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In addition to regulating mast cell homeostasis, the activation of KIT following ligation by stem cell factor promotes a diversity of mast cell responses, including cytokine production and chemotaxis. Although we have previously defined a role for the mammalian target of rapamycin complex 1 in these responses, it is clear that other signals are also required for maximal KIT-dependent cytokine production and chemotaxis. In this study, we provide evidence to support a role for glycogen synthase kinase 3β (GSK3β) in such regulation in human mast cells (HuMCs). GSK3β was observed to be constitutively activated in HuMCs. This activity was inhibited by knockdown of GSK3β protein following transduction of these cells with GSK3β-targeted shRNA. This resulted in a marked attenuation in the ability of KIT to promote chemotaxis and, in synergy with FceRI-mediated signaling, cytokine production. GSK3β regulated KIT-dependent mast cell responses independently of mammalian target of rapamycin. However, evidence from the knockdown studies suggested that GSK3β was required for activation of the MAPKs, p38, and JNK and downstream phosphorylation of the transcription factors, Jun and activating transcription factor 2, in addition to activation of the transcription factor NF-κB. These studies provide evidence for a novel prerequisite priming mechanism for KIT-dependent responses regulated by GSK3β in HuMCs. The Journal of Immunology, 2010, 184: 564–572.

Mast cells are tissue-resident cells of hematopoietic origin that play a role in innate and acquired immune responses (1). Mast cell growth, development, and survival are driven by stem cell factor (SCF)-dependent activation of its receptor KIT (2). In addition to its role in mast cell homeostasis, however, SCF can also regulate other mast cell functions. In this respect, SCF-mediated activation of KIT potently induces mast cell chemotaxis (3, 4) and adhesion to extracellular matrix (5), supporting a role for SCF in mast cell homing to their tissues of residence in vivo. Furthermore, SCF-mediated KIT activation, particularly in conjunction with aggregation of the FceRI, also promotes the generation of multiple cytokines and chemokines (5–7).

KIT is a member of the growth factor receptors with inherent tyrosine kinase activity family (8, 9). Dimerization of KIT, following SCF binding, activates its inherent tyrosine kinase activity resulting in phosphorylation of specific tyrosine residues in the cytoplasmic tail of KIT allowing recruitment of critical adaptor and signaling molecules (10). These receptor–proximal events lead to the initiation of multiple downstream signaling processes, eventually culminating in transcriptional regulation (8, 9, 11). Despite a comprehensive understanding of these immediate signaling events elicited by activated KIT, it is unclear how these events subsequently differentially control the diverse group of responses mediated by KIT. In exploring this differential regulation, we recently described, however, that the mammalian target of rapamycin complex (mTORC1) cascade, which is activated downstream of PI3K following challenge of either mouse or human mast cells with SCF (12), contributes to the regulation of SCF-mediated chemotaxis and SCF/Ag-mediated cytokine production (12). Nevertheless, because a substantial portion of these responses remained following rapamycin-induced inhibition of mTORC1 signaling, it was concluded that other signaling pathways apart from those regulated by mTORC1 participate in the regulation of SCF-mediated chemotaxis and transcriptional activation, leading to cytokine and chemokine generation (12).

In this study, we present evidence to support a role for glycogen synthase kinase 3β (GSK3β) in such regulation. GSK3β is a ubiquitously expressed serine/threonine kinase, which has been reported to play a role in the regulation of diverse cellular responses including cell growth, tumorigenesis, cell migration, and cytokine generation (13–15). However, it is not fully understood how GSK3β regulates these responses. In studies that use knockdown of GSK3β expression, we now demonstrate that GSK3β activation is a prerequisite signal for SCF-mediated chemotaxis and SCF/Ag-mediated cytokine generation. Thus, this may constitute a novel priming mechanism for specific mast cell responses. The regulation of the chemotactic response by GSK3β appears dependent on its modulation of JNK and p38-dependent pathways, whereas the regulation of cytokine generation by GSK3β may be explained by the differential regulation of transcriptional control downstream of JNK and p38.

Materials and Methods

Mast cell culture

Primary human mast cells (HuMCs) were derived from CD34+ peripheral blood progenitor cells (16) obtained from normal volunteers following informed consent under a protocol approved by the National Institutes of...
The Journal of Immunology

Health internal review board. The cells were developed in StemPro-34 culture medium containing StemPro-34 supplement (Invitrogen, Carlsbad, CA), t-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), recombinant human (rHu) IL-3 (30 ng/ml, first week only), rHuIL-6 (100 ng/ml), and rHuSCF (100 ng/ml) (PeproTech, Rocky Hill, NJ). Experiments were conducted 7–9 wk after the initiation of HuMC cultures.

