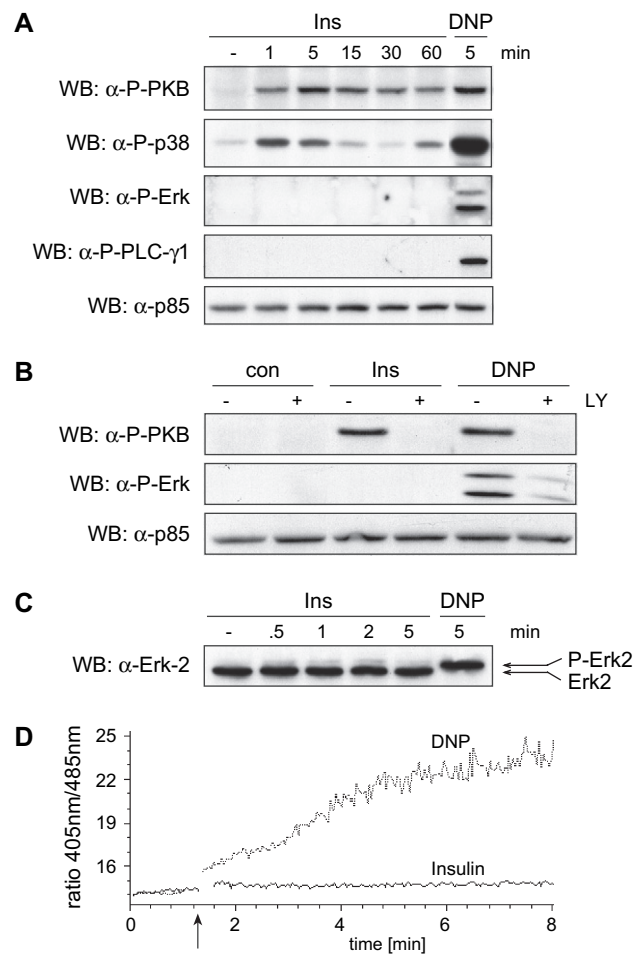


Figure 1. Insulin induces phosphorylation of PKB, but not Erk, in BMMCs. (A) IgE-preloaded BMMCs were either left unstimulated or stimulated with Ins (1 μ M) or Ag (DNP; 20 ng/mL) for the indicated times. Postnuclear supernatants were analyzed by Western blotting against P-PKB (S473) (top panel), P-38 (T180/Y182) (second panel from top), P-Erk (T202/Y204) (third panel from top), P-PLC- γ 1 (Y783) (second panel from bottom), and p85 (loading control; bottom panel). (B) BMMCs were pretreated with vehicle (DMSO) or 10 μ M LY294002 (LY) for 30 minutes before being left unstimulated (con) or stimulated for 5 minutes with Ins or Ag. Postnuclear supernatants were assessed by anti-P-PKB (top panel), anti-P-Erk (middle panel), and anti-p85 (loading control; bottom panel) Western blotting. (C) BMMCs were stimulated with Ins for the indicated times. Postnuclear supernatants were analyzed by anti-Erk2 immunoblotting. Erk2 and P-Erk2 are indicated by arrows. (D) IgE-preloaded BMMCs were stimulated with Ag (20 ng/mL) or Ins (1 μ M) and calcium mobilization measured. The arrow marks the time point of stimulus addition. Comparable results were obtained in separate experiments.

further corroborated by the inhibition of Ins- and Ag-stimulated PKB phosphorylation by the PI3K inhibitor, LY294002 (Fig. 1B). Since Ag-triggered PKB phosphorylation at S473 has been shown to possess a PKC- β -dependent signaling component [23], we compared the effect of Ins and IgE+Ag on PKC- $\beta^{+/+}$ and PKC- $\beta^{-/-}$ BMMCs and found that, in contrast to IgE+Ag, where a partial dependence could be observed, Ins-induced PKB phosphorylation was not dependent on PKC- β (data not shown). Ins also stimulated the phosphorylation of p38 at T180/Y182 but this was more transient than Ins-induced PKB phosphorylation (Fig. 1A). Also, while the level of Ins- and IgE+Ag-induced PKB phosphorylation was comparable, p38 stimulation by Ag was far stronger than by Ins (Fig. 1A). Intriguingly, while Fc ϵ R1 engagement, as expected, induced a robust phosphorylation of Erk at T202/Y204, Ins stimulation did not result in any detectable Erk phosphorylation, even after prolonged exposure of the membrane (Fig. 1A). Moreover, using a very sensitive anti-Erk2 antibody, we could not detect a significant mobility shift of Erk2 after Ins stimulation, though Ag stimulation resulted in the shift of the entire Erk2 population (Fig. 1C). The absence of Erk phosphorylation was not due to activation of the PI3K pathway negatively feeding back onto the Erk pathway, since inhibition of PI3K by LY294002 did not yield any Erk phosphorylation (Fig. 1B). In fact, LY294002 treatment reduced Ag-mediated Erk phosphorylation (Fig. 1B). In contrast to Ag, Ins also did not activate PLC- γ 1, assessed using an anti-phospho-PLC- γ 1 antibody (pY783) (Fig. 1A). This finding was consistent with our inability to detect significant calcium mobilization in response to Ins (Fig. 1D). These data indicate that Ins stimulates a very distinct subset of signaling pathways from IgE+Ag in BMMCs.

