Thymic stromal lymphopoietin (TSLP) is a cytokine that plays diverse roles in the regulation of immune responses. TSLP requires a heterodimeric receptor complex consisting of IL-7 receptor α subunit and its unique TSLP receptor (gene symbol CRLF2) to transmit signals in cells. Abnormal TSLP signaling (e.g. overexpression of TSLP or its unique receptor TSLPR) contributes to the development of a number of diseases including asthma and leukemia. However, a detailed understanding of the signaling pathways activated by TSLP remains elusive. In this study, we performed a global quantitative phosphoproteomic analysis of the TSLP signaling network using stable isotope labeling by amino acids in cell culture. By employing tetravalent antibody in addition to anti-phosphotyrosine antibodies as enrichment methods, we identified 4164 phosphopeptides on 1670 phosphoproteins. Using stable isotope labeling by amino acids in cell culture-based quantitation, we determined that the phosphorylation status of 226 proteins was modulated by TSLP stimulation. Our analysis identified activation of several members of the Src and Tec families of kinases including Btk, Lyn, and Tec by TSLP for the first time. In addition, we report TSLP-induced phosphorylation of protein kinases such as Ptk6 (SHP-1) and Ptk11 (Shp2), which has also not been reported previously. Co-immunoprecipitation assays showed that Shp2 binds to the adapter protein Gab2 in a TSLP-dependent manner. This is the first demonstration of an inducible protein complex in TSLP signaling. A kinase inhibitor screen revealed that pharmacological inhibition of PI-3 kinase, Jak family kinases, Src family kinases or Btk suppressed TSLP-dependent cellular proliferation making them candidate therapeutic targets in diseases resulting from aberrant TSLP signaling. Our study is the first phosphoproteomic analysis of the TSLP signaling pathway that greatly expands our understanding of TSLP signaling and provides novel therapeutic targets for TSLP/TSLPR-associated diseases in humans. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.017764, 1–22, 2012.
tions involve children with Down syndrome (21–28). Most of alterations occur in 5–7% of all B-ALL and in 60% of B-ALL in ALL). A number of groups have demonstrated that specifically B-progenitor acute lymphoblastic leukemia (B-... 26).

Most recently, TSLPR has been implicated in oncogenesis, specifically B-progenitor acute lymphoblastic leukemia (B-ALL). A number of groups have demonstrated that CRLF2 alterations occur in 5–7% of all B-ALL and in 60% of B-ALL in children with Down syndrome (21–28). Most of CRLF2 alterations involve IGH@-CRLF2 rearrangements or PAR1 deletion resulting in overexpression of CRLF2. Other CRLF2 alterations include rearrangements to other, as yet unknown, part...

Clearly, an understanding of TSLP signaling will accelerate in the development of specific therapies in diseases where the TSLP/TSLPR axis plays a key role in pathogenesis. It is known that TSLP can activate the JAK-STAT pathway by inducing the phosphorylation of two members of the Janus kinase family, Jak1 and Jak2, and six members of Stat transcription factor family, STAT1, 3, 4, 5a, 5b, and 6 (29, 30). TSLP requires Jak1 and Jak2 to activate Stat5 (31). TSLP is also known to increase the phosphorylation of ERK1/2, JNK1/2, AKT, ribosomal protein S6, and 4E-BP1 (12, 29, 32, 33). However, the knowledge of TSLP signaling obtained from biochemical experiments is scattered and the detailed signal transduction pathways responsible for various biological effects of TSLP still remain elusive.

Stable isotope labeling by amino acids in cell culture (SILAC) is a well-established method for labeling cellular proteome that allows precise MS-based protein quantitation (34–36). SILAC-based quantitation of the phosphoproteome in cells was first reported by Ibarrola et al. using antiphosphotyrosine antibodies to enrich tyrosine phosphorylated proteins (37). This strategy has been employed to dissect tyrosine phosphorylation-mediated signaling pathways including EGF (38), EphB2 (39), Her2/neu (40), c-Src (41), and divergent growth factors in mesenchymal stem cell differentiation (42). However, one of the drawbacks in the tyrosine-phosphorylated protein enrichment methods by antiphosphotyrosine antibodies is the lack of information about phosphorylation sites in the identified proteins (43). A number of phosphopeptide enrichment methods including immobilized metal affinity chromatography (IMAC) (44, 45), titanium dioxide (TiO2)-based phosphopeptide enrichment (46, 47), strong cation exchange (SCX) chromatography (48, 49), and antiphosphotyrosine antibody-based enrichment of tyrosine phosphorylated peptides (50) have been developed to pinpoint the phosphorylation sites in the phosphoproteome. These enrichment methods have also been combined with the SILAC strategy to quantitate phosphorylation changes in various biological systems. For example, Gruhler et al. combined SCX/IMAC phosphopeptide enrichment with SILAC to study the pheromone-regulated phosphorylation in yeast (51) and Nguyen et al. combined IMAC with SILAC and label-free quantitation methods to study temporal dynamics of the phosphoproteome in T-cell receptor signaling (52). Olsen and colleagues combined SILAC with TiO2-based enrichment to characterize the EGFR-mediated temporal changes of the phosphoproteome in HeLa cells (53). Rigbolt and colleagues also combined SCX/TiO2 with SILAC to characterize the temporal changes of the phosphoproteome during human embryonic stem cell differentiation (54). Guha et al. used antiphosphotyrosine antibodies to enrich tyrosine-phosphorylated peptides and quantitated the changes of the tyrosine phosphoproteome in cells expressing lung cancer-specific alleles of EGFR and KRAS by SILAC (55). Rubbi et al. combined antiphosphotyrosine antibodies with SILAC to reveal crosstalk between Bcr-Abl and negative feedback mechanisms controlling Src signaling (56). Thus, SILAC-based quantitative phosphoproteomic approaches are a powerful tool to dissect phosphorylation-mediated signaling pathways.