Lentivirus short hairpin RNA transfection of 293T cells and transduction of HuMCs

The following GSK3ß-targeted short hairpin (sh)RNAs were purchased from Sigma-Aldrich (St. Louis, MO): CCGGGTGTGGATCAGTTGGTATATT (control nontarget control vector). The packaging vector (MissionLentiviral CCAGCCACTACAGAATTTAACTCGAGTTAAATTCTGTGTAGTTTG-10552) (construct A); CCGGCCAATCTAAGTTGGAATCTCGAGTTTCTACCAACTGATCCACACTTTTT (TRCN0000039998) (construct C); CCGGC- CCAAAAAATCAAAGAATTTAATCGTGAATATCCTGTGTTGGTGTTTGG (TRCN0000039999) (construct D); CCGGCCGCAACAGAAGAGTGTATTACATGGTCTTTTATTCCCTGTGTTGGTGTTTGG (TRCN0000039990) (control vector). These constructs were cotransfected into 293T cells using FuGENE6 transfection reagent (Roche, Indianapolis, IN) in Opti-MEM medium. The transfected 293T cells were grown in DMEM containing FBS (10%), t-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Following 16–19 h of transfection, medium was removed and replaced with fresh DMEM. After 62–65 h of transfection, virus was collected by centrifugation (25,000 rpm, 1 h 40 min, 4°C), then the resulting pellet resuspended in 3 ml prewarmed complete StemPro medium.

Expression and knockdown of GSK3ß in human mast cells

HuMCs were incubated overnight in complete Stem Pro medium containing human myeloma IgE (100 ng/ml; Calbiochem, La Jolla, CA), biotinylated within the National Institute of Allergy and Infectious Diseases Core Facility. The cells were then starved in cytokine-free StemPro medium for 4 h (for chemotaxis assay and corresponding cell lysate preparations), then the cells were activated by the addition of SCF (30 ng/ml). For cytokine release studies, HuMCs (1 × 106/ml) were sensitized in complete StemPro culture medium overnight and the next day washed in culture medium and triggered concurrently via KIT with SCF (30 ng/ml) and via the FcεRI with streptavidin (SA) (100 ng/ml) for 6 h. Some experiments, the cells were pretreated with the mTOR inhibitor, rapamycin (100 nM) or the PI3K inhibitor, wortmannin (100 nM), (Calbiochem) for 20 min prior to activation.

Real-time PCR analysis

HuMCs (2–3 × 105/sample) were sensitized overnight with biotinylated human IgE (100 ng/ml) in complete StemPro medium. The following day, cells were washed with the same medium three times to remove excess IgE, then the cells were stimulated with SA (100 ng/ml) and SCF (30 ng/ml) for 4 h. Total RNA was isolated from each preparation using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of total cellular RNA was treated for genomic DNA contamination and reverse transcribed using SA Biosciences Reverse Transcription reagents and oligo(dT) (SA Biosciences, Frederick, MD). Gene expression was analyzed using real-time PCR on an ABI7500 SDS system (Applied Biosystems, Foster City, CA). A common cytokine PCR array was purchased from SA Biosciences, and real-time PCR was performed according to manufacturer’s instructions. All reactions (two different HuMCs donors) were performed in triplicate for 40 cycles. The relative fold expression levels of cytokines was calculated as follows: for each sample the threshold cycle (Ct) was determined and normalized to the average of five different housekeeping genes in the KIT (ΔCt). The ΔCt of treated or untreated cells was then subtracted from untreated control shRNA-transduced cells (ΔΔCt) and the relative fold expression was calculated using the equation 2ΔΔCt.

Cytokine quantitation

Cell-free supernatants from activated cells were harvested and cytokine content was measured by using DuoSet ELISA System (R&D Systems, Minneapolis, MN).