SHIP is transiently tyrosine-phosphorylated in Ins-treated BMMCs

In typical Ins-responsive cell types, like liver, muscle, and fat cells, Ins-induced activation of the SH2-containing inositol 5'-phosphatase, SHIP2 [24]. It was therefore tempting to speculate that SHIP1 may be playing this role in BMMCs and we thus investigated whether SHIP1 was phosphorylated in response to Ins. As shown in Figure 2A, when BMMCs were stimulated with Ins and the postnuclear supernatants immunoprecipitated with an anti-phosphotyrosine antibody (anti-Ptyr), a very transient induction of a number of proteins, including a 145-kDa protein, was detected (Fig. 2A). Two of the proteins that co-precipitated with anti-Ptyr were identified as SHIP1 and the adaptor protein Shc (Fig. 2A). To verify that SHIP1 itself was tyrosine phosphorylated after Ins stimulation, postnuclear supernatants from Ins-stimulated BMMCs were subjected to immunoprecipitation with anti-SHIP1 antibodies and Western analysis with anti-Ptyr antibodies. This verified that SHIP1

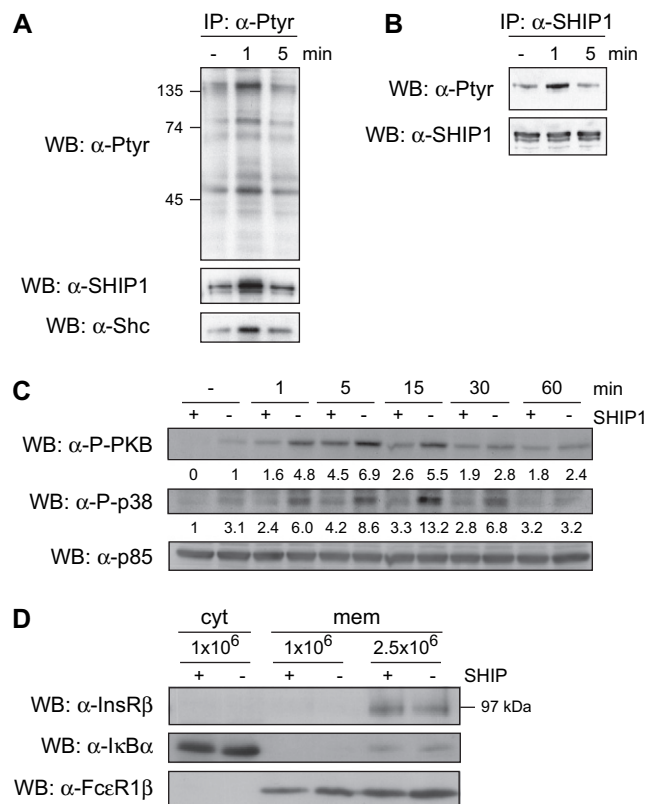


Figure 2. Insulin stimulates the tyrosine phosphorylation of SHIP1. (A) BMMCs were left untreated or stimulated with Ins (1 μ M) for the indicated times. Postnuclear supernatants were subjected to immunoprecipitation with anti-Ptyr antibodies and precipitates were analyzed by anti-Ptyr (top panel), anti-SHIP1 (middle panel), and anti-Shc (bottom panel) Western blotting. (B) Postnuclear supernatants from BMMCs treated as in (A) were subjected to anti-SHIP1 immunoprecipitation and precipitates were assessed by anti-Ptyr (upper panel) and anti-SHIP1 (loading control; lower panel) immunoblotting. (C) SHIP1 $^{+/+}$ and SHIP1 $^{-/-}$ BMMCs were stimulated with Ins for the indicated times and postnuclear supernatants analyzed by anti-P-PKB (top panel), anti-P-p38 (middle panel), and anti-p85 (loading control; bottom panel) immunoblotting. Densitometry was performed, and relative expression levels are indicated under each band. Comparable results were obtained in different experiments ($n > 3$). (D) SHIP1 $^{+/+}$ and SHIP1 $^{-/-}$ BMMCs were subjected to cytosol-membrane fractionation and equivalents of 1×10^6 cells (cytosol (cyt) and membrane (mem)) or 2.5×10^6 cells (mem) were separated by SDS-PAGE followed by anti-InsR β (top panel), anti-I κ B α (cytosol control; middle panel), and anti-Fc ϵ R1 β immunoblotting (membrane control; bottom panel).

was indeed transiently tyrosine phosphorylated in Ins-treated BMMCs (Fig. 2B).