Previously, we undertook a systematic mutagenesis approach to evaluate the role of tyrosine residues in the TSLP receptor complex in mediating TSLP-induced cellular proliferation (57). In contrast to IL-4, 7, or 9 signaling, our data revealed that any single cytoplasmic tyrosine residue of either human IL-7Ra chain or TSLPR could mediate TSLP-induced cellular proliferation. Mutation of all four cytoplasmic tyrosine residues of human TSLP receptor complex to phenylalanine was required to completely abolish TSLP-dependent cell proliferation and Stat5a phosphorylation delineating the importance of tyrosine phosphorylation in TSLP signaling. Here, we applied a SILAC-based strategy to study the phosphoproteomic changes induced by TSLP in cells. By using TiO2- and antiphosphotyrosine antibody-based phosphopeptide enrichment, we identified 4,164 phosphopeptides on 1,670 phosphoproteins. Using SILAC-based quantitation, we not only identified Jak2 and Stat5 along with several known signaling molecules in TSLP signaling, but also established that members of the Src and Tec families of non-receptor tyrosine kinases are activated upon TSLP stimulation. Using co-immunoprecipitation, we showed that Ptpn11 (Shp2), a novel signaling intermediate identified in TSLP signaling, can form a protein complex with Gab2 in a TSLP-dependent manner. Using a panel of kinases inhibitors, we observed that pharmacological inhibition of PI-3 kinase, Jak family kinases, Src family kinases, or Btk suppressed TSLP-dependent cellular proliferation, which is consistent with the results from our
quantitative phosphoproteomic analysis. These data suggest that these kinases could be potential therapeutic targets for abnormal TSLP signaling induced diseases.

**EXPERIMENTAL PROCEDURES**

**Reagents**—4G10 anti-phosphotyrosine (HRP conjugated) and anti-Gab2 antibodies were purchased from Millipore (Billerica, MA). Anti-Btk and anti-p-Btk (Y551) antibodies were from BD Bioscience (San Jose, CA). Anti-PI-3kinase (14B10, H-70), antiphospho-Stat3 and anti-Stat3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Phospho-Stat6 and anti-Stat6 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Titanspheres (TiO2, 5 μm beads) were from GL Sciences Inc. (Torrance, CA). TPCK-treated trypsin was from Worthington Biochemical Corp. (Lakewood, NJ). Recombinant mouse IL-3 and recombinant human TSLP were from R&D Systems (Minneapolis, MN), RPMI 1640 with and without Lysine and Arginine, fetal bovine serum (FBS), L-glutamine, and antibiotics were purchased from Invitrogen (Carlsbad, CA). Silic acid, 125I-Lysine and 13C6-Arginine, were from Cambridge Isotope Laboratories (Andover, MA). PCI-32765 was from Haoyuan Chemexpress (Shanghai, China). A-674563 was from Synkinase (San Diego, CA). Ki-20227 was from Symansis (Auckland, New Zealand). TG-101348 was from Active Technology (Danvers, MA). Titanspheres (TiO2, 5 μm) and anti-phosphotyrosine mouse mAb (P-Tyr-100) beads for immunoaffinity purification of phosphopeptides were from Cell Signaling Technology (Danvers, MA).

**EXPERIMENTAL PROCEDURES**

**Immunopurification**—Protein extracts were diluted in 20 mM HEPES pH 8.0 to a final concentration of 2 μg/ml, adjusted to 7.2, 10 mM sodium phosphate, 50 mM NaCl and subjected to centrifugation at 2000 × g at room temperature for 5 min. Before IAP, P-Tyr-100 beads (Cell Signaling Technology) were washed with IAP buffer twice at 4 °C and the pH of the supernatant containing peptides was adjusted to 7.2 by adding 1 M Tris Base. For IAP, the supernatant was incubated with P-Tyr-100 beads (Cell Signaling Technology) at 4 °C for 30 min and the beads were washed three times with IAP buffer and then twice with water. Peptides were eluted twice from beads by incubating the beads with 0.1% TFA at room temperature.

**Strong Cation Exchange Chromatography and TiO2-Based Phosphopeptide Enrichment**—Peptides were fractionated by strong cation exchange (SCX) chromatography as described earlier (49). Briefly, 10 mg of lyophilized peptides mixture was resuspended in 1 ml of SCX solvent A (5 mM KH2PO4 pH 2.7, 30% ACN) and fractionated by SCX chromatography on a PolySULPHOETHYL A (5 μm, 200 Å) column (200 × 9.4 mm; PolyLC Inc., Columbia, MD) by employing an increasing gradient of SCX solvent B (5 mM KH2PO4 pH 2.7, 30% ACN, 350 mM KC1) on an Agilent 1100 LC system. For each experiment, a total of 90 fractions were initially collected, which were pooled into fractions of various sizes (13, 16, or 22 fractions for each respective experiment), and dried using speedvac (Eppendorf). Each fraction was subjected to TiO2-based phosphopeptide enrichment as described by Larsen et al. (46). Briefly, TiO2 beads were incubated with DHB solution (80% ACN, 1% TFA, 3% 2,5-dihydroxybenzoic acid (DHB)) for 2–4 h at room temperature. Each fraction was resuspended in DHB and incubated with pretreated TiO2 beads (5 mg) overnight at room temperature. Phosphopeptide-bound TiO2 beads were washed three times with DHB solution and twice with 40% ACN. Peptides were eluted three times with 40 μl of 2% ammonia into 10 μl of 20% TFA.

**Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)**—LC-MS/MS analysis of phosphopeptides enriched by IAP was carried out using a reversed phase liquid chromatography system interfaced with an LTQ-Orbitrap Velos mass spectrometer in a “high-high” mode, where mass spectra of both precursor and product ions were acquired in a high resolution Orbitrap analyzer (Thermo Scientific). The peptides were loaded onto an analytical column (10 cm × 75 μm, Magic C18 AQ 5 μm). For identification and quantification of phosphopeptides, the higher mass accuracy and sensitivity of FT-MS was utilized. Phosphopeptide-bound TiO2 beads were washed three times with DHB solution and twice with 40% ACN. Peptides were eluted three times with 40 μl of 2% ammonia into 10 μl of 20% TFA.

**Phosphopeptide-bound TiO2 beads were washed three times with DHB solution and twice with 40% ACN. Peptides were eluted three times with 40 μl of 2% ammonia into 10 μl of 20% TFA.
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search algorithms against a mouse RefSeq database (version 40 containing 31,183 protein entries) through the Proteome Discoverer platform (version 1.3, Thermo Scientific). For both algorithms, the search parameters included a maximum of two missed cleavages; carbamidomethylation at cysteine as a fixed modification; N-terminal acetylation, deamidation at asparagine and glutamine, oxidation at methionine, phosphorylation at serine, threonine and tyrosine and SILAC labeling (13C6) at lysine and arginine as variable modifications. For the MS data acquired on the LTQ-Orbitrap Velos mass spectrometer, the monoisotopic peptide tolerance was set to 10 ppm and MS/MS tolerance to 0.1 Da. For the MS data acquired on the LTQ-Orbitrap XL ETD mass spectrometer, the monoisotopic peptide tolerance was set to 20 ppm and MS/MS tolerance to 0.5 Da. The false discovery rate was set to 1% at the peptide level. The ratio of heavy/light for each phosphopeptide-spectrum match (phosphoPSM) was calculated by the quantitation node and the probability of phosphorylation for each Ser/Thr/Tyr site on each peptide was calculated by the PhosphoRS node in the Proteome Discoverer (version 1.3, Thermo Scientific). We chose a 1.5-fold cutoff for increased phosphorylation and a 0.67-fold cutoff for decreased phosphorylation. The MS and MS/MS spectra for all ratios corresponding to heavy/light >1.5 or <0.67 were manually verified.

Motif Analyses—The surrounding sequence (seven amino acid residues on either side) for each identified phosphorylation site was extracted from the RefSeq database. For phosphorylation sites that were localized at the region of the N- or C-termini, the surrounding sequence could not be extended to in this fashion were excluded from further motif analysis. PhosphoSitePlus (PSP) Logo Generator was used to plot the frequency of amino acid residues on each position (http://www.phosphosite.org/sequenceLogoAction.do). The motif-x algorithm (http://motif-x.med.harvard.edu) was used to extract motifs (60). The significance threshold was set to p < 0.02. The minimum occurrence of motif was set to 10. All phosphosites identified in this study (including phosphosites that showed no change in response to TSLP) were used as background for this motif enrichment analysis.

Pathway Analysis—Molecular function of phosphoproteins was obtained from Ingenuity (https://analysis.ingenuity.com) and Panther Classification System (61). Subnetwork analysis of TSLP-up-regulated phosphoproteins was performed using the Ingenuity Systems Pathway Analysis (IPA) platform (https://analysis.ingenuity.com). The list of RefSeq ID of TSLP-up-regulated phosphoproteins was uploaded to the IPA platform and sorted according to their interconnection information stored in the Ingenuity Pathways Knowledge Base. The most interconnected proteins were used as seeds to construct subnetworks.

Western Blot Analysis—Exponentially growing Ba/F3-IT cells were washed six times with PBS and cultured in RPMI 1640 medium and deprived of IL-3 for 6 h. The cells were left untreated or stimulated with a cytokine (recombinant human TSLP (200 ng/ml) or IL-3 (1 ng/ml) at 37 °C for the time indicated in the figures. Cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM sodium orthovanadate in the presence of protease inhibitors) followed by centrifugation. The supernatant was resolved by SDS-PAGE and Western blotted with anti-Ptpn11 or anti-Gab2 antibodies. After washing, the immunoprecipitates were resolved by SDS-PAGE and assayed by Western blotting with anti-phosphothreonine antibodies (4G10) followed by reprobing with anti-Ptpn11 or anti-Gab2 antibodies.

