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Activation of the PI3K pathway increases TLR-induced TNF-α and IL-6 but reduces IL-1β production in mast cells

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ABSTRACT

Recognition of bacterial constituents by mast cells (MCs) is dependent on the presence of pattern recognition receptors, such as Toll-like receptors (TLRs). The final cellular response, however, depends on the influence of multiple environmental factors. In the current study we tested the hypothesis that the PI3K-activating ligands insulin-like growth factor-1 (IGF-1), insulin, antigen, and Steel Factor (SF) are able to modulate the TLR4-mediated production of proinflammatory cytokines in murine MCs. Costimulation with any of these ligands caused increased LPS-triggered secretion of IL-6 and TNF-α, but attenuated the production of IL-1β, though all three cytokines were produced in an NFκB-dependent manner. The pan-specific PI3K-inhibitor Wortmannin reverted the altered production of these cytokines. In agreement, MCs deficient for SHIP1, a negative regulator of the PI3K pathway, showed augmented secretion of IL-6/TNF-α and reduced production of IL-1β in response to LPS alone. The differential effects of IGF-1 on TLR4-mediated cytokine production were also observed in the context of TLR2 and IL-33 receptor-mediated MC activation. Importantly, these effects were seen in both bone marrow-derived and peritoneal MCs, suggesting general relevance for MCs. Using pharmacological and genetic tools, we could show that the p110δ isoform of PI3K is strongly implicated in SF-triggered suppression of LPS-induced IL-1β production. Costimulation with antigen was affected to a lesser extent. In conclusion, NFκB-dependent production of proinflammatory cytokines in MCs is differentially controlled by PI3K-activating ligand/receptor systems.

1. Introduction

Mast cells (MCs) are well-established mediators of immunoglobulin E-associated immune responses, including allergic disorders, but also play a critical role in the defense against invading microorganisms and certain parasites [1]. Pathogens can be recognized by MCs via pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs). Several members of the TLR family, e.g. TLR2 and TLR4, are expressed by bone marrow-derived MCs (BMMCs) [2]. Stimulation of MCs with TLR ligands induces the production of various proinflammatory cytokines without initiating degranulation [3,4]. The TLR4 complex recognizes lipopolysaccharide (LPS, endotoxin), the main cell wall component of Gram-negative bacteria. Of note, MCs do not express membrane-bound CD14 and thus are activated exclusively by R-chemotypes of LPS and lipid A [5,6]. TLR2 forms heterodimers with TLR1 or TLR6, which recognize a diverse set of ligands, including lipoproteins and lipoteichoic acid [7]. The TLRs are part of the Toll-like receptor/interleukin-1 receptor (TIR) superfamily whose members contain the same cytoplasmic protein interaction motif. This so-called TIR domain initiates similar signaling pathways downstream of the different receptors of the TIR superfamily. An additional member of this superfamily expressed by MCs is the IL-33 receptor (formerly called ST2) [8,9].

A signaling pathway immensely important for MC development, homeostasis, and activation is the phosphoinositide 3-kinase (PI3K) pathway [10]. PI3K class I comprises several regulatory and catalytic subunits, which can pair to yield different functional heterodimers [11]. PI3K is activated downstream of many signaling receptors (e.g. FcεR1, c-kit, TLRs, insulin receptor, and IGF-1R [12–15]) and phosphorylates the plasma membrane-localized phosphatidylinositol-4,5-bisphosphate to

Abbreviations: BMMC, bone marrow-derived mast cell; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1 beta; IL-33, interleukin-33; IL-6, interleukin-6; LPS, lipopolysaccharide; MC, mast cell; MD-2, myeloid differentiation factor 2; PI, propidium iodide; PMC, peritoneal mast cell; TIR, Toll-like receptor/Il-1 receptor; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.

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yield the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [16]. Particular importance for the catalytic PI3K subunit p110δ in MC signaling has been shown using $p110\delta^{D910A}$ knock-in BMMCs, which were greatly impaired in antigen-induced (Ag) degranulation [17]. The PI3K pathway is negatively regulated by the SH2-containing inositol-5′-phosphatase (SHIP1). Deletion of SHIP1 leads to a hyperactive phenotype in BMMCs, enhancing Ag-mediated degranulation and production of proinflammatory cytokines [12,18–20]. Moreover, SHIP1−/− BMMCs even degranulate under conditions in which WT BMMCs do not, coining the term 'gatekeeper' of MC degranulation for SHIP1 [12,13].

In most experimental studies that address signal transduction mechanisms, stimulation of a single receptor system is performed. However, under physiological conditions every cell is surrounded by a milieu of different stimuli, which co-activate multiple receptors. Thus, a response to a given stimulus may be modulated by additional ligands and the final response of a cell will be determined by the net effect of all integrated signals. For instance, in previous studies we have shown that combining SF and Ag in BMMC stimulation leads to a synergism in MC effector functions [21]. Moreover, TLR2 ligands can synergize with sub-optimal amounts of Ag in the stimulation of proinflammatory cytokine release [4,22].

In this study we costimulated MCs via members of the TIR superfamily (TLR4, TLR2, IL-33R) and via receptors known to induce a strong activation of the PI3K pathway (IGF-1R, FcεR1, c-kit) and analyzed the effects on the production of different NFκB-dependent proinflammatory cytokines. While IL-6 and TNF-α secretion was increased by costimulation when compared to TIR ligand stimulation alone, IL-1β production was decreased. This modulation was PI3K-dependent and could be suppressed by using the pan-PI3K inhibitor Wortmannin. Finally, using an inhibitor specific for the p110δ isoform of PI3K and BMMCs from p110δ null mice expressing a kinase-dead mutant of p110δ, this PI3K isoform was shown to be crucial for the respective regulation downstream of the receptor tyrosine kinase c-kit. These data show that the activation of the PI3K pathway differentially controls the production of well-known pro-inflammatory cytokines in MCs.

2. Materials and methods

2.1. Cell culture

According to procedures established by Razin et al. [23], bone marrow cells (1×10^6/ml) from 6 to 8 week old male mice (129/Sv) were cultured (37 °C, 5% CO_2) as single cell suspensions in RPMI 1640 medium containing 15% FCS, 1% X63Ag8–653-conditioned medium, as a source of IL-3 [24], 2 mM L-glutamine, 1×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 mg/ml streptomycin. At weekly intervals, the non-adherent cells were reseeded at 5×10^5 cells/ml in fresh medium. By 4–6 weeks in culture, greater than 99% of the cells were positive for c-kit and FcεR1 as assessed by phycoerythrin-labeled anti-c-kit antibodies (Pharmingen, Mississauga, Canada) and FITC-labeled rat anti-mouse IgE antibodies (Southern Biotechnology, Birmingham, AL, USA), respectively. SHIP+/+ and −/− BMMCs were *in vitro* differentiated using the same protocol but starting from bone marrow cells of 6 to 8 week old SHIP1+/+ and −/− littermates (129/Sv x C57BL/6) [25]. Btk+/+ and −/− BMMCs were *in vitro* differentiated from bone marrow cells of 6 to 8 week old Btk+/+ and −/− littermates (C57BL/6). BMMCs in which the endogenous p110δ is catalytically inactive ($p110\delta^{D910A}$) and respective WT cells were *in vitro* differentiated from bone marrow cells of $p110\delta^{D910A}$ and WT mice (C57BL/6) [26]. Both WT and $p110\delta^{D910A}$ BMMCs expressed comparable levels of MC markers FcεR1 and c-kit (data not shown). This was also true for SHIP1+/+, SHIP1−/−, Btk+/+, and Btk−/− BMMCs. L138.8A MC line [27,28] was cultured (37 °C, 5% CO_2) as single cell suspensions accordingly. Peritoneal MCs from WT mice (129/Sv) were prepared and cultured according to Malbec et al. [29].