SHIP1 attenuates PKB and p38 activation in Ins-stimulated BMMCs

Since we found that SHIP1 became tyrosine phosphorylated after Ins treatment of BMMCs, we asked if the hemopoietic-specific SHIP1 carried out the role of SHIP2 in MCs. Specifically, SHIP1 $^{+/+}$ and SHIP1 $^{-/-}$ BMMCs were stimulated with Ins for different times and total cell lysates subjected to Western analysis with anti-phospho-PKB

and anti-phospho-p38 antibodies. As shown in Figure 2C, Ins stimulated a stronger and more sustained phosphorylation of PKB and p38 in SHIP1-deficient BMMCs, indicating that SHIP1 is responsible for attenuating the PI3K pathway in Ins-stimulated BMMCs. Importantly, increased responsiveness to Ins of the SHIP1^{-/-} BMMCs was not due to increased expression of the InsR (Fig. 2D). Paralleling the results in wild-type BMMCs, Ins was not able to stimulate the phosphorylation of Erk or PLC- γ 1 in SHIP1^{-/-} BMMCs and consequently there was no calcium mobilization observed after Ins treatment of these cells (data not shown). Moreover, even though SHIP1^{-/-} BMMCs are much more prone to Ag-triggered cytokine production [22], an event dependent on Erk activation, we did not observe significant Ins-induced IL-6 or tumor necrosis factor- α (TNF- α) secretion in these cells (data not shown). Of note, the observed hyper-responsive phenotype of Ins-stimulated SHIP1^{-/-} BMMCs manifests even in the presence of SHIP2 [25].

IGF-1 and Ins activate the same restricted set of signaling pathways in BMMCs

It is known that Ins can bind not only to its two alternatively spliced receptors (IRa and IRb), but also to hybrid receptors consisting of the IR and the IGF-1R [26]. Since IGF-1 is also capable of binding to the same hybrid receptors, as well as to the IGF-1R, we compared the effects of IGF-1 and Ins on BMMCs. Specifically, IgE-preloaded BMMCs were stimulated with IGF-1, Ins, or Ag for different times and the postnuclear supernatants were subjected to Western analysis using anti-phospho-PKB and Erk antibodies. As can be seen in Figure 3A, IGF-1, like Ins, induced a robust phosphorylation of PKB and no detectable phosphorylation of Erk in BMMCs. Ag, on the other hand, as expected, activated both signaling pathways (Fig. 3A). We also performed an Ins and IGF-1 dose-response analysis to determine how sensitive BMMCs were towards these stimuli. Whereas only 13×10^{-4} μ M of IGF-1 was sufficient to induce a significant PKB activation, 0.1 μ M of Ins was required to stimulate a comparable response (Fig. 3B). In separate experiments, 0.01 μ M of Ins were found to still induce low-level PKB phosphorylation (data not shown). As expected, we found that SHIP1 also negatively regulated IGF-1-induced PKB phosphorylation, shown by the use of wild-type and SHIP1^{-/-} BMMCs (Fig. 3C).

Ins, IGF-1, and Ag trigger

PAK activation and H₂O₂ production

The preferred activation of the PI3K pathway by Ins and IGF-1 prompted us to investigate this pathway in more detail. PI3K has been implicated in the activation of the small GTPase Rac [27], which in turn has been demonstrated to activate p21-activated kinase (PAK) [28] as well as to contribute to activation of the multicomponent NADPH oxidase [29]. To determine whether Ins or IGF-1 could