For co-immunoprecipitation experiments, exponentially growing Ba/F3-IT cells were washed six times with PBS and cultured in RPMI 1640 medium without IL-3 for 6 h. The cells were left untreated or stimulated with recombinant human TSLP (200 ng/ml) at 37 °C for 15 min. Cells were lysed in modified RIPA buffer followed by centrifugation. The supernatant was subjected to immunoprecipitation using anti-Ptpn11 or anti-Gab2 antibodies. After washing, the immunoprecipitates along with cell lysates were resolved by SDS-PAGE and Western blotted with anti-Gab2 antibodies and reprobed with anti-Ptpn11 antibodies. Similarly, the Gab2 immunoprecipitates along with cell lysates were resolved by SDS-PAGE and Western blotted with anti-Ptpn11 antibodies and reprobed with anti-Gab2 antibodies.

Growth Assays—Exponentially growing Ba/F3-IT cells were washed six times with PBS, cultured in RPMI 1640 medium without IL-3 for 6 h, and then stimulated with recombinant human TSLP in the absence or presence of the Btk kinase inhibitor, PCI-32765 as the indicated doses. Two days after the addition of TSLP, the viable cells were counted using a cell counter (Beckman Coulter, Fullerton, CA, USA). The results (except where indicated) are expressed as fold-changes in cell number as compared with the number of cells grown in TSLP-containing media without the Btk kinase inhibitor. All experiments were carried out in triplicate and mean and S.E. were calculated.

Kinase Inhibitor Assays—Exponentially growing Ba/F3-IT cells were washed six times with PBS, cultured in RPMI1640 without IL-3 for 6 h and resuspended in RPMI 1640 medium containing 20 ng/ml TSLP at the concentration of 80,000 cells/ml. Kinase inhibitors were dissolved in DMSO with the stock concentration of 10–100 mM. Cells were incubated with graded concentrations of each kinase inhibitor (final concentrations were 10,000, 3333, 1111, 370, 123, 41, or 14 nM). Cells were seeded at a density of 1000 cells/well in a final volume of 50 μl of media containing 10 ng/ml of TSLP into a 384 well plate using an EpMotion 5075 liquid transfer device (Eppendorf, Hauppauge, NY). Three days after plating, cell viability was determined using a colorimetric MTS assay (CellTiter 384® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI)). All absorbance values were normalized to the mean of 45 wells containing no inhibitor. EC50 values on TSLP-dependent cellular proliferation were calculated using second-degree polynomial regression curve fit through eight data points (average of no inhibitor wells and seven serial dilution points).

RESULTS

It has been well documented that TSLP can induce Stat3 and Stat5a phosphorylation. We have previously established a TSLP-responsive cell line by co-expressing human IL-7Ra/TSLPR in an IL-3-dependent pre-B-cell line, Ba/F3 (designated as Ba/F3-IT cells) (57). We first carried out time course experiments of TSLP-induced Stat3 and Stat5a phosphory-
To determine the peak time for TSLP signaling, as shown in Fig. 1A, both Stat3 and Stat5a phosphorylation was induced at 5 min and reached a plateau at 15 min after TSLP stimulation. Thus, we decided to use 15 min as the duration of TSLP activation. However, the whole-cell lysates did not show any detectable difference in the tyrosine phosphorylation profile between unstimulated and TSLP-stimulated Ba/F3-IT cells (Fig. 1B) suggesting that TSLP-induced tyrosine phosphorylation is not as dramatic as that induced by growth factors that are ligands for receptor tyrosine kinases.

Quantitative Phosphoproteomic Analysis of TSLP Signaling—To identify novel signaling molecules in the TSLP signaling network, we used a SILAC-based quantitative phosphoproteomic approach that combined two different phosphopeptide enrichment methods with high resolution mass spectrometry (Fig. 2). After stimulation of “heavy” cells with TSLP for 15 min, the “heavy” and “light” cell populations were grown in heavy medium were stimulated with TSLP for 15 min. After lysis, the samples were mixed, digested with trypsin, desalted on a C_{18} column and subjected to lyophilization. One portion of the peptide mixture was incubated with antiphosphotyrosine antibodies for enrichment of tyrosine-phosphorylated peptides. The other part of the peptide mixture was fractionated by SCX and phosphopeptides were enriched on TiO_{2} beads. The enriched phosphopeptides were analyzed by LC-MS/MS. The resulting high resolution mass spectra reveal TSLP-induced changes in the phosphorylation status on each site.
TiO$_2$-based phosphopeptide enrichment methods have shown that tyrosine phosphorylation constitutes 1–5% of the entire phosphoproteome identified in eukaryotes (53, 54, 62). Therefore, to increase the number of identifications of tyrosine phosphorylation, antiphosphotyrosine antibodies were also used to enrich tyrosine phosphorylated peptides. The enriched peptides were analyzed on an LTQ-Orbitrap Velos mass spectrometer in a “high-high” mode. We also used TiO$_2$ to enrich phosphopeptides. For this, the peptides were first fractionated by SCX and then subjected to TiO$_2$-based enrichment. The enriched peptides from each fraction were analyzed on an LTQ-Orbitrap XL ETD mass spectrometer.