2.2. Reagents

R-form LPS from *S. minnesota* mutant R595 and S-form LPS from *S. abortus equi* were extracted and purified as described [30–32] and were a gift from M. Freudenberg and C. Galanos (MPI for Immunobiology, Freiburg, Germany). The synthetic lipopeptide FSL-1 was obtained from Echaz Microcollections (Tübingen, Germany). IL-33 was purchased from Axxora Deutschland GmbH (Grünberg, Germany). Human recombinant IGF-1 was obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany) and insulin, monoclonal IgE with specificity for DNP (SPE-7) as well as DNP-HSA containing 30–40 mol DNP per mol albumin were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). DMSO was purchased from Carl Roth GmbH & Co (Karlsruhe, Germany), Wortmannin and LY294002 from Calbiochem-Novabiochem (LaJolla, CA, USA), and Bay-11-7082 from Biomol GmbH (Heidelberg, Germany). D001 was from Vitas-MLab, Apeldoom, the Netherlands. Polyclonal anti-p85 (#06-195) antibodies were purchased from Upstate/Biomol (Hamburg, Germany). Monoclonal anti-P-Erk antibodies (T202/Y204) (12D4) were obtained from Nanotools (Teningen, Germany) and polyclonal anti-P-PKB antibodies (S473) from Cell Signaling Technology (Frankfurt a. M., Germany).

2.3. Cytokine ELISAs

Mouse IL-6 ELISAs, mouse IL-1β ELISAs (BD Pharmingen, Heidelberg, Germany), and mouse TNF-α ELISAs (R&D Systems, Wiesbaden-Nordenstadt, Germany) were performed according to the manufacturer's instructions. IL-6 and TNF-α were measured in supernatants. IL-1β was measured from cell lysates due to a lack of secretion of this cytokine from MCs within 3–4 h after stimulation with the studied ligands. Levels of cytokines varied between experiments due to genetic background or age of the cells. Qualitative differences or similarities between WT and mutant cells, however, were consistent throughout the study.

2.4. Mast cell stimulation and Western blotting

IgE-preloaded (0.15 μg/ml overnight) cells were washed with PBS and resuspended in RPMI/0.1% BSA. Cells were adapted to 37 °C for 20 min and stimulated with the indicated concentrations of the stimuli. After stimulation for different lengths of time, cells were pelleted and solubilized with 0.5% NP-40 and 0.5% deoxycholate in phosphorylation solubilization buffer at 4 °C [33]. After normalizing for protein content, the postnuclear supernatants were subjected directly to SDS-PAGE and Western blot analysis. Densitometric analysis was performed with the software MultiGauge V3.2 from Fujifilm.

2.5. Statistical analysis

All values in the figures 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. P values of $^*<0.05$, $^{**}<0.005$, and $^{***}<0.0005$ were considered statistically significant.

3. Results

3.1. IGF-1 costimulation enhances LPS-triggered TNF-α and IL-6 secretion, but attenuates IL-1β production

We demonstrated recently that IGF-1 is capable of promoting survival of murine BMMCs via the activation of the PI3K signaling pathway [15]. Since MCs play prominent roles in various inflammatory conditions [1], we analyzed whether IGF-1 might influence the production of proinflammatory cytokines in response to the TLR4 ligand, LPS. BMMCs were left untreated or stimulated either with 1 μg/ml LPS, with 100 ng/ml IGF-1 or with a combination of both for 4 h. IGF-1 alone did not induce any

significant production of IL-6, TNF-α, or IL-1β (Fig. 1A). As expected, LPS stimulation induced a substantial production of all cytokines measured (Fig. 1A). Interestingly, when BMMCs were costimulated with LPS and IGF-1, secretion of IL-6 and TNF-α were markedly enhanced, whereas the production of IL-1β was attenuated compared to stimulation with LPS alone (Fig. 1A). Titrations of both LPS and IGF-1 in combination were performed with LPS and IGF-1 concentrations ranging from 0.1 to 10 μg/ml and 1 to 300 ng/ml, respectively. Titrating IGF-1 against one concentration of LPS (0.3 μg/ml) revealed that the maximum effect (upregulation of IL-6 and TNF-α as well as suppression of IL-1β production) was achieved with 100 ng/ml IGF-1 (data not shown). Variation in LPS concentration with costimulation by IGF-1 (100 ng/ml) had a significant influence on the production of all cytokines measured with the biggest differences between 0.3 and 1 μg/ml LPS (Fig. 1B). These data demonstrate that IGF-1 is able to differentially affect LPS-induced production of proinflammatory cytokines in MCs. Comparable data were obtained using insulin instead of IGF-1 (data not shown).

3.2. IGF-1-induced activation of PI3K is responsible for modulation of LPS-triggered cytokine responses

We showed previously that in BMMCs IGF-1 preferentially activates the PI3K pathway [15]. Thus, we sought to determine the role of PI3K in the observed regulation of proinflammatory cytokine production. Wortmannin was used as an inhibitor of PI3K activity [34] and BMMCs were incubated with vehicle (DMSO) or increasing concentrations of Wortmannin and then stimulated with LPS alone or costimulated with LPS and IGF-1. Wortmannin significantly attenuated the effects of IGF-1 costimulation. IL-6 and TNF-α secretion were decreased dose-dependently, while IL-1β production was increased with rising Wortmannin concentrations (Fig. 2A). These effects of Wortmannin were also observed in cells stimulated only with LPS (Fig. 2A), reflecting the ability of LPS to stimulate the PI3K pathway in BMMCs (data not shown). These results indicate that the PI3K pathway plays a critical role in the modulation of LPS-induced cytokine production by IGF-1 and, intriguingly, it positively regulates IL-6/TNF-α secretion but negatively regulates IL-1β production. A central signaling element activated by LPS is NFκB [35] and the production of IL-6, TNF-α, and IL-1β is reported to be dependent on NFκB activation [36–38]. To verify the role of NFκB, the effect of the IKKβ inhibitor Bay-11-7082 was investigated. The production of all measured proinflammatory cytokines was suppressed by Bay-11-7082 in a dose-dependent manner irrespective of the mode of stimulation (Fig. 2B). Thus, our results suggest that the PI3K pathway can act as both a positive and a negative modulator of NFκB activation. Of note, whereas the attenuation of IL-6 and TNF-α production by Wortmannin was also observed using a second PI3K inhibitor,