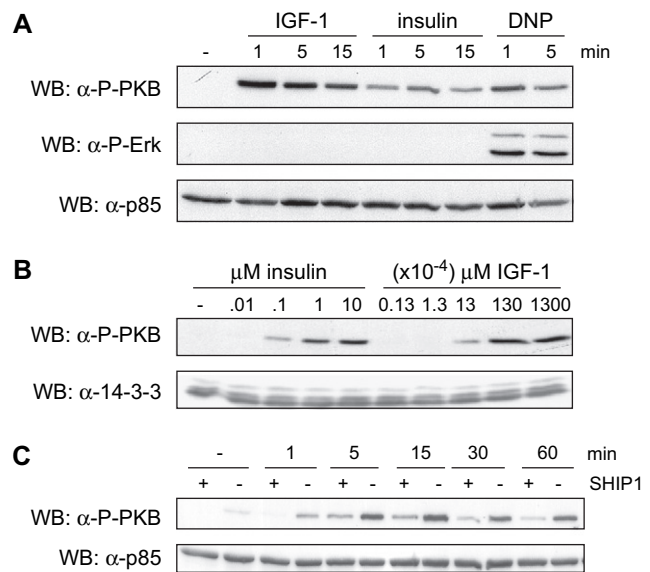


Figure 3. IGF-1 induces the same restricted set of signaling pathways in BMMCs as insulin. (A) IgE-preloaded BMMCs were stimulated with IGF-1 (0.013 μ M), Ins (1 μ M), or Ag (200 ng/mL DNP-HSA) for the indicated times. Postnuclear supernatants were analyzed by anti-P-PKB (top panel), anti-P-Erk (middle panel), and anti-p85 (loading control; bottom panel) Western blotting. (B) BMMCs were treated with the indicated concentrations of Ins and IGF-1 for 5 minutes. Postnuclear supernatants of 1×10^6 cells were analyzed by anti-P-PKB (upper panel) and anti-14-3-3 (loading control; lower panel) Western blotting. (C) SHIP1^{+/+} and SHIP1^{-/-} BMMCs were stimulated with IGF-1 (0.013 μ M) for the indicated times and postnuclear supernatants analyzed by anti-P-PKB (upper panel) and anti-p85 (loading control; lower panel) Western blotting.

stimulate PAK1 phosphorylation in BMMCs, we treated these cells with these two growth factors for 5 minutes and carried out Western analysis with anti-phospho-PAK1 antibodies. As shown in Figure 4A, both Ins and IGF-1 stimulated PAK1 phosphorylation in BMMCs and this phosphorylation was dependent on PI3K activity since it was inhibited by two structurally unrelated PI3K inhibitors, wortmannin and LY294002 (Fig. 4A). Moreover, we found that both Ins and IGF-1 stimulated H₂O₂ production in BMMCs, albeit to a lesser degree than Ag (Fig. 4B). Interestingly, whereas Ag-induced H₂O₂ production exhibited a strong dependence on PI3K, only a marginal influence of PI3K inhibition was found for Ins- or IGF-1-stimulated H₂O₂ generation (Fig. 4B). Since we had shown previously that the PI3K pathway plays an essential role in BMMC degranulation [5], we asked whether Ins or IGF-1 promoted Ag-triggered degranulation. As shown in Figure 4C, Ins alone did not induce BMMC degranulation and only modestly promoted degranulation induced by a suboptimal dose of Ag (2 ng/mL). Similarly, IGF-1 was capable of slightly, but consistently, enhancing BMMC degranulation in response to suboptimal as well as optimal concentrations of Ag (Fig. 4D). In contrast to degranulation, no impact of Ins or IGF-1 on IL-6 and TNF- α secretion was observed (data not shown).