From triplicate antiphosphotyrosine enrichment and triplicate TiO$_2$-based enrichment experiments, a total of 54 LC-MS/MS runs were carried out. Mass spectral data were processed and searched against databases using MASCOT (58) and SEQUEST (59) algorithms through the Proteome Discoverer platform. Using a false discovery rate (FDR) cutoff of 1%, a target-decoy analysis generated 19,485 phosphopeptide-spectrum-matches (phosphoPSMs) (Supplemental Table S1). By removing redundant phosphopeptides, we identified a total of 4,164 phosphopeptides derived from 1,670 phosphoproteins (Supplemental Table S2). Of these phosphopeptides, 3,121 were mapped to a single protein, 887 to more than one protein product of a single gene and 157 corresponded to protein products of more than one gene. As shown in Fig. 3A and 3B, we identified 3,347 phosphopeptides harboring serine phosphorylation and 737 phosphopeptides harboring threonine phosphorylation, the majority of which were obtained from TiO$_2$-based enrichment method. As shown in Fig. 3C, we also identified 424 tyrosine-phosphorylated peptides mainly from antiphosphotyrosine-based enrichment method. The PhosphoRS node in the Proteome Discoverer calculates the probability of phosphorylation at each potential amino acid residue (i.e. Ser, Thr, and Tyr). By setting a PhosphoRS probability cutoff of 75%, 3,197 peptides contained unambiguous localization of phosphorylation sites whereas 967 had ambiguous localization of phosphorylation sites. We identified a total of 4,505 phosphosites, of which 3,441 were phosphoserine sites, 633 phosphothreonine sites, and 431 phosphotyrosine sites (Figs. 3D, 3E, and 3F). Clearly, the strategy to combine anti-pTyr with TiO$_2$-based phosphopeptide enrichment dramatically increased the percentage of tyrosine-phosphorylated peptides among the identified phosphopeptides (Fig. 4A) and expanded the coverage of phosphotyrosine sites in the phosphoproteome (Fig. 4D).

The relative abundance of phosphopeptides was quantitated based on the area under the MS peaks using the quantitation node in the Proteome Discoverer platform (Supplemental Tables S1). We chose a 1.5-fold cutoff for increased phosphorylation and a 0.67-fold cutoff for decreased phosphorylation. After manual confirmation of the ratios from paired SILAC MS peaks, we identified 192 phosphopeptides as up-regulated and 92 as down-regulated upon TSLP stim-

![Fig. 3. Overlap of phosphopeptides and phosphosites identified from antiphosphotyrosine- and TiO$_2$-based phosphopeptides enrichment methods.](image-url)
These TSLP-regulated phosphopeptides correspond to 226 proteins. Of these proteins, 147 have phosphorylation sites up-regulated (Fig. 4E) and 86 have phosphorylation sites down-regulated by TSLP (Fig. 4F). Seven proteins contain both TSLP-up-regulated and down-regulated phosphorylation sites.

We observed that a higher proportion of all up-regulated phosphorylation events involved phosphotyrosines (33%) (Fig. 4E) than the down-regulated events (10%) (Fig. 4F).

We obtained functional annotation of identified phosphoproteins from PANTHER (61) and Ingenuity (https://analysis.ingenuity.com). As depicted in supplemental Fig. S1A, phosphoproteins identified from our analysis have a broad spectrum of molecular functions whereas kinases occupy a significant portion of the phosphoproteome identified. More interestingly, as shown in supplemental Fig. S1B, kinases and phosphatases constitute a much larger portion of the TSLP-up-regulated phosphoproteome while as shown in supplemental Fig. S1C, only phosphatases have increased coverage in the TSLP-down-regulated phosphoproteome.

We next analyzed the motifs surrounding phosphorylation sites regulated by TSLP. We first used the PhosphoSitePlus (PSP) logo generator to illustrate the frequency of amino acid residues on both sides of phosphoserine/threonine/tyrosine sites. The PSP logo revealed that the proline residue appears more often at +1 position of up-regulated phosphoserine and phosphothreonine sites (supplemental Figs. S2A and S2C).

We used the motif-x algorithm to identify phosphorylation motifs in TSLP signaling. The motif-x algorithm uses position-weighted matrices to extract the motifs that are significantly overrepresented in the input data sets compared with the background data set (60). By comparing with the background dataset derived from the whole phosphoproteome identified in our analysis, the motif-x algorithm identified five phosphorylation motifs from the up-regulated phosphopeptides (supplemental Fig. S3 and supplemental Table S4A) and two from down-regulated phosphopeptides (supplemental Fig. S4 and supplemental Table S4B). Motifs from up-regulated phosphorylation include two leucine directed (L at −2 or −1 position, supplemental Figs. S3A and S3B), two proline-directed (P at −7 or +1 position, supplemental Figs. S3C and S3D), and one isoleucine-directed motif (I at +3 position, supplemental Fig. S3E) whereas motifs from down-regulated phosphorylation events include one lysine-directed (K at +6 position, supplemental Fig. S4A) and one alanine-directed (A at −4 position, supplemental Fig. S4B).