Chemotaxis

Chemotaxis assays were performed using Transwell polycarbonate membranes (8-µm pore size) (Corning, Corning, NY). HuMCs (1 × 10⁶/well) were incubated in cytokine-free StemPro medium for 4 h and then resuspended in cytokine-free StemPro medium containing 0.5% BSA. The cell suspension (100 µl) was placed in the upper chamber and preincubated in the bottom chamber containing 600 µl cytokine-free StemPro medium for 30 min at 37°C. After 30 min, the inserts were replaced into the bottom chambers with or without SCF (30 ng/ml). After 4 h, the migrated cells were collected in the bottom chamber and counted under microscopy.

Immunoblotting

Cell lysates were prepared as described previously (18). Aliquots of lysates were loaded onto a 4–12% NuPage BisTris gel (Invitrogen) and following electrophoresis, proteins were transferred onto nitrocellulose membranes. The proteins were probed with following phospho-(p)-specific Abs from Cell Signaling Technology (Beverley, MA): p-AKT (S473), p-GSK3b (S9), p-JNK (T183 and Y185), p-map kinase kinase (MKK) 3/6 (S189 and Y192), p-ERK1/2 (T202/Y204), and p-GSK3b (T71), p-NFkB (S536), p-NFkB (S276), total JNK, total p38, and total NF-κBp65. p-Clun (S73) was from Upstate Biotechnology (Lake Placid, NY), and p-GSK3α/β (Y279/Y216) was from Invitrogen. Total Syk, total KIT, total Lyn, and total clun Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Immune reactive proteins were visualized by probing with HRP-conjugated secondary antibodies and then by ECL (PerkinElmer, Wellesley, MA). Quantification of changes in protein phosphorylation were performed using a Quantity One scanner (Bio-Rad, Hercules, CA).

Statistical analysis

Data are represented as the mean ± SE. The statistical analyses were performed by unpaired Student’s t test. Differences were considered significant when p < 0.05. The n values represent experiments from multiple preparations.

Results

Expression and knockdown of GSK3β in human mast cells

Disruption of the GSK-3β gene in mice leads to an embryonically lethal phenotype (19), therefore, to explore the role of GSK3b in human mast cell function, we elected to use a gene knockdown approach. To achieve this, HuMCs were stably transduced with GSK3ß-targeted shRNA, using a lentivirus system. Five (A–E) different constructs were examined for their ability to selectively knockdown GSK3β expression. As a control, we used a scrambled shRNA construct purchased from Sigma-Aldrich. The level of expression of GSK3ß in the cells treated with the control-scrambled shRNA was not substantially different from that observed in untreated cells (data not shown). Of the shRNAs targeting GSK3ß, four decreased GSK3ß levels to varying degrees within the cells following transfection. Of these, two (A and B) were selected for further characterization based on their differential abilities to reduce GSK3ß expression in HuMCs (A: 41 ± 3%; B: 69 ± 4% reduction) (Fig. 1A). These constructs had little effect on the expression of 2561.
through KIT and FcεRI, we observed no consistent increase in the phosphorylation of this residue. Similarly, the GSK3β substrate, GS, was constitutively phosphorylated at S641 (Fig. 2A, 2C). However, in contrast to the phosphorylation of GSK3β, this phosphorylation was slightly elevated in cells activated through KIT and FcεRI. Surprisingly, in light of this observation, we also observed that the phosphorylation of the inhibitory S9 residue of GSK3β was enhanced in the activated HuMCs (Fig. 2A, 2D).

Similar responses to the above were observed in HuMCs activated via either FcεRI or KIT alone (data not shown).

As expected, because of the effective reduction in total GSK3β protein in the GSK3β knockdown HuMCs, the phosphorylation of GSK3β-Y216 and GSK3β-S9 was substantially reduced in both quiescent and activated HuMCs (Fig. 2). The phosphorylation of GS at S641, however, was also reduced in these cells. In contrast, and by means of a negative control, we observed no decrease in the SCF/SA-dependent phosphorylation of AKT, a surrogate marker for P13K activation, in these cells (Fig. 2A, 2E). Taken together, these data suggest that, in HuMCs, GSK3β is active under resting conditions and that, upon cell activation through FcεRI and KIT, this permits an increase in phosphorylation of the GSK3β substrate GS. These responses can be effectively reduced in the HuMCs following shRNA-induced knockdown of GSK3β.