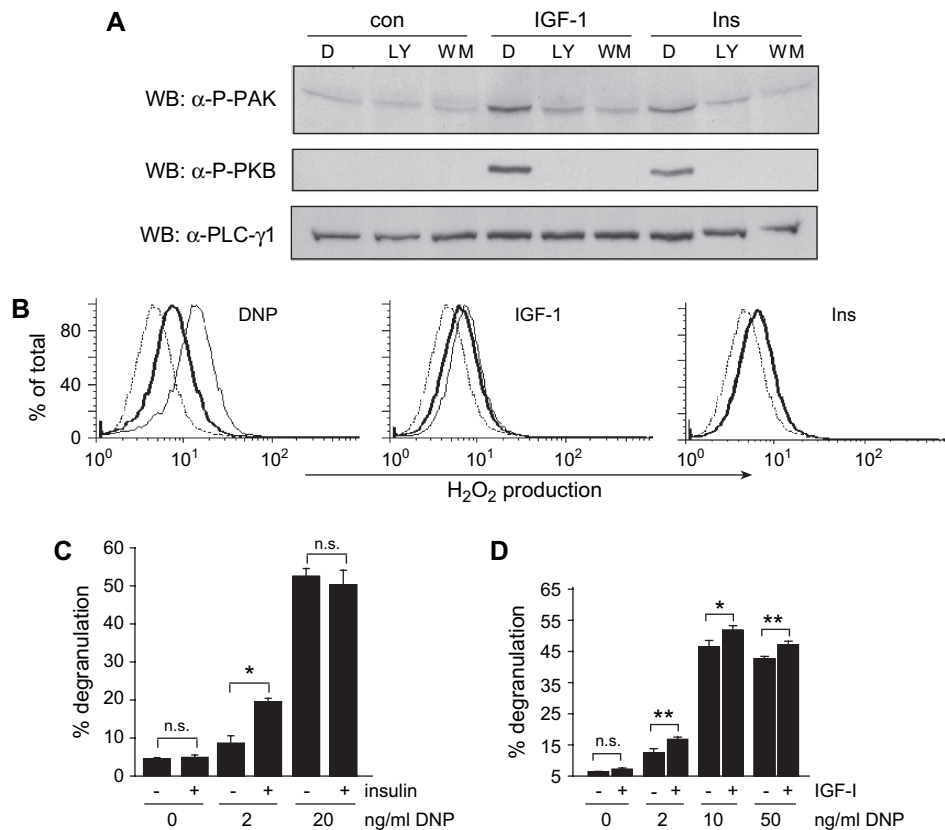


Figure 4. Insulin and IGF-1 induce PAK phosphorylation and H_2O_2 production. **(A)** BMMCs were pretreated with vehicle (DMSO, (D)), 25 μ M LY294002 (LY), or 50 nM wortmannin (WM) for 30 minutes before being left unstimulated (con) or stimulated for 5 minutes with IGF-1 (0.013 μ M) or Ins (1 μ M). Postnuclear supernatants were assessed by anti-P-PAK (top panel), anti-P-PKB (middle panel), and anti-PLC- γ 1 (loading control; bottom panel) immunoblotting. **(B)** BMMCs were loaded with IgE overnight and incubated with 100 nM wortmannin or vehicle for 30 minutes in the presence of the dye H_2DCFDA . Cells were then either left unstimulated (dashed lines) or treated with Ag (DNP-HSA, 20 ng/mL), IGF-1 (0.013 μ M), or Ins (1 μ M) and H_2O_2 production analyzed by FACS (thin solid lines = vehicle + stimulus; thick solid lines = wortmannin + stimulus). $n = 3$; p values for induction of H_2O_2 production: con vs DNP, < 0.0001 ; con vs IGF-1, 0.0002; con vs Ins, < 0.0001 . p values for inhibition by wortmannin: DNP vs DNP+wortmannin, 0.0006; IGF-1/Ins vs IGF-1/Ins+wortmannin, n.s. **(C)** IgE-preloaded BMMCs were left unstimulated or stimulated with Ins (1 μ M) alone or the indicated concentrations of Ag (DNP-HSA) $\pm$ Ins for 30 minutes. Subsequently, degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD. Comparable results were obtained in two independent experiments. *, p value = 0.0255. **(D)** IgE-preloaded BMMCs were left unstimulated or stimulated with IGF-1 (0.013 μ M) alone or the indicated concentrations of Ag $\pm$ IGF-1 for 30 minutes. Subsequently, degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of triplicates $\pm$ SD. Comparable results were obtained in 3 different experiments. p values: 2 ng/mL DNP vs 2 ng/mL DNP+IGF-1, 0.0052; 10 ng/mL DNP vs 10 ng/mL DNP+IGF-1, 0.0186; 50 ng/mL DNP vs 50 ng/mL DNP+IGF-1, 0.0044.

Differences in the Rac dependence of

PKB activation in Ag- and IGF-1-stimulated BMMCs

To prove that H_2O_2 production originated from Rac-dependent NADPH oxidase, Rac-glucosylating toxins from different *Clostridium* strains were tested. As shown in Figure 5A, treatment of BMMCs with TB1470 from *Clostridium difficile* or lethal toxin (LT) from *Clostridium sordellii* (data not shown), which are known to possess comparable specificity [30], suppressed IGF-1- and Ag-induced H_2O_2 production (Fig. 5A). Interestingly, whereas both LT and TB1470 inhibited PAK phosphorylation in Ins-, IGF-1-, or Ag-stimulated BMMCs, only in Ag-triggered cells PKB phosphorylation was abolished using these toxins (Fig. 5B and data not shown). This demonstrated that the Rac dependency for PKB activation in BMMCs strongly depended on the stimulus used.

Ins and IGF-1 promote BMMC survival

Since we observed the preferential activation of the PI3K pathway in Ins- or IGF-1-stimulated BMMCs resulting in strong phosphorylation of PKB, and PKB is well known to directly phosphorylate and inactivate transcription factors of the forkhead box class O (FOXO) family in a PI3K-dependent manner [31], we assessed FOXO1 phosphorylation in response to Ins or IGF-1. Indeed, we found that Ins and IGF-1 stimulate phosphorylation of FOXO1 (Fig. 6A). FOXO1 has been reported to be involved in cell-cycle regulation [31] and thus we analyzed MC survival in response to Ins and IGF-1. Specifically, BMMCs were cultured in either IL-3-deprived medium as a negative control, IL-3-containing medium as a positive control, or IL-3-deprived but Ins- or IGF-1-containing medium. After 72 hours, cell cultures were analyzed by FACS using