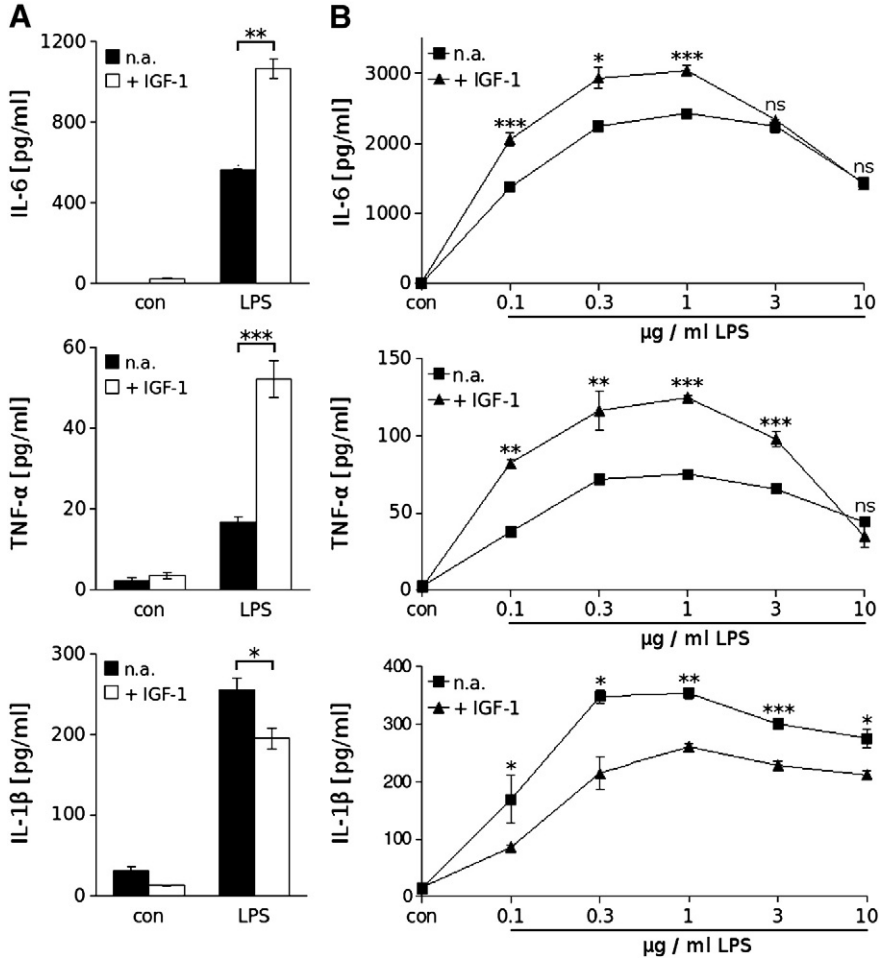

Fig. 1. LPS-induced cytokine production in BMMCs is modulated by IGF-1. (A) WT BMMCs were left untreated (con) or stimulated with LPS (1 μg/ml), IGF-1 (100 ng/ml), or a combination of both for 4 h. The addition of both LPS and IGF-1 was performed at the same time. Subsequently, the amounts of IL-6 (top panel), TNF-α (middle panel), and IL-1β (bottom panel) were determined by ELISA. Each bar represents the mean of triplicates ± SD. Comparable results were obtained in several experiments using different BMMC cultures ($n \geq 5$). (B) WT BMMCs were left untreated (con) or stimulated with varying LPS concentrations (0.1 to 10 μg/ml) with or without IGF-1 (100 ng/ml) for 4 h. IL-6 (top panel), TNF-α (middle panel), and IL-1β (bottom panel) were determined. Each point represents the mean of triplicates ± SD. Comparable results were obtained in 2 different experiments. Statistical significance refers to the differences between stimulation with LPS alone vs. LPS plus IGF-1 n.a., nothing added.