**Effect of shRNA-induced knockdown of GSK3β on mast cell cytokine production**

Having successfully established knockdown of GSK3β activity in HuMCs, we next investigated the role of GSK3β in mast cell cytokine generation. For these studies, the cells were again costimulated through KIT, via SCF, and the FcεRI, through sensitizing with biotinylated IgE, and challenging with SA as the response to either stimulus alone is not marked (20). To initially screen the effect of GSK3β knockdown on multiple cytokines, cells were stimulated for an optimal period of 4 h, based on previous kinetic studies (25), then cytokine gene expression was determined by a commercially available array. In these studies, we observed a marked increase in mRNA’s for multiple cytokines, including those for GM-CSF, IL-8, and IL-13 (Fig. 3A–C and Supplemental Material). These responses were markedly attenuated in GSK3β knockdown cells compared with the control cells (Fig. 3A–C). On the basis of this initial screen, we examined the amounts of GM-CSF and IL-8 protein present in the supernatants of control and GSK3β knockdown HuMCs challenged with SCF in the presence of SA. As demonstrated in Fig. 3D and 3E, there was a marked reduction in SCF/SA-induced generation of GM-CSF and IL-8 in the GSK3β knockdown HuMCs compared with controls. The relative inhibition produced by the two constructs (Fig. 3D, 3E) correlated with their relative abilities to knockdown GSK3β protein levels (Fig. 1A). This close correlation was further illustrated by plotting the relative expression of GSK3β protein levels to that of IL-8 production (Fig. 3F).

**Effect of shRNA-induced knockdown of GSK3β on mast cell chemotaxis**

In addition to stimulating cytokine generation in mast cells, SCF is a potent chemotactic agent for HuMCs (4). To thus explore whether SCF-mediated chemotaxis was similarly dependent on GSK3β, we next examined the relative ability of HuMCs to migrate in response to SCF following GSK3β knockdown. From Fig. 3G, it can be seen that GSK3β knockdown HuMCs displayed a reduced capacity to migrate toward SCF compared with control cells. The extent of inhibition of migration again correlated to the extent of knockdown of GSK3β observed in these studies, with GSK3β shRNA B producing a greater knockdown of GSK3β and migration than that produced by GSK3β shRNA A (Fig. 3G).
GSK3β regulates mast cell responses independently of mTOR

We next examined how GSK3β may regulate mast cell cytokine production and chemotaxis. GSK3β has been reported to phosphorylate the mTOR regulator, tuberin (TSC2), in HEK293T cells (26), thereby influencing the activation of the mTORC1 and mTORC2 cascades. Our previous studies in HuMCs demonstrated that the mTORC1 cascade, downstream of PI3K, contributes to SCF/SA-mediated cytokine production and SCF-mediated chemotaxis in human mast cells (12). The mTORC1 cascade is initiated by the PI3K-dependent activation of AKT, which phosphorylates and inactivates TSC1 and TSC2 (tuberin), negative regulators of mTOR activation. This results in the sequential phosphorylation and activation of mTOR and its downstream substrates: the transcriptional regulators p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (27, 28). In contrast, the mTORC2 cascade leads to the phosphorylation (S473) and activation of AKT (29–31). We therefore investigated whether the reduced cytokine production in the GSK3β knockdown HuMCs may be a consequence of inhibition of the mTORC1- or mTORC2-regulated signaling cascades.

As before, GSK3β knockdown significantly reduced GSK3β expression in HuMCs (Fig. 4A, 4B). However, there were minimal differences in the basal and SCF/SA-induced phosphorylation of mTOR, p70S6K, and 4E-BP1 in the GSK3β knockdown HuMCs cells compared with controls (Fig. 4A–E). Furthermore, as discussed earlier, there was also no difference in the resting or SCF/SA induced, mTORC2-dependent phosphorylation of AKT(S473) in the GSK3β knockdown HuMCs when compared with control cells (Fig. 2A, 2E). Similarly, the mTORC1 inhibitor, rapamycin, failed to block the phosphorylation of GSK3β (S9, Y216) (Fig. 4F) despite a marked inhibition of GSK3β (S9 only) by the PI3K inhibitor wortmannin. Taken together, these data support the conclusion that GSK3β regulates SCF/SA-induced mast cell responses independently of both the mTORC1 and mTORC2 cascades. We therefore next examined whether GSK3β regulated human mast cell cytokine production and chemotaxis by an alternative mechanism.