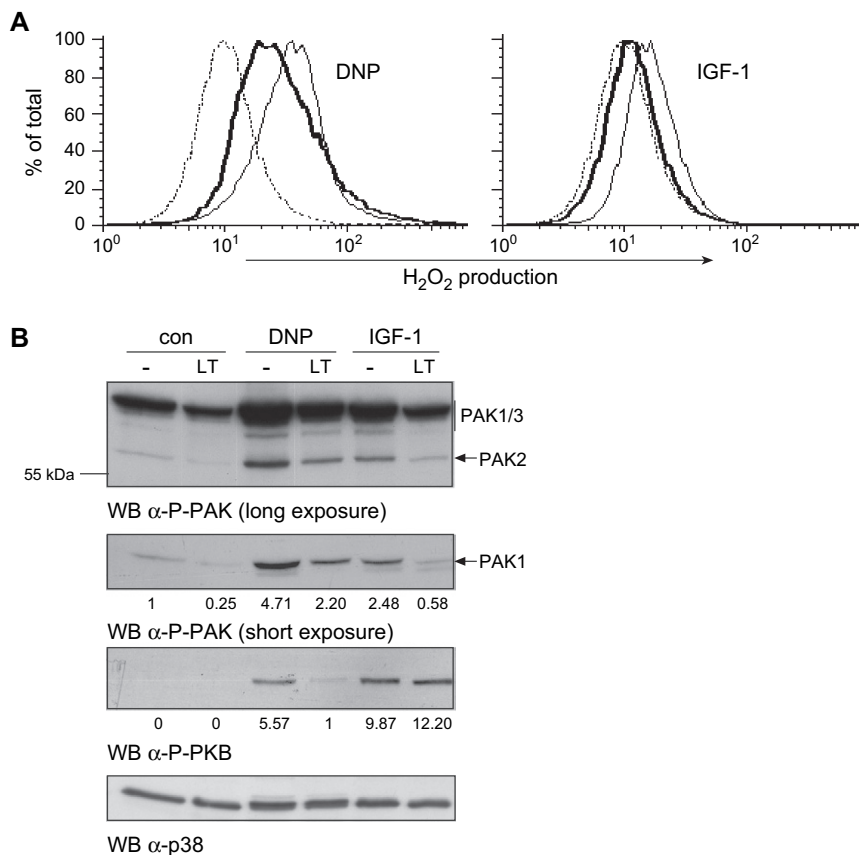


Figure 5. Differences in Rac dependence of PKB activation in Ag- and IGF-1-stimulated BMMCs. **(A)** IgE-loaded BMMCs were left untreated or incubated with 40 ng/mL TB1470 for 4 hours. Cells were then either left unstimulated (dashed lines) or treated with Ag (DNP-HSA, 20 ng/mL) or IGF-1 (0.013 μ M) and H₂O₂ production analyzed by FACS (thin solid lines = vehicle + stimulus; thick solid line = toxin + stimulus). **(B)** IgE-loaded BMMCs were left untreated or pretreated with 40 ng/mL LT (which has the same specificity as TB1470) for 4 hours. Cells were then left unstimulated (con) or stimulated for 5 minutes with Ag (DNP-HSA, 20 ng/mL) or IGF-1 (0.013 μ M) and postnuclear supernatants assessed by anti-P-PAK (upper two panels), anti-P-PKB (third panel from top), anti-P-Erk (second panel from bottom), and anti-p38 (loading control; bottom panel) immunoblotting. Densitometry was performed, and relative expression levels are indicated under each band. Comparable results were obtained in different experiments ($n > 3$).

propidium iodide and annexin V positivity. Though IL-3 was the most efficient pro-survival factor, as expected, both Ins and IGF-I significantly promoted survival of BMMCs (Fig. 6B). Since the assays were carried out in the absence of FCS, synergistic effects between Ins or IGF-I and serum could be excluded. The dominant activation of the PI3K pathway by IGF-I prompted us to finally address the PI3K dependence of this pro-survival action using two structurally unrelated PI3K inhibitors, wortmannin and LY294002. Since long-term incubation with these inhibitors is detrimental to the cells, a 12-hour incubation period was chosen. Whereas the pro-survival effect of IGF-I was already observed after this short-term period, addition of the PI3K inhibitors diminished this effect in a dose-dependent manner (Fig. 6C). These data suggest that activation of the PI3K/PKB pathway in the absence of activation of the Erk pathway is sufficient to promote survival of BMMCs.

Discussion

Data from epidemiological studies indicate an inverse relationship between atopic disorders such as asthma and type I diabetes mellitus [11–13]. It has been hypothesized that this is due to an imbalance of Th1/Th2 cytokine secretion [32]. Interestingly, bronchial segments isolated from diabetic rats showed a 50% reduction in the number of degranulated MCs and in histamine release, compared to healthy rats [16]. Since these data were consistent with the possibility that Ins directly modulates MC function, we investigated the effects of Ins and IGF-I on BMMC signaling and biological properties.