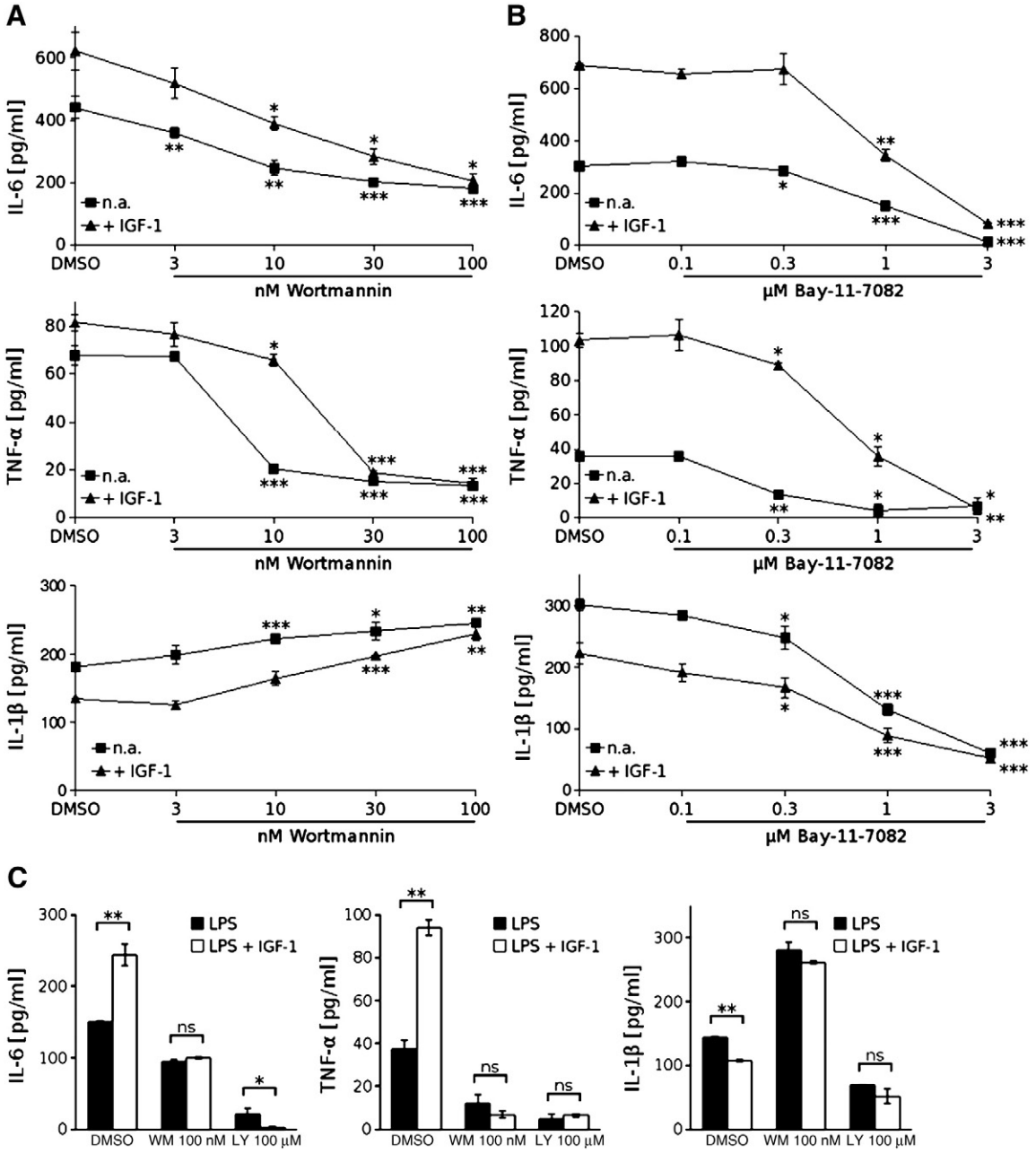

Fig. 2. Inhibition of PI3K blocks the IGF-1-induced costimulatory effects on cytokine production. (A) WT BMMCs were incubated either with DMSO or Wortmannin (3 to 100 nM) for 20 min. They were then stimulated with LPS alone (0.3 μg/ml) or IGF-1 (100 ng/ml) plus LPS for 4 h. Subsequently, the amounts of IL-6 (top panel), TNF-α (middle panel), and IL-1β (bottom panel) were determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3). (B) WT BMMCs were incubated either with DMSO or Bay-11-7082 (0.1 to 3 μM) for 20 min. They were then stimulated with LPS alone (0.3 μg/ml) or IGF-1 (100 ng/ml) plus LPS for 4 h. IL-6 (top panel), TNF-α (middle panel), and IL-1β (bottom panel) were determined. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3). Statistical significance refers to the differences between control (DMSO)-treated vs. Wortmannin/Bay-11-7082-treated cells. (C) WT BMMCs were incubated either with DMSO, 100 nM Wortmannin (WM), or 100 μM LY294002 (LY) for 20 min. They were then stimulated with LPS alone (0.3 μg/ml) or IGF-1 (100 ng/ml) plus LPS for 4 h. Subsequently, IL-6 (left panel), TNF-α (middle panel), and IL-1β (right panel) were determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3) n.a., nothing added.

LY294002, Wortmannin-induced enhancement of IL-1β production could not be confirmed by treatment with LY294002 (Fig. 2C). In contrast, LY294002 suppressed IL-1β production in response to LPS alone and the combination of LPS and IGF-1. This is very likely attributable to off-target effects of LY294002 at elevated concentrations, as it has been reported that LY294002 is a somewhat less specific PI3K inhibitor than Wortmannin [39,40]. Since TNF-α and IL-6 production was subject to comparable regulatory mechanisms throughout our study, we concentrated on regulation of TNF-α and IL-1β in our further investigations.

3.3. SHIP1 differentially controls LPS-triggered proinflammatory cytokine production

So far, our data indicate that regulators of the PI3K pathway potentially influence the outcome of LPS stimulation and the effect of IGF-1 costimulation in MCs. The inositol-5-phosphatase SHIP1 is known as a potent suppressor of PI3K-induced signaling by hydrolyzing PIP_3 [12,41]. Thus, SHIP1 deficiency should result in enhanced and decreased LPS-triggered TNF-α and IL-1β production, respectively. Indeed, this was the case (Fig. 3A). Interestingly,

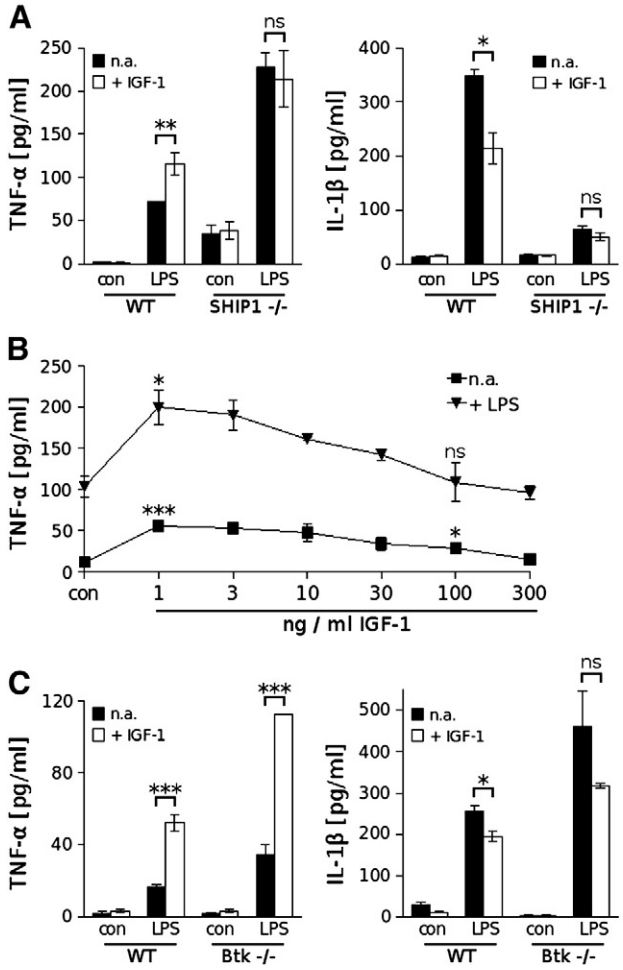

Fig. 3. SHIP1 affects LPS-induced proinflammatory cytokine production. (A) WT and SHIP1−/− BMMCs were left untreated (con) or stimulated with LPS (0.3 μg/ml), IGF-1 (100 ng/ml), or a combination of both for 4 h. Subsequently, the amounts of TNF-α (left panel) and IL-1β (right panel) were measured by ELISA. Each bar represents the mean of triplicates ± SD. Comparable results were obtained in two experiments using MCs from different cultures. (B) SHIP1−/− BMMCs were left untreated (con) or stimulated with varying IGF-1 concentrations (1 to 300 ng/ml) +/− LPS (0.3 μg/ml) for 4 h. TNF-α was determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in two different experiments. Statistical significance refers to the differences between stimulations with IGF-1 and the corresponding controls. (C) WT and Btk−/− BMMCs were left untreated (con) or stimulated with LPS (1 μg/ml), IGF-1 (100 ng/ml), or a combination of both for 4 h. Each bar represents the mean of triplicates ± SD. Subsequently, the amounts of TNF-α (left panel) and IL-1β (right panel) were measured n.a., nothing added.

whereas IGF-1 costimulation showed the expected effects in WT BMMCs, SHIP1−/− BMMCs produced comparable levels of cytokines in response to LPS alone and LPS plus IGF-1 (Fig. 3A). This suggests that LPS-induced activation of the PI3K pathway cannot be augmented by IGF-1 in SHIP1−/− BMMCs. To test whether the missing effect of IGF-1 costimulation in SHIP1−/− BMMCs was due to an altered sensitivity to IGF-1, a titration of IGF-1 with these cells was performed. SHIP1−/− BMMCs were stimulated for 4 h with 0.3 μg/ml LPS plus IGF-1 concentrations that ranged from 1 to 300 ng/ml. Indeed, TNF-α production showed a significant dependence on the IGF-1 concentration tested with a maximal cytokine production observed at only 1 ng/ml IGF-1 in both the stimulation with IGF-1 alone and the costimulation with LPS (Fig. 3B). These results demonstrate that IGF-1 can modulate LPS-induced cytokine production in a PI3K-dependent manner even in SHIP1−/− cells. Furthermore, IGF-1-mediated modulation of cytokine production is independent of SHIP1 activation.