Regulation of the MAPK activation by GSK3β in human mast cells

The MAPKs, p38, and JNK, regulate transcriptional activation pathways that contribute to mast cell function, including cytokine generation (32). Thus, we next investigated whether the MAPK cascade represented a key intermediary step in the regulation of GSK3β-regulated mast cell cytokine production. As can be seen in Fig. 5A, in contrast to the lack of effect on the mTOR cascades, the increase in both JNK (Fig. 5A, 5B) and p-38 (Fig. 5A, 5C) phosphorylation in response to SCF/SA was reduced in the GSK3β knockdown HuMCs compared with the control treated...
These and potentially other transcription factors, which are known to regulate the phosphorylation of multiple transcription factors including Jun and ATF2. The MAPK pathway leads to the phosphorylation and, thus, regulation of many transcription factors by GSK3β. The GSK3β subunit was also significantly reduced in the GSK3β knockdown HuMCs compared with control-treated cells. Furthermore, the phosphorylation of the p65 NF-κB subunit was also significantly reduced in the GSK3β knockdown cells compared with control-treated cells (Fig. 6A, D). Taken together, these data support the conclusion that GSK3β activation is a prerequisite signal for the MAPK-dependent activation of c-Jun and ATF2, and also NF-κB p65 activity, which in turn regulates SCF/SA-induced cytokine production.

**Discussion**

In this study, we present evidence that supports a prerequisite role for GSK3β in KIT-mediated mast cell chemotaxis and KIT/FceRI-mediated enhanced gene expression leading to cytokine production in HuMCs. As reported in other cell types (14, 21, 22), in quiescent HuMCs, GSK3β was determined to be constitutively activated. This conclusion was supported by the observed basal phosphorylation of the activating tyrosine residue (Y216) in GSK3β, the phosphorylation of its substrate GS at S641, and the reduction of these phosphorylation states in the GSK3β knockdown cells (Fig. 2). Although we did not observe a consistent increase in phosphorylation of GSK3β at the Y216 position in cells. There was no effect on total p38 or JNK protein levels under these conditions (Fig. 5A). The SCF/SA-induced phosphorylation of MKK3/6, upstream of p38, was however also markedly reduced in the GSK3β knockdown-HuMCs (Fig 5A, 5D).

Because the MAPKs JNK and p38 have also been shown to regulate SCF/KIT-induced mast cell migration (33–35), we next determined the phosphorylation status of these proteins under the conditions used for the chemotaxis studies, i.e., stimulation with SCF after a 4-h period of starvation in SCF-depleted media. As shown in Fig. 5E–I, SCF-mediated MKK3/6, JNK, and p38 activation, but not total p38 or JNK protein, were significantly reduced in the GSK3β knockdown cells, compared with control-treated cells under these conditions. In contrast, SCF-induced AKT activation was unaffected compared with control-treated cells (Fig. 5E, 5I).

From these data, we conclude that GSK3β regulates the activation of the MAPKs p38 and JNK, thus providing an explanation for the reduced SCF-induced chemotaxis observed in the GSK3β knockdown HuMCs. Furthermore, GSK3β-dependent, p38- and JNK-mediated transcription activation would also provide an explanation for the reduced SCF/SA-induced cytokine production of served in the GSK3β knockdown HuMCs. We thus next examined the SCF/SA-induced phosphorylation of specific transcription factors in the GSK3β knockdown HuMCs.

**Transcription factors regulation by GSK3β**

The MAPK pathway leads to the phosphorylation and, thus, regulation of multiple transcription factors including Jun and ATF2. These and potentially other transcription factors, which are known to be phosphorylated in mast cells, may contribute to mast cell cytokine production and other transcriptional-dependent processes. We therefore examined whether GSK3β regulated the phosphorylation of Jun, ATF2, and NF-κB. As can be seen in Fig. 6, the phosphorylation of the AP-1 transcription factors c-Jun (Fig. 6A, 6B) and ATF2 (Fig. 6A, 6C) in response to SCF/SA stimulation was greatly reduced in GSK3β knockdown HuMCs compared with control-treated cells. Furthermore, the phosphorylation of the p65 NF-κB subunit was also significantly reduced in the GSK3β knockdown cells compared with control-treated cells (Fig. 6A, 6D). Taken together, these data support the conclusion that GSK3β activation is a prerequisite signal for the MAPK-dependent activation of c-Jun and ATF2, and also NF-κB p65 activity, which in turn regulates SCF/SA-induced cytokine production.
response to SCF and/or SA, under the conditions used to examine chemotaxis and cytokine production, there was an apparent increase in the phosphorylation of GS at S641 under these conditions, which was reduced in the GSK3β knockdown. The constitutive Y216 phosphorylation of GSK3β may be due to the cells being maintained in SCF. Indeed, when the cells were starved of SCF for a prolonged period of time (overnight) prior to stimulation, we were able to observe an SCF-dependent increase in the phosphorylation of this residue. Thus, the phosphorylation of this residue may be directly dependent on KIT. Regardless, our results suggest that triggering of mast cells through KIT and/or FcεRI facilitates the ability of GSK3β to phosphorylate its substrate(s) without necessarily increasing its constitutive activity, a potential mechanism of action that is elaborated upon below.