In terms of signaling, we found that both Ins and IGF-1 activated the PI3K pathway in BMMCs and this pathway was negatively regulated by SHIP in these cells. Intriguingly, while Ins and IGF-1 are well-known activators of Erk in typical Ins-responsive cells [33], they did not activate Erk in BMMCs. This inability to activate Erk in

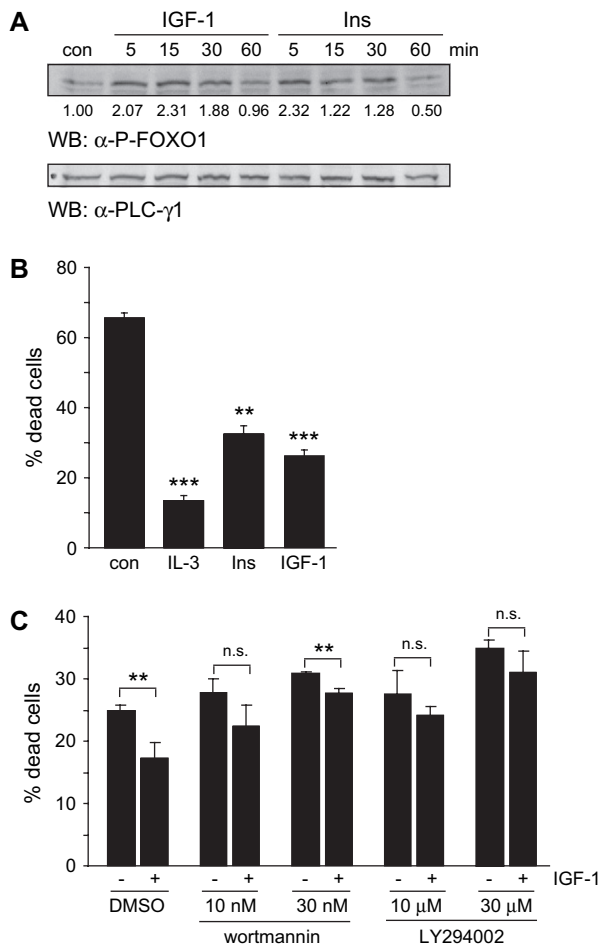


Figure 6. Ins and IGF-1 promote BMMC survival. **(A)** BMMCs were treated for the indicated times with 0.013 μ M IGF-1 or 1 μ M Ins and post-nuclear supernatants analyzed by anti-P-FOXO1 (S256) (upper panel) and anti-p85 immunoblotting (loading control; lower panel). Densitometry was performed, and relative expression levels are indicated under each band. Comparable results were obtained in different experiments ($n = 3$). **(B)** BMMCs were grown for 72 hours in medium alone or in medium containing IL-3 (30 ng/mL), Ins (1 μ M), or IGF-I (0.013 μ M). Cells were assessed by FACS with respect to propidium iodide and annexin V positivity. Propidium iodide single-positive plus propidium iodide and annexin V double-positive cells were regarded as dead cells. Each bar is the mean of triplicates $\pm$ SD. Comparable results were obtained in 3 different experiments. p values: con vs IL-3, < 0.0001 ; con vs Ins, < 0.0069 ; con vs IGF-1, < 0.0001 . **(C)** BMMCs were grown for 12 hours in medium supplemented with 0.1% BSA (-) or with 0.1% BSA plus IGF-I (0.013 μ M) in the presence of DMSO (vehicle control) or the indicated concentrations of wortmannin or LY294002. Cells were assessed as described under **(D)**. Each bar is the mean of triplicates $\pm$ SD. Comparable results were obtained in 2 different experiments. p values: DMSO vs IGF-1, 0.0072; 30 nM wortmannin vs 30 nM wortmannin + IGF-1, 0.0063.

BMMCs is not due to SHIP1 outcompeting Grb2 for binding to Shc [19] and thus reducing Sos recruitment to the plasma membrane since we did not observe Erk activation in SHIP1^{-/-} BMMCs in response to Ins (data not shown). Nor is it likely due to PKB-mediated phosphorylation of Raf at a negative regulatory site which can inhibit the acti-

vation of the Erk cascade [34], since LY294002, at concentrations that completely blocked Ins-induced PKB activation, did not increase Erk phosphorylation. On the other hand, it has been suggested that PLC- γ is necessary for Ins-induced Erk activation [35] and we did not observe activating phosphorylation of PLC- γ or calcium mobilization in BMMCs in response to Ins. As well, Erk activation is often dependent on the activation of different PKC isotypes [36] and the activation of certain PKC isotypes (i.e., *conventional* and *novel*) is dependent on PLC- γ -mediated calcium mobilization and/or diacylglycerol generation [37]. Alternatively, or in addition, IGF-1-induced Erk activation in human mammary epithelial cells was recently reported to be dependent on the expression of the epidermal growth factor receptor, whereas PKB activation was not [38]. Therefore, it is possible that in MCs there is no suitable receptor tyrosine kinase to be transactivated by the IGF-1R and thus the Erk pathway is not activated. Whatever the reason, the missing Erk activation might limit the response of BMMCs to Ins and IGF-I since there is neither IL-6 nor TNF- α production in Ins- or IGF-I-stimulated BMMCs (data not shown).