3.4. Btk is not involved in the PI3K-dependent effects of IGF-1 on LPS-triggered cytokine production

The PI3K-dependent cytoplasmic tyrosine kinase Btk is known to play an important positive role in MCs in response to antigen stimulation [42]. Conversely, Btk negatively regulates LPS-induced secretion of IL-6 and TNF-α in MCs [43]. Thus, suppression of Btk function might be one possibility how IGF-1 enhances the outcome of LPS stimulation. To analyze this, we performed LPS plus IGF-1 costimulation experiments with WT and Btk−/− BMMCs. As expected, IGF-1 costimulation enhanced LPS-triggered TNF-α secretion and attenuated IL-1β production in WT BMMCs (Fig. 3C). As reported recently, LPS-induced TNF-α production was higher in Btk−/− BMMCs compared to WT cells (Fig. 3C; [43]). The same pattern was observed for the production of IL-1β (Fig. 3C). However, comparable effects of IGF-1 on LPS-triggered cytokine production were observed in WT and Btk−/− BMMCs, indicating that Btk is not involved in the IGF-1-driven modulation of LPS signaling.

3.5. IGF-1 differentially influences cytokine production induced by LPS, lipopeptide, and IL-33 in peritoneal mast cells

To ensure that the effects of costimulation with LPS plus IGF-1 were not restricted to the BMMCs, PMCs, an accepted model for connective tissue-type MCs [29], were analyzed. As was found for BMMCs, IGF-1 differentially influenced LPS-triggered cytokine production in PMCs (Fig. 4). As TLR4 is part of the family of Toll/IL-1 receptors, we asked whether IGF-1 costimulation-dependent modulation of signaling is a common trait of these receptors. TLR2 (receptor for bacterial lipopeptides) and the IL-33 receptor represent two further members of this family expressed on MCs [4,9]. To determine the potential of IGF-1 to affect lipopeptide- as well as IL-33-induced cytokine production, PMCs were stimulated solely with either the lipopeptide FSL-1 or IL-33 and in combination with IGF-1. The results of the subsequent IL-6 (data not shown), TNF-α, and IL-1β measurements followed the same trends as obtained with the combination of LPS and IGF-1 (Fig. 4). Costimulation with IGF-1 increased IL-6 and TNF-α, but decreased IL-1β production (Fig. 4). Comparable effects were obtained in BMMCs (data not shown). These results show that LPS-, lipopeptide-, and IL-33-induced cytokine production in both PMCs and BMMCs was affected in the same differential manner by costimulation with IGF-1. Since PMCs are mature serosal-type MCs with a clear *in vivo* equivalent [29], these data underline the relevance of our findings for MCs *in vivo*.

3.6. Differential modulation of LPS-triggered proinflammatory cytokine production by antigen via the activation of the PI3K pathway

Given that both IGF-1-induced enhancement of LPS-triggered TNF-α secretion and attenuation of IL-1β production is dependent on the PI3K pathway, we hypothesized that other PI3K-inducing ligand/receptor systems are also capable of influencing LPS-induced proinflammatory cytokine production in a similar manner. A promising candidate receptor is the high-affinity receptor for IgE (FcεR1), since triggering of this receptor by IgE/antigen complexes results in a strong activation of the PI3K pathway [18]. Moreover, costimulation of BMMCs with LPS/lipopeptides and antigen has been shown to yield a marked increase in IL-6 and TNF-α secretion compared to the single stimuli [4,22]. Indeed, costimulation of BMMCs with LPS and IgE/antigen lead to enhanced secretion of TNF-α while LPS-induced production of IL-1β was suppressed significantly by additional stimulation with antigen (Fig. 5A). Intriguingly, antigen alone did not trigger production of IL-1β (Fig. 5A). Inhibition of PI3K by Wortmannin suppressed secretion of TNF-α, but enhanced production of IL-1β in response to LPS as well as LPS plus antigen (Fig. 5B). These results corroborate the hypothesis that LPS-triggered cytokine production of MCs is susceptible to differential

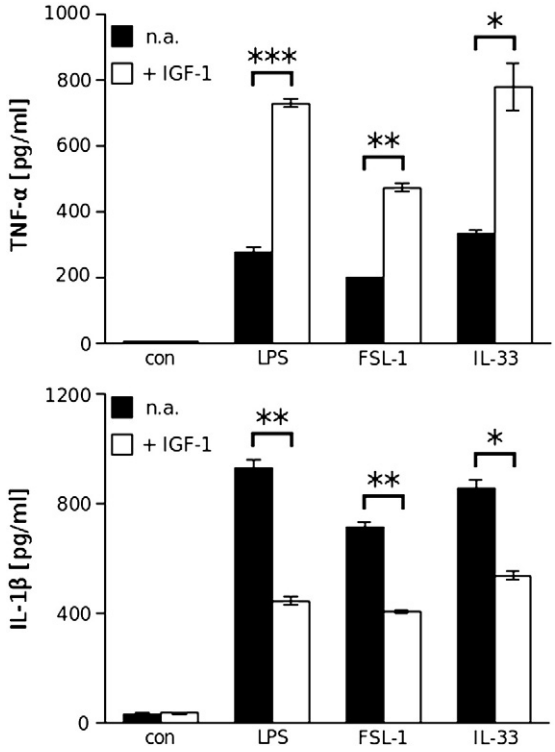

Fig. 4. IGF-1 modulates cytokine production in response to different TIR ligands in PMCs. WT PMCs were left untreated or stimulated with LPS (1 μg/ml), FSL-1 (0.5 μg/ml) or IL-33 (2 ng/ml) alone or in combination with IGF-1 (100 ng/ml) for 4 h. TNF-α (upper panel) and IL-1β (lower panel) were determined by ELISA. Each bar represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3) n.a., nothing added.

regulation by costimulation of MCs via PI3K-activating receptors, e.g. IGF-1R and FcεR1.

3.7. Constitutive activation of the PI3K pathway in mast cells suppresses LPS-induced Il-1β production

In line with the fact that the activation of c-kit by SF results in strong activation of the PI3K pathway in MCs [44], costimulation of BMMCs with LPS and SF resulted in reduced IL-1β production compared to LPS stimulation alone (Fig. 6A). Gain-of-function point mutations of c-kit are found in more than 90% of patients with systemic mastocytosis, resulting in ligand-independent, constitutive activation of c-kit signaling [45]. Consequentially, we sought to determine whether such constitutive activation of c-kit might downregulate LPS-triggered IL-1β production. The IL-3-dependent L138.8A MC line is derived from spontaneously immortalized BMMCs and contains an activating c-kit mutation on one allele [27,28]; (personal communication, L. Hültner). Compared to normal BMMCs, L138.8A MCs showed residual PI3K-dependent PKB phosphorylation even in the absence of IL-3 indicating constitutive activation of the PI3K pathway (Fig. 6B). SF stimulation of both L138.8A MCs and BMMCs resulted in comparable phosphorylation of PKB and Erk1/2 (Fig. 6B). In accordance with the constitutive activation of the PI3K pathway in L138.8A MCs and in contrast to normal BMMCs, L138.8A MCs only produced IL-1β in response to LPS in the presence of the PI3K inhibitor Wortmannin (Fig. 6C). These data implicate altered cytokine responses to microbial patterns by MCs carrying a c-kit gain-of-function mutation.