In addition to the phosphorylation of (Y216) in GSK3β, however, we observed that the inhibitory S9 residue GSK3β was also phosphorylated in a PI3K-dependent manner following SCF/SA challenge (Figs. 2D, 4F). This phenomenon has also been reported in monocytes, dendritic cells, and T cells, following exposure to TLR2, TLR4, TLR5, and TLR9 agonists, Escherichia coli, and viral peptide respectively (36–38). In our study, however, the observed increased phosphorylation of GS, at least at early time points, would suggest that downregulation of GSK3β activation may occur latently to the constitutive activation. We have previously demonstrated that PI3K, and signals dependent on PI3K activity, are delayed responses compared with other signals initiated upon FcεRI or KIT activation (12). Thus, it is likely that any response due to downregulating GSK3β activity would be chronologically secondary to those regulated by GSK3β activation. Nevertheless, these data do suggest that the ability of GSK3β to phosphorylate its substrates may depend on the net balance between positive and negative regulation of GSK3β activity.

The marked reduction in the ability of SCF/SA to enhance IL-8, IL-13, and GM-CSF mRNA levels and IL-8 and GM-CSF secretion, associated with the diminution of GSK3β activity in the GSK3β knockdown HuMCs (Fig 3F), strongly supports a requirement for GSK3β activity in the regulation of KIT/FcεRI-mediated cytokine production. This conclusion is further supported by the close statistical correlation between the degree of GSK3β knockdown and IL-8 secretion. Similarly, the close correlation between GSK3β knockdown and reduction in SCF-induced chemotaxis in the GSK3β knockdown HuMCs also provides evidence for a prerequisite role for GSK3β in the SCF-induced chemotactic response.

There are conflicting reports regarding the role of GSK3β in cytokine production in other cells of hematopoietic lineage.

**FIGURE 4.** GSK3β regulates SCF/SA-mediated mast cell responses independently of mTOR. HuMCs, transduced with scrambled shRNA (shContr) or shRNA for GSK3β (shGSK3β-B), were sensitized overnight and then stimulated with SA (100 ng/ml) and SCF (30 ng/ml) for 10 min as described in Materials and Methods. Whole-cell extracts were prepared and immunoblotted with anti-GSK3β, anti-p-mTOR(S2448), anti-p-70S6K(T389), and anti-p-4E-BP1(T37, T46) Abs (A). Data in B–E were generated by scanning the blots from three to five independent experiments and normalized to the response obtained at 10 min with SCF/SA stimulation (n = 3–5, *** < 0.001 for comparison with SCF/SA response in shContr-transduced HuMCs by Student’s t test). In F, HuMCs were pretreated with rapamycin (100 nM) or wortmannin (100 nM) 20 min prior to stimulation with SCF or SA at the time indicated. Whole-cell extracts were prepared and immunoblotted with anti-p-AKT(S473), anti-p-GSK3β (Y216), anti-p-GSK3β(S9), or anti-p-GS(S641) Abs. Protein loading of the samples was normalized by probing for Syk.
Treatment of monocytes with GSK3β inhibitors such as LiCl and/or SB216763, or with GSK3β-targeted small interfering RNA, has been reported to inhibit TLR2-, 4-, 5-, and 9-dependent release of IL-1β, IL-6, TNF-α, IL-12, and IFN-γ but to enhance TLR-dependent production of IL-10 (36). GSK3β inhibitors were also reported to inhibit *E. coli*-induced IL-12, IL-6, and TNF-α, but not IL-10, release from dendritic cells (37). In contrast, in T cells, GSK3β inhibitors were observed to enhance viral peptide-induced IL-2 production, whereas overexpression of GSK3β in T cells downregulated the response (38). This apparent dichotomy in the GSK3β-dependent regulation of cytokine generation in the various cell types may reflect the potential for GSK3β to both negatively and positively regulate transcriptional signaling pathways for cytokine production. Indeed, it is possible that, in addition to