Protein Kinases and Phosphatases in TSLP Signaling—Protein kinases and phosphatases are critical for regulating reversible phosphorylation of proteins in signaling pathways. Their enzymatic activities are also often regulated by phos-
phorylation. Notably, of the 226 proteins regulated by TSLP, 23 are protein kinases and eight are protein phosphatases (Fig. 5, Table I and supplemental Table S3). Some of these kinases have been implicated in TSLP signaling during the preparation of this manuscript. Although Stat5 was shown to be phosphorylated by TSLP stimulation almost a decade ago (2, 63), only recently it has been shown that two members of the Janus kinase family, Jak1 and Jak2, are phosphorylated.
Phosphatases contain phosphorylation sites up-regulated by TSLP signaling. Of these, 11 kinases and eight phosphatases have not previously been implicated in TSLP stimulation. Here, we detected an increase in phosphorylation of the conserved T203/Y205 residues in ERK1/2, the conserved T183/Y185 residues in JNK1/2 and the conserved Y570 site on Jak2. Previously, Brown et al. have shown that TSLP can induce the phosphorylation of ERK1/2, JNK1/2 and the conserved Y570 residues in Jak2 upon TSLP stimulation (29–31). Through the use of quantitative mass spectrometry, we were able to detect increased phosphorylation at the Y570 site on Jak2. Previously, Brown et al. have shown that TSLP can induce the phosphorylation of ERK1/2, JNK1/2 and the conserved Y518 residues in Tec kinase (Fig. 6B), which is known to correlate with increased Btk phosphorylation at Y987 NSFNNPApYYVLEGVPHQLLPLEPPSLAR 6.63 ± 0.08

### TABLE I

| Gene Symbol | Gene Name | Protein | Site<sup>a</sup> | Phosphopeptide Sequence | TSLP stimulated/unstimulated (Mean ± S.D.)<sup>b</sup> |
|-------------|-----------|---------|------------------|-------------------------|--------------------------------------------------|
| 1 Akt1      | RAC-alpha ser/thre/one-protein kinase (PKB/Akt) | S126     |                | GSPPpSDNSGAEEMEVLAKPK | 0.66 ± NA |
| 2 Btk       | BTK protein tyrosine kinase | Y551     |                | YVLDDEpYTSSVGSKFPVR | 2.79 ± 0.23 |
| 3 Chek1     | Chk1 protein ser/thre/one kinase | T279     |                | ApTSGMGSESSSGFSK | 1.80 ± NA |
| 4 Csnk1d    | Casein kinase 1 (CKI) delta | S356     |                | LRGTQEAVPPTLPPTSHANTpSPRP | 0.65 ± NA |
| 5 Fes       | Fes/Fps protein tyrosine kinase | Y713     |                | ISDFGMSREEDGlpYAASAGLR | 12.58 ± NA |
| 6 Lyn       | Lyn protein tyrosine kinase | Y397<sup>*</sup> |                | VIEDEMpYTAR | 8.16 ± 4.10 |
| 7 Mapk1     | Mitogen-activated protein kinase 1 (Erk2) | T183, Y185 |                | VADPDHDHTGFLpTEpYVATR | 68.82 ± 30.03 |
| 8 Mapk3     | Mitogen-activated protein kinase 3 (Erk1) | T208     |                | IADPEHDHTGFtEYpYpVATR | 100 ± NA |
| 9 Mapk9     | Mitogen-activated protein kinase 9 (JNK2) | T183, Y185 |                | TACTNFMMpTpPYVVTR | 2.66 ± 0.37 |
| 10 Mapk12   | Mitogen-activated protein kinase 12 (p38 gamma) | T183, Y185 |                | TACTNFMTmTPPyVVTR | 1.59 ± 0.38 |
| 11 Stk10    | Serine/thre/one-protein kinase 10 (Lok) | T950     |                | LSEEAEPRpTpPSK | 2.54 ± NA |
| 12 Tec      | Tec protein tyrosine kinase | Y518<sup>a</sup> |                | YVLDDQpYTSSSGAKFPVK | 2.11 ± 0.11 |
| 13 Ptn6     | Tyrosine protein phosphatase non-receptor type 6 (SHP-1) | Y538<sup>a</sup> |                | GQESeEpGNITYPPAVR | 6.43 ± 0.29 |
| 14 Ptn7     | Tyrosine protein phosphatase non-receptor type 7 | T66      |                | HKEEpYNVHSV | 2.47 ± 0.11 |
| 15 Ptn11    | Tyrosine protein phosphatase non-receptor type 11 (Shp2) | Y546<sup>a</sup> |                | SGLTVEpCSVNpTPR | 1.90 ± NA |
| 16 Ptn18    | Tyrosine protein phosphatase non-receptor type 18 | Y381     |                | GASAgtGPGAPRTSTDTPlpYSQVAPR | 1.70 ± 0.08 |
| 17 Pi4k2a   | Phosphatidylinositol 4-kinase type 2-alpha | S47      |                | VAAAGSpSPPCpGHDREr | 0.41 ± 0.07 |
| 18 Pik3ca   | PI-3-kinase p110 subunit alpha | Y246     |                | LCVELepYGQk | 100 ± NA |
| 19 Inpp1    | Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2 (Shp2) | Y987 |                | NSFNNPApYYVLEGVPHQLPLEPPSLAR | 6.63 ± 0.08 |

*<sup>a</sup>: The site of phosphorylation on the protein was obtained from the longest isoform of the corresponding gene.

*: The site of phosphorylation on the protein was obtained from the longest isoform of the corresponding gene.