3.8. The p110δ isoform of PI3K is responsible for SF-mediated repression of LPS-induced IL-1β production

The class I subset of PI3Ks comprises several regulatory and catalytic subunits [16] [11]. Since Wortmannin does not differentiate between the

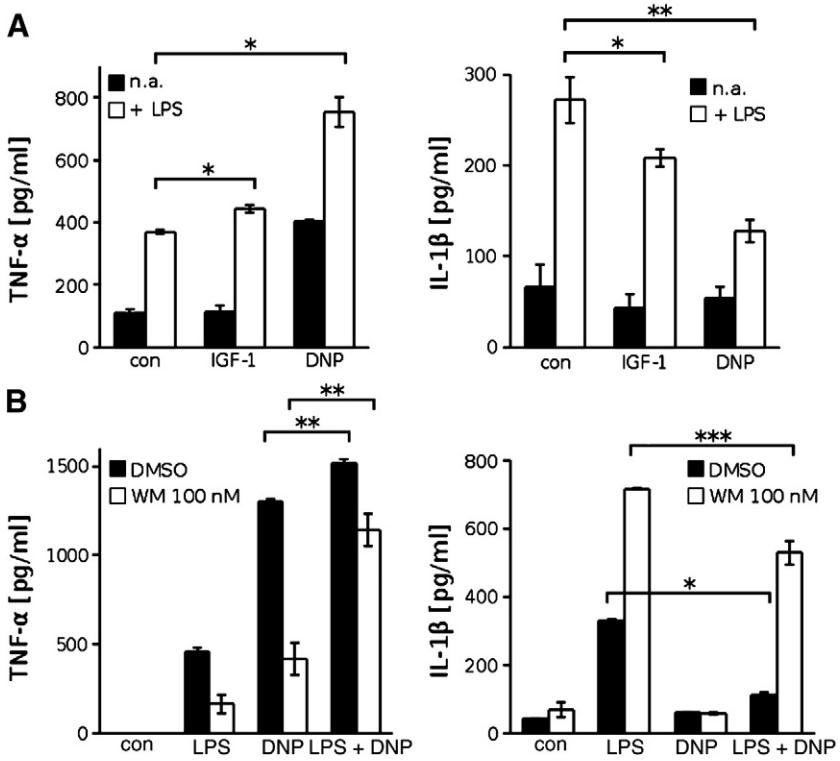

Fig. 5. PI3K-mediated modulation of LPS-induced IL-1β production by antigen-triggered FcεR1 crosslinking. (A) WT BMMCs preloaded with IgE were left untreated (con) or stimulated with IGF-1 (100 ng/ml) or antigen (DNP-HSA (DNP); 20 ng/ml) with or without the addition of LPS (0.3 μg/ml) for 4 h. Subsequently, TNF-α (left panel) and IL-1β (right panel) were determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3). (B) IgE-loaded WT BMMCS were incubated with DMSO or 100 nM Wortmannin (WM) for 20 min. They were then stimulated with LPS (0.3 μg/ml), DNP-HSA (DNP; 20 ng/ml), or a combination of both for 4 h. Subsequently, the amounts of TNF-α (left panel) and IL-1β (right panel) were determined. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3) n.a., nothing added.

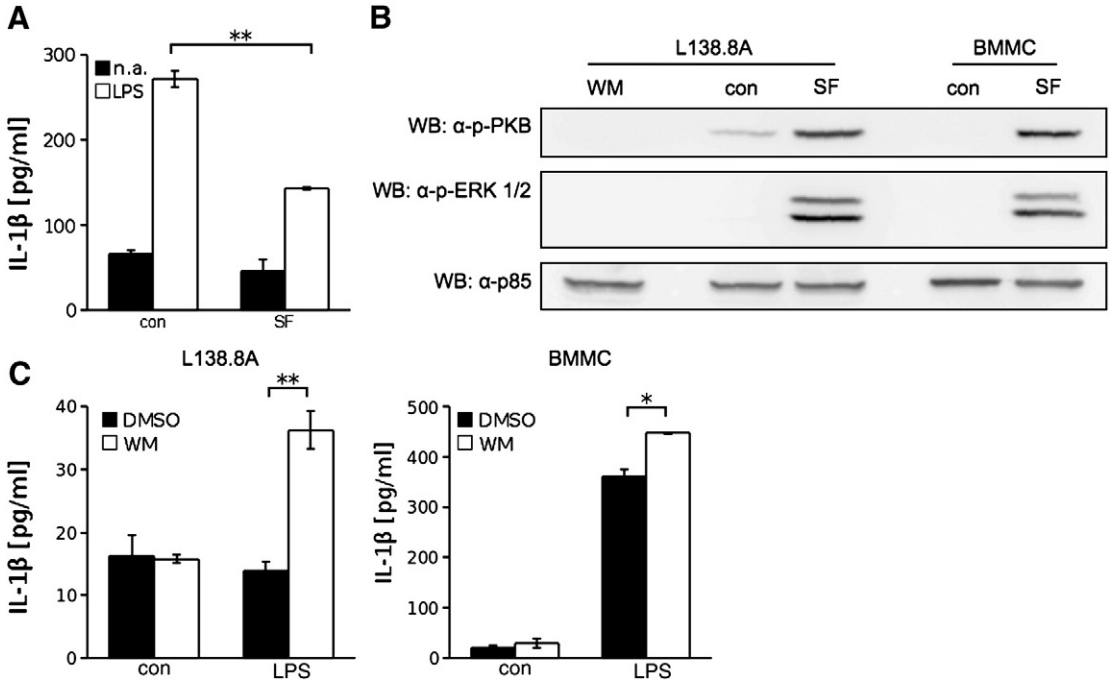

Fig. 6. IL-1β production of L138.8A BMMCs in response to LPS is prevented by constitutive activity of PI3K and can be rescued by Wortmannin. (A) WT BMMCs were left untreated (con) or stimulated with SF (100 ng/ml) with or without the addition of LPS (0.3 μg/ml) for 4 h. Subsequently, the extent of IL-1β production was determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3). (B) WT BMMCs and L138.8A BMMCs were left untreated or stimulated with 100 ng/ml Steel Factor (SF) for 1 min. As an additional treatment, unstimulated L138.8A BMMCs were pretreated with 100 nM Wortmannin (WM) for 20 min. Postnuclear supernatants were analyzed by immunoblotting with antibodies against phospho-PKB (top panel), phospho-ERK 1/2 (middle panel) and p85 (bottom panel). (C) WT BMMCs (right panel) and L138.8A BMMCs (left panel) were incubated either with DMSO or 100 nM WM for 20 min. They were then stimulated with 3 μg/ml LPS for 4 h. Subsequently the amounts of TNF-α and IL-1β were determined by ELISA. Each point represents the mean of triplicates ± SD n.a., nothing added.