![FIGURE 5.](http://www.jimmunol.org/)

**FIGURE 5.** GSK3β regulates SCF/SA- and SCF-mediated MAPK activity in HuMCs. HuMCs, transduced with scrambled shRNA (shContr) or shRNA for GSK3β (shGSK3β-B), were sensitized overnight and then stimulated with SCF (30 ng/ml) (A) or starved for 4 h then stimulated with SCF (30 ng/ml) (E) for 2 min as described in Materials and Methods. Whole-cell extracts were prepared and immunoblotted (A and E) with anti-p-JNK (T183, Y185), total JNK, anti-p-MKK3/6 (S189, S207), anti-p-p38 (T180), total p-38, or p-AKT (S473) Abs. Protein loading of the samples was normalized by probing for Syk (A and E). Data in B–D and F–I were generated by scanning the blots in three to five independent experiments and normalized to the response obtained with SCF/SA stimulation (B–D), or SCF response (F–I), in shContr-transduced HuMCs (n = 3–5; *p < 0.05, **p < 0.001 and ***p < 0.0001, by Student’s t test).

![FIGURE 6.](http://www.jimmunol.org/)

**FIGURE 6.** GSK3β regulates SCF/SA-mediated AP1 transcription factors and NF-κB activity in HuMCs. HuMCs, transduced with scrambled shRNA (shContr) or shRNA for GSK3β (shGSK3β-B), were sensitized overnight and then stimulated with SA (100 ng/ml) and SCF (30 ng/ml) for 30 min as described in materials and methods. Whole-cell extracts were prepared and immunoblotted with anti-p-cJun (S73), total cJun, anti-p-ATF2(T71), or anti-p-NF-κB(S536) Abs (A). Protein loading of the samples was normalized by probing for Syk (A). Data in B–D were generated by scanning in three to four independent experiments and normalizing to the responses obtained with SA/SCF stimulation in shContr-transduced HuMCs (*p < 0.05, **p < 0.001, and ***p < 0.0001, by Student’s t test).
in our present study, however, although SCF/SA-induced phosphor-
cells (32) and HuMCs (6). Similarly, both JNK and p38 have been
transcription factors in both mouse bone marrow-derived mast
have been shown to regulate cytokine production mediated by AP1
pathways and, particularly, in the respective downstream tran-
GSK3b
k
b

regulating positive signals, negative signaling pathways may also
be regulated by GSK3β in mast cells. In this respect, it has been
suggested that the ability of AKT to enhance cytokine generation
through NF-κB activation in mouse mast cells may be due to
downregulation of GSK3β activity (39). Whether this may also be
true for HuMCs is unclear from the current study; however, the
induced phosphorylation of the inhibitory GSK3β S9 residue in
response to SCF/SA in HuMCs was reduced by the PI3K inhibitor
wortmannin (Fig. 4F).

There has emerged no common mechanistic explanation as to
how GSK3β may be exerting its regulatory influence on cytokine
generation and other processes in hematopoietic cells. As we have
previously demonstrated that the mTORC1 cascade contributes to
KIT/FcεRI-mediated cytokine production and KIT-mediated mast
cell chemotaxis (12), and as GSK3β has been proposed to regulate
the mTOR pathway through phosphorylation of tuberin (26), the
scenario existed that, in the HuMCs, GSK3β may be acting via
regulation of mTOR pathways. However, the observations that
the KIT/FcεRI-mediated phosphorylation of components of the
mTORC1 and mTORC2 cascades was not reduced in the GSK3β
knockdown HuMCs (Fig. 4), argues against this possibility. It has
been proposed that the contrasting roles for GSK3β in TLR-
cytokine production in monocytes may be explained by opposing
regulation of the transcription factors CREB and NF-κB through
competition for binding to a common coactivator protein CREB
binding protein (36). According to this model, GSK3β inhibition
would increase CREB activation allowing CREB to compete with
the p65 subunit of NF-κB for binding to CREB binding protein.
In our present study, however, although SCF/SA-induced phosphor-
ylation of the p65 subunit of NF-κB was observed to be signifi-
cantly reduced in the GSK3β knockout HuMCs (Fig. 6A, 6D),
we did not consistently observe an increase in CREB activity in
these cells (data not shown). Regardless, these data are in agree-
ment with other studies showing that GSK3β is required for NF-
κB activation (19).