<sup>b</sup> NA: Not Available because only one phosphopeptide containing the site was quantitated.

on two conserved tyrosine residues in their kinase domains upon TSLP stimulation (29–31). Through the use of quantitative mass spectrometry, we were able to detect increased phosphorylation at the Y570 site on Jak2. Previously, Brown et al. have shown that TSLP can induce the phosphorylation of ERK1/2 in leukemic cells (33) and Arima et al. have shown that TSLP can induce the phosphorylation of ERK1/2, JNK1/2 and p38 in human dendritic cells. Here, we detected an increased phosphorylation of the conserved T203/Y205 residues in ERK1/2, the conserved T183/Y185 residues in JNK1/2 and the conserved Y570 residues in Jak2 upon TSLP stimulation.

Not surprisingly, we identified 13 protein kinases and eight protein phosphatases that have not previously been implicated in TSLP signaling. These, 11 kinases and eight phosphatases contain phosphorylation sites up-regulated by TSLP. The majority of the newly identified kinases belong to the Tec and Src family of nonreceptor tyrosine kinases. Bruton's tyrosine kinase (Btk) is a member of the Tec family of nonreceptor tyrosine kinases that plays a crucial role in B-cell development, whose mutations cause X-linked agammaglobulinemia, an immunodeficiency characterized by the failure to produce mature B lymphocytes because of a failure of Ig heavy chain rearrangement (64). Our mass spectrometric analysis identified an increased phosphorylation of Y551 in Btk (Fig. 6A), which is known to correlate with increased kinase activity (65). We also observed TSLP-induced increase in phosphorylation of Y518 in Tec kinase (Fig. 6B), which is located within its tyrosine kinase domain and conserved among Tec family kinases. Lyn, a member of the Src family of nonreceptor tyrosine kinases, is mainly expressed in hematopoietic cells and neural tissues and plays a critical role in...
Fig. 6. Novel kinases/phosphatases identified in TSLP signaling pathway. A–H, phosphorylation status of tyrosine residues of nonreceptor tyrosine kinases—Btk, Tec, Lyn, Fes, and Hck/Src/Yes1/Fyn/Lck—and nonreceptor tyrosine phosphatases—Ptpn6 and Ptpn18—and PI-3 kinase catalytic subunit were up-regulated as evidenced by MS spectra showing the changes in the relative abundance of phosphopeptides.
B-cell receptor signaling. Here, we observed that Y397, a positive regulatory site of its kinase activity (66, 67), was phosphorylated upon TSLP stimulation (Fig. 6C). Fps/Fes (Fps, Fujinami poultry sarcoma; Fes, feline sarcoma) is one member of the nonreceptor protein tyrosine kinase family in which Fer is the only other member. It has been shown that Fps/Fes kinase is involved in normal hematopoiesis as well as growth factor and cytokine receptor signaling pathways (68).

As shown in Fig. 6D, TSLP increased the phosphorylation status of Y713 in Fps/Fes, which is located in its kinase domain and plays a positive role in regulating its kinase activity (69, 70). Further, we detected a TSLP-induced increase in the phosphorylation status of the phosphopeptide IIED-NEpYTAR/LIEDNEpYTAR containing a conserved tyrosine site in the kinase domain of the Src family (Fig. 6D). This peptide contains a Src kinase substrate motif pY[A/G/S/T/E/D] (60) and is shared among several members of the Src family kinases, Hck, Src, Yes1, Fyn, and Lck. Taken together, our data clearly establish that the Tec and Src non-receptor tyrosine protein kinase families are indeed involved in TSLP signaling.

In addition to these tyrosine kinases, we also discovered that phosphorylation of several serine/threonine kinases (e.g. Chk1 and Stk10) was regulated by TSLP (Fig. 5, Table I). Chk1 (gene symbol Chek1) is a downstream effector kinase of ATR. The ATR/Chk1 module primarily responds to the DNA damage, such as single strand breaks (71). Stk10 is a member of the Ste20 family of serine/threonine protein kinases and similar to the polo-like kinase kinases. It is highly expressed in hematopoietic cells (72).

Besides protein kinases, we also observed 8 protein phosphatases to be regulated by TSLP, including four non-receptor protein-tyrosine phosphatases - Ptpn6, Ptpn7, Ptpn11 and Ptpn18. Protein tyrosine phosphatase non-receptor type 6 (Ptpn6), also known as SHP-1, is predominantly expressed in hematopoietic cells (73) and plays a negative regulatory role in GM-CSF and erythropoietin signaling pathways (74). Mice deficient in Ptpn6 exhibit a plethora of perturbations in the hematopoietic and immune systems (75, 76). Here, we observed that addition of TSLP increased the phosphorylation of Y536 in SHP-1 (Fig. 6F and Table I), which increases the phosphatase activity of SHP-1 (77, 78). Ptpn18, also designated as fetal liver phosphatase 1, is not a well-studied phosphatase and consists of a protein tyrosine phosphatase domain followed by a carboxyl-terminal domain of 160 amino acids. It is predominantly expressed in hematopoietic cells, especially in hematopoietic progenitor cells (79, 80). Gensler et al. have shown that Ptpn18 may play a negative role in Her2 signaling (81). Our proteomic data showed that the phosphorylation of Y381 in Ptpn18 was enhanced by TSLP (Fig. 6G and Table I). Ptpn11, also known as Shp2, contains two SH2 domains at the N terminus, a catalytic protein-tyrosine phosphatase (PTP) domain in the middle, and a proline-rich motif at the C terminus. As a positive regulator of most receptor tyrosine kinase and cytokine receptor signaling pathways, Shp2 plays a key role in the activation of the Ras-Erk cascade downstream of these receptors (84, 85). In addition, a role of Shp2 in regulating PI-3 kinase/Akt, RhoA, NF-κB, and NFAT pathways has been reported (84–86). We characterized Shp2 in greater detail and it is discussed below.