Recently, dependence of PKB activation on PKC- β in Ag-stimulated BMMCs has been reported [23]; however, we found this was not the case following Ins or IGF-1 stimulation. Additional differences were found with respect to PI3K-regulated signaling events between Ag and Ins or IGF-1 stimulation. For example, while Ag-triggered H₂O₂ generation was attenuated by both PI3K and Rac inhibition, Ins- or IGF-1-triggered H₂O₂ production was only significantly reduced by Rac inhibition. Moreover, while we found that PKB activation was dependent on Rac activation in Ag-stimulated BMMCs, in keeping with a previous report demonstrating that PKB activation is downstream of Rac in Ag-stimulated RBL-2H3 cells [18], this dependence on Rac was not seen with Ins- or IGF-1-induced PKB activation, suggesting significant stimulus-dependent differences in the control of PKB activation in BMMCs.

In adipose cells, H₂O₂ production in response to Ins has been shown to contribute to signal amplification and propagation via oxidation/inhibition of the protein tyrosine phosphatase, PTP1B [39], and the NADPH oxidase, Nox4, has been implicated in this process [39]. In light of these data, the Rac dependence of PKB activation in response to Ag might be explained in another way. Specifically, PI3K and NADPH oxidase could constitute a positive feedback cycle resulting in the production of H₂O₂ and the consequent inhibition of protein tyrosine phosphatases (and thus the amplification of the initial signal after receptor engagement). Consistent with this, we found that inhibition of NADPH oxidase by diphenyleneiodonium chloride [40] suppressed Ag- and Ins/IGF-1-induced PKB and PAK phosphorylation in BMMCs (data not shown).

In order to study the impact of Ins and IGF-1 signaling on MC effector functions, we analyzed its effect on

Ag-triggered BMMC degranulation. Related to this, we have shown previously that activation of the PI3K pathway is a crucial step for degranulation and that the level of PI3K activation controls the extent of degranulation [5,7,41,42]. However, the degranulation process needs at least triggering amounts of mobilized calcium ions and this might be why in the current study we did not detect degranulation in wild-type or SHIP1^{-/-} BMMCs in response to Ins or IGF-1 alone. Nonetheless, both Ins and IGF-1 promoted, albeit modestly, mediator release in combination with suboptimal doses of Ag. It is thus tempting to speculate that MCs in the vicinity of Ins- or IGF-1-producing cells (e.g., MCs located in the pancreatic fat tissue) might produce a more robust inflammatory response in the presence of Ag than mast cells more distal to these cells. In SHIP1^{-/-} BMMCs, degranulation was not increased by Ins or IGF-1 costimulation (data not shown), probably due to the fact that FcεR1-mediated PI3K activation is already markedly elevated even at suboptimal Ag levels [5,7,42].

Although Ins and IGF-I did not induce Erk activation in BMMCs, they did promote cell survival after withdrawal of the MC growth factor IL-3, perhaps in part via PI3K-mediated phosphorylation of the transcription factor, FOXO1. Phosphorylation of FOXO1 results in its nuclear exclusion and this typically enhances cell survival [31]. A further mechanism might be sustained glycolytic activity and mitochondrial integrity via PKB activation [43]. However, PI3K-dependent translocation of the glucose transporter GLUT4 from intracellular membranes to the plasma membrane [10] may not be involved since a Western analysis of GLUT4 expression in BMMCs and cardiac myocytes suggested no significant GLUT4 expression in BMMCs (data not shown). While there is next to no literature as yet on the effects of Ins or IGF-1 on mast cells, one report in 1992 suggested that IGF-1 could prevent DNA fragmentation and apoptotic cell death in IL-3-depleted BMMCs [44]. However, very high levels of IGF-1 (1 μg/mL = 0.13 μM) were used in this study and the survival assays were carried out in the presence of 10% FCS, making it difficult to judge the sole effect of IGF-I.

One of the remaining issues is which type of IR is mediating the effects observed in BMMCs. Selective Ins binding is complicated by tissue-specific alternative splicing of exon 11 that directs synthesis of two IR isoforms (IRa and IRb), and by posttranslational assembly of hybrids between these isoforms and the homologous IGF-1R. Hybrid receptors composed of αβ dimers from the IGF-1R and the IRb selectively bind IGF-1, whereas hybrid receptors composed of IGF-1R and IRa bind IGF-1 and Ins with similar affinities [26]. Since IRa is expressed more strongly in hematopoietic cells and neurons, whereas IRb is more highly expressed in typical Ins-responsive tissues [45], it might well be that the comparable effects seen in BMMCs after stimulation with Ins or IGF-1 are due to binding of these ligands to the same receptor hybrid consisting of IGF-1R and IRa.

In summary, we present evidence that BMMCs are responsive to Ins and IGF-1. Although they stimulate a robust activation of the PI3K pathway, in a SHIP1-controlled manner, they do not activate the Erk pathway. Moreover, both promote BMMC survival and slightly enhance Ag-triggered MC degranulation. These effects might contribute to the inverse relationship observed between type I diabetes mellitus (i.e., low levels of Ins) and atopic disorders like asthma and allergies.

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