The most remarkable defects that we observed, however, in the
GSK3β knockout HuMCs were in the p38 and JNK MAPK
pathways and, particularly, in the respective downstream tran-
scription factors ATF2 and c-Jun. JNK activity has previously
been shown to regulate cytokine production mediated by AP1
transcription factors in both mouse bone marrow-derived mast
cells (32) and HuMCs (6). Similarly, both JNK and p38 have been
previously described to regulate mast cell chemotaxis (33–35).
Therefore, the reduced SCF/SA-induced cytokine production and
SCF-induced chemotaxis observed in the GSK3β knockout
HuMCs may be explained by defective JNK and p38 signaling in
these cells (Fig. 5A–D).

How GSK3β may act as a prerequisite signal for the regulation of
these pathways may be explained by the unique manner in which
GSK3β phosphorylates its substrates. As discussed, GSK3β sub-
strates require prior phosphorylation by a secondary kinase at amino
acids 4–5 COOH-termini to the GSK3β phosphorylation sites for
optimal GSK3β-mediated phosphorylation. Thus, although GSK3β
is active in resting conditions, it cannot optimally phosphorylate its
substrates, until upon FcεRI or KIT kinase activation, the GSK3β
substrates become phosphorylated as a consequence of the activa-
tion of one of the kinases downstream of these receptors. This would
then allow GSK3β to optimally phosphorylate its target signaling
proteins and hence transduce the signals required for gene expres-
sion leading to cytokine production and the processes required for
chemotaxis (Fig. 7). Of potential relevance may be the presence of
two highly conserved SxxxS/T sequences in MKK3 and MKK6,
which are responsible for the phosphorylation and activation of p38,
and in MKK4 and MKK7, which are responsible for the phos-
phorylation and activation of JNK. Multiple such sequences are also
found in MEK kinase 1 and MEK kinase 4, upstream kinases of
MKK4 and MKK7. Thus, phosphorylation of these sites by GSK3β
following initial phosphorylation by a “priming” serine/threonine
kinase may provide a mechanism by which constitutive activation
of GSK3β may regulate the activation of p38 and JNK and subsequent
downstream transcription factors. In support of this conclusion, we
observed that SCF and SA/SCF-mediated MKK3/6 phosphorylation
was markedly reduced in the GSK3β knockout HuMCs (Fig. 5).

In summary, in this study, we have presented evidence to support
the conclusion that GSK3β is a prerequisite signal for KIT-
mediated chemotaxis and KIT/FcεRI-mediated cytokine pro-
duction in human mast cells. The regulation of cytokine genera-
tion by GSK3β could be explained by the differential regulation of
transcriptional control downstream of JNK and p38, as well as
transcriptional control of NF-κB p65 subunit, whereas the regu-
lation of the chemotactic response by GSK3β may be explained
by its modulation of JNK- and p38-dependent pathways. As with
other cells types, it is, however, possible that as-yet undefined
inhibitory pathways both regulating GSK3β activation and

FIGURE 7. Potential model by which constitutively
activated GSK3β may regulate HuMC cytokine pro-
duction and chemotaxis. Under resting conditions,
GSK3β is constitutively active, because of phosphor-
ylation of the Y216 residue, but is unable to optimally
phosphorylate its substrates as they require initial
phosphorylation by a “priming” kinase to allow these
reactions to take place. Upon SCF/SA challenge,
GSK3β substrates are phosphorylated by priming
kinases, thus allowing GSK3β to phosphorylate and
activate these substrates. This leads to activation of
the JNK and p38 MAPK pathways, thereby initiation of to
chemotaxis, and downstream transcription factors and
NF-κB leading to cytokine production. GSK3β activity
is terminated upon phosphorylation at the S9 position
as a consequence of activation of PI3K. On the basis of
other reports, it is possible that GSK3β may also regu-
late an inhibitory pathway for NF-AT activation (39),
leading to cytokine production.
regulated by GSK3β activity may play a role in human mast cell biology. Thus, GSK3β may act as a central regulator for the precise control of the signaling processes required for mast cell chemotaxis and cytokine production.

**Disclosures**

The authors have no financial conflicts of interest.

**References**