different catalytic subunits of PI3K further definition of the responsible PI3K isoform requires isoform-specific inhibitors and/or respective transgenic models. Previous work has demonstrated a central role of the p110δ isoform of PI3K with respect to antigen- as well as SF-triggered MC activation [17]. In a first set of experiments, we compared the effects of Wortmannin and the p110δ-specific inhibitor D-001 on the regulation of IL-1β production. Whereas Wortmannin enhanced LPS-induced IL-1β production as well as reduced antigen- and SF-induced suppression of LPS-induced IL-1β production, D-001 only exerted a marked effect in SF + LPS-stimulated BMMCs (Fig. 7A). Further, we analyzed BMMCs from WT and p110δ knock-in mice, in which the endogenous p110δ is kinase-dead as the result of germline knock-in mutation ($p110\delta^{D910A}$) [26]. These cells were stimulated with SF, antigen, or LPS and subsequently PI3K-dependent PKB phosphorylation was measured by immunoblotting. Densitometry revealed differential dependence of the used stimuli on the p110δ isoform (SF > antigen > LPS) with respect to phosphorylation of PKB (Fig. 7B). Finally, WT and $p110\delta^{D910A}$ BMMCs were stimulated with LPS, antigen, SF, or combinations thereof (LPS + antigen and LPS + SF) and the production of IL-1β was analyzed. In accordance with the grade of dependence on p110δ activity (Fig. 7B), SF-mediated reduction of LPS-induced IL-1β production was reverted strongest in $p110\delta^{D910A}$ cells while antigen-mediated reduction was less affected and there was no difference in response to sole LPS in WT and $p110\delta^{D910A}$ BMMCs (Fig. 7C). The data indicate that SF-induced suppression of LPS-triggered IL-1β production is clearly dependent on the p110δ isoform of PI3K, and that additional isoforms of PI3K might be involved in response to different ligands.

4. Discussion

TLR-mediated activation of effector functions by microbial constituents is critical to the induction of innate and regulation of adaptive immune responses [46]. Such immune responses need to be kept under tight control. Both inefficient and excessive responses can be fatal to the host. In MCs, TLR signaling has been shown to be positively regulated by concomitant antigen stimulation. In particular, the production of proinflammatory cytokines (IL-6 and TNF-α) is increased [4,22]. On the other hand, TLR-mediated cellular activation can be suppressed by additional surface molecules, e.g. SIGIRR and RP105 [47,48].

In this study we demonstrate that IGF-1, which by itself is unable to induce cytokine production in MCs, differentially affects proinflammatory cytokine production in response to stimulation of TIR domain containing receptors (TLR4, TLR2, and IL-33R). When compared to TIR stimulus alone, additional stimulation with IGF-1 leads to increased IL-6 and TNF-α but decreased IL-1β production. This effect can be measured in serosal-type (PMCs) and mucosal-type (BMMCs) MCs.

Since IL-6, TNF-α, and IL-1β production is known to be dependent on NFκB activation [36–38], it seems remarkable that the addition of a single factor, IGF-1, is sufficient to differentially regulate expression of these cytokines. The observed oppositional regulation is mediated by the PI3K pathway, since inhibition of PI3K by Wortmannin abolishes the IGF-1-induced effects on IL-6, TNF-α, and IL-1β production. To exclude that IL-1β, the cytokine whose production was increased by PI3K inhibition, was induced independently of NFκB in MCs, we analyzed the effect of the IKKβ inhibitor, Bay-11-7082. However, the production of all cytokines investigated (IL-6, TNF-α, and IL-1β) in response to LPS alone or LPS plus IGF-1 was inhibited in a dose-dependent manner by suppression of the NFκB pathway. This suggests that one or more PI3K-dependent signaling element(s) differentially regulate NFκB-dependent production of IL-6/TNF-α and IL-1β in MCs. Interestingly, well-known MC stimuli, such as antigen and SF, which are reported to strongly activate the PI3K pathway and increase TLR-mediated IL-6/TNF-α production [10,22], are down-regulating IL-1β production in response to TLR stimulation as well in a PI3K-dependent manner, indicating a common mechanism.

This strongly suggests that factors intracellularly controlling the PI3K pathway, such as PI3K-activating proteins (e.g. adaptor proteins) and PIP_3-hydrolyzing phosphatases (e.g. SHIP1 and PTEN), might

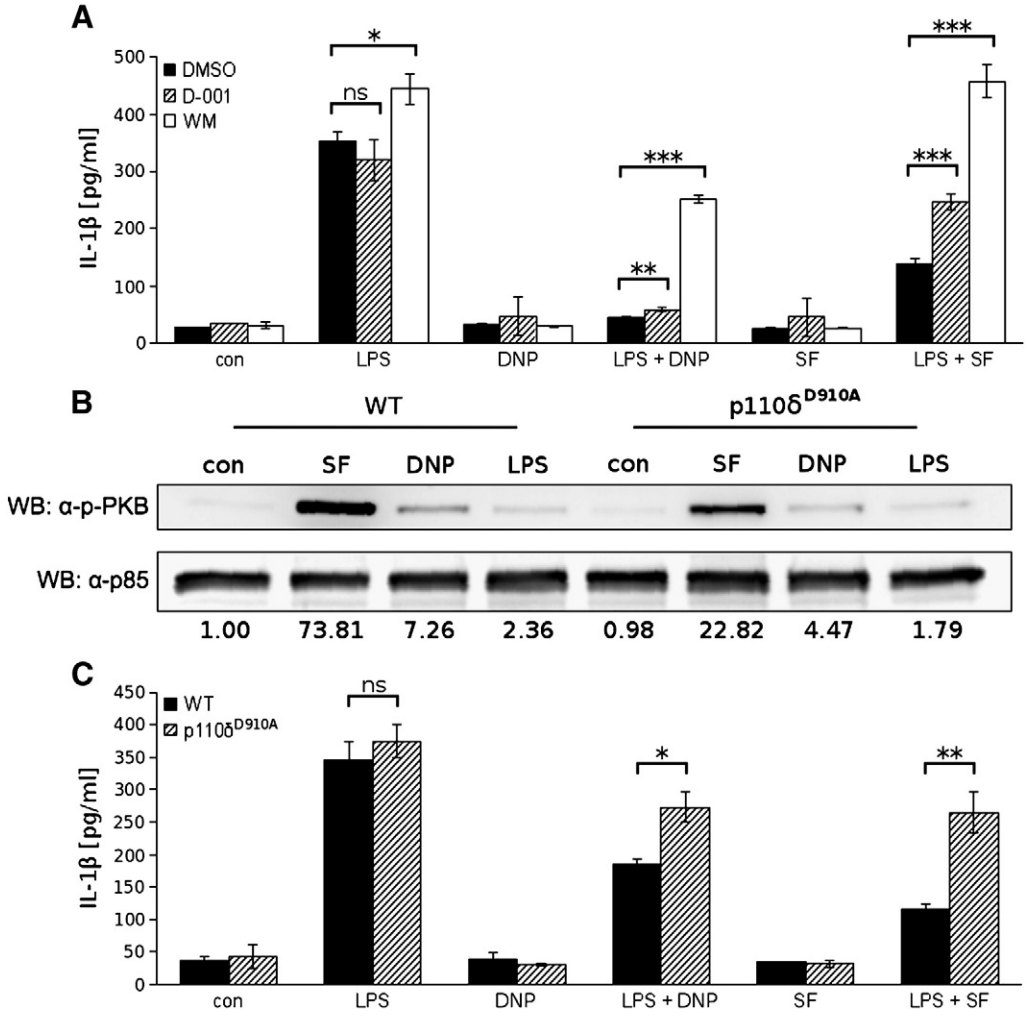

Fig. 7. The p110δ isoform of PI3K is responsible for SF-mediated repression of LPS-induced IL-1β production. (A) WT BMMCs were pretreated with either DMSO, Wortmannin (WM) or D-001 for 20 min and subsequently stimulated with 1 μg/ml LPS, 20 ng/ml DNP, 100 ng/ml Steel Factor (SF) and combinations of both LPS and DNP or SF for 4 h. The amounts of IL-1β were determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3). (B) WT and p110δ KI BMMCs were left untreated or stimulated with either 10 ng/ml SF or 5 ng/ml DNP for 1 min or 1 μg/ml LPS for 1 h. Postnuclear supernatants were analyzed by immunoblotting with antibodies against phospho-PKB (upper panel), and p85 (lower panel). Densitometry was performed and relative expression levels are indicated. (C) WT and $p110\delta^{D910A}$ BMMCs were left untreated or stimulated with 1 μg/ml LPS, 20 ng/ml DNP, 100 ng/ml SF and combinations of both LPS and DNP or SF for 4 h. Subsequently, the amounts of IL-1β were determined by ELISA. Each point represents the mean of triplicates ± SD. Comparable results were obtained in different experiments (n ≥ 3).

affect not only the amount, but also the composition of proinflammatory cytokines produced in response to TLR stimulation. In this line, SHIP1-deficiency resulted in augmented secretion of IL-6 and TNF-α, but reduced production of IL-1β in response to LPS. These effects were reversible by treatment with the PI3K inhibitor Wortmannin (data not shown). This further corroborated our finding that the activation of the PI3K pathway differentially affects the production of proinflammatory cytokines in response to TLR ligands. Thus, differential SHIP1 expression/activity in humans might contribute not only to quantitative, but also qualitative differences in proinflammatory cytokine responses in the course of disease processes. In this respect, a somatic mutation in the SHIP1 gene resulting in reduced catalytic activity of the SHIP1 protein has been found in a patient with acute myeloid leukemia [49]. Moreover, analysis of highly allergic donor basophils revealed a negative correlation between the amount of SHIP1 protein and histamine release [50].

The most intriguing result of our study concerns the downregulation of TLR-mediated IL-1β production by ligands, which cause strong activation of the PI3K pathway in MCs, such as IGF-1, antigen, and SF [10,15], and at the same time are unable to induce IL-1β production by themselves. On the contrary, costimulation of MCs with LPS and antigen yields increased IL-6/TNF-α secretion compared to the single stimuli [22]. This indicates that in a situation characterized by allergy plus infection not all MC cytokine responses are simply upregulated, but rather the cytokine pattern in the respective tissue is altered. In this line, MCs from mastocytosis patients expressing gain-of-function mutants of c-kit, e.g. D816V, might also react to microbial patterns with a different cytokine pattern compared to MCs from healthy individuals. This is strongly suggested by our data using the L138.8A MC line containing an activating c-kit mutation on one allele [27,28]. Given that MCs are involved in shaping the interstitial milieu via their secretory functions, changes in the activation state of the PI3K pathway in MCs might have strong impact on different tissue functions.

It is not possible to state at this time why the activation of the PI3K pathway by different ligand/receptor systems has opposite effects on TLR-mediated TNF-α/IL-6 and IL-1β production. As a potential mechanism for the downregulation of IL-1β production, a recent study in macrophages has shown that the transcription factor FoxO1 can bind to an insulin-responsive element within the IL-1β promotor and, in conjunction with NFκB, increases the production of IL-1β in RAW264.7 macrophages [51]. While the authors showed overexpression of FoxO1 altered IL-1β production, both IL-6 and TNF-α levels remained unaffected, suggesting a possible mechanism for the independent regulation of IL-1β in our study. The activation of PKB via PI3K leads to phosphorylation of

FoxO1 and prevents its translocation to the nucleus [52]. Thus, it is possible that the downregulation of IL-1β production we observe in BMMCs and PMCs in response to ligands, which cause strong PI3K activation, is due to phosphorylation of FoxO1. Consistent with this, IGF-1, antigen, and SF have been shown to negatively regulate forkhead transcription factors in MCs [15,53,54].

While our results obtained by using the pan-specific PI3K inhibitor Wortmannin and SHIP1−/− BMMCs show the importance of PI3K for the regulatory mechanism described in this study, it does not reveal the nature of the PI3K isoform involved. Due to progress in the development of isoform-specific pharmacological inhibitors as well as generation of various transgenic mouse models, the definition of specific roles of the different PI3K isoforms is possible [16] [11]. However, within the PI3K signaling system there is strong redundancy most likely owing to the crucial biological role of PI3K signaling. As an example, p110α could compensate in the absence of p110δ to promote early B cell development in the bone marrow and B cell survival in the spleen [55]. Moreover, p110β was shown to be functionally redundant with p110γ with respect to PI3K signaling downstream of G-protein-coupled receptors [56]. Such strong redundancies imply difficulties in defining the responsible isoform (s) for a given signaling situation. Nevertheless, using p110δ-specific pharmacological inhibitors and transgenic BMMCs expressing a catalytically inactive p110δ mutant (D910A) we were able to demonstrate an important role of this isoform for the SF-induced suppression of LPS-triggered IL-1β production. The picture obtained is somewhat clearer using the inhibitor and this might be due to adaptation processes of the p110δ null BMMCs during the *in vitro* differentiation of BMMCs.

Finally, it is important to note that TLR-mediated production of proinflammatory cytokines (IL-6 and TNF-α) in monocytes/macrophages is also regulated by the PI3K pathway, however in a contrary fashion to what is reported here in MCs. In these cells, inhibition of PI3K results in enhanced production, whereas deletion of the PIP_3 phosphatases, SHIP1 or PTEN, leads to a marked attenuation of proinflammatory cytokine production [57,58]. This demonstrates that adoption of knowledge about regulation and function of signaling pathways from one cell type to another might lead to faulty assumptions and advocate the comparable analysis of differential cell types.

5. Conclusion

In conclusion, we show that different PI3K-activating ligand/receptor systems, like IGF-1/IGF-1R, antigen/FcεR1, and SF/c-kit, are able to differentially regulate TLR-mediated production of NFκB-dependent proinflammatory cytokines in MCs. Secretion of IL-6 and TNF-α is enhanced, whereas the production of IL-1β is suppressed. Moreover, we pinpoint the particular role of the p110δ isoform of PI3K for the respective SF/c-kit-driven regulation.

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