**TSLP Induces the Interaction Between Shp2 and Gab2**—As shown in Figs. 7A and 7B, SILAC-based quantitation showed that addition of TSLP increased the phosphorylation of Y546 in Shp2 implicating Shp2 in TSLP signaling. We sought to...
determine temporal dynamics of TSLP-induced Shp2 phosphorylation on Y546. As shown in Fig. 7C, upon TSLP stimulation, Shp2 phosphorylation on Y546 was induced at a very early time point (1 min), reached a peak at 15 min and then attenuated. By immunoprecipitation followed by Western blotting, as shown in Fig. 8A, the bands in the upper panel revealed different phosphotyrosine profiles of the Shp2 protein complex before and after TSLP stimulation. This suggests that after addition of TSLP, a number of proteins, especially those molecules around molecular weight of ~95 kDa, undergo tyrosine phosphorylation and bind to Shp2. In humans, PTPN11 is a bona fide oncogene mutated in various types of cancers. Both germline and somatic mutations have been shown to be associated with Noonan and LEOPARD syndromes as well as different types of hematologic malignancies, most notably juvenile myelomonocytic leukemia (87). Recent studies have also implicated TSLP/TSLPR signaling in leukemogenesis (23, 25–28). Interestingly, of the known Shp2-binding proteins, Gab2 is known to be involved in leukemogenesis as a critical determinant of the lineage and severity of BCR-ABL-induced leukemia (88). Gab2 is a 95 kDa protein which contains an N-terminal Pleckstrin homology (PH) domain and a number of known tyrosine phosphorylation sites mediating its interactions with other signaling molecules. Therefore, we sought to examine the interaction between Shp2 and Gab2. As shown in Fig. 8A, the bands in the upper panel revealed different phosphotyrosine profiles of the Shp2 protein complex before and after TSLP stimulation. This suggests that after addition of TSLP, a number of proteins, especially those molecules around molecular weight of ~95 kDa, undergo tyrosine phosphorylation and bind to Shp2.

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PI-3 Kinase Subnetwork in TSLP Signaling—Previously, Arim et al. showed that the PI-3 kinase inhibitor LY294002 blocked the phosphorylation of Akt and Erk1/2 in response to TSLP (29) implicating PI-3 kinase in TSLP signaling. Here, we provide direct evidence that Y146 in the PI-3 kinase catalytic subunit is phosphorylated upon TSLP stimulation (Fig. 5, Fig. 6H, and Table I). Interestingly, our data also showed that two enzymes involved in phosphoinositide signaling—phosphatidylinositol 4-kinase type 2-alpha (PI4KIIα) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2 (Ship2)—were also regulated by TSLP (Fig. 5 and Table I). PI4KIIα, one of the two type II PI 4-kinases, can phosphorylate phosphatidylinositol at the D-4 position thereby generating the substrate for PI3-kinase to synthesize PIPs as second messengers (82). Ship2, like Ship1, contains an N-terminal SH2 domain, a central catalytic domain and a C-terminal proline-rich region. It is known that Ship2 acts downstream of receptors by removing a phosphate from the 5/11032 position of the PI-3 kinase products PIP3 (83). More interestingly, network analysis showed that a PI-3 kinase subnetwork was indeed enriched for hypersensitivity response and inflammatory response in our data set, consistent with the role of TSLP signaling in allergic inflammation (Fig. 9). Overall, our analysis suggests that PI-3 kinase and/or its products (PIPs) play an important role in TSLP signaling.

Btk Protein Tyrosine Kinase Plays an Important Role in TSLP Signaling—As described above, SILAC-based quantitation illustrated that TSLP can induce increased phosphorylation of Y551 in Btk—a critical site for Btk enzymatic activity (65) (Fig. 6A). We further examined temporal changes in the phosphorylation status of Y551 in Btk after the addition of TSLP. Very interestingly, as shown in Fig. 10A, TSLP can induce phosphorylation of Y551 in Btk after 1 min of treatment suggesting that Btk may be an upstream kinase in the TSLP signal transduction pathway. Thus we sought to determine the roles of Btk in TSLP signaling. PCI-32765 is an irreversible Btk inhibitor that binds covalently to a cysteine residue (Cys-481) in the active site of Btk, thus inhibiting its enzymatic activity (89). Davis et al. have shown that inhibition of Btk by PCI-32765 can suppress cellular proliferation driven by chronic B-cell receptor signaling in human large B-cell lymphoma (90). More recently, Herman et al. examined the in vitro activity of PCI-32765 against chronic lymphocytic leukemia patient cells (91). Their results showed that PCI-32765 exhibited a dose-dependent induction of cytotoxicity in these CLL cells (EC50 = 10 μM). Here, we first examined the inhibitory effect of PCI-32765 on TSLP-dependent cellular proliferation. As shown in Fig. 10B, our growth assays revealed that EC50 of Btk to inhibit TSLP-induced cellular proliferation by PCI-32765 is ~ 5 μM suggesting that
Btk might play a critical role in TSLP-dependent cellular proliferation (92). We next examined the inhibitory role of PCI-32765 on TSLP-induced phosphorylation on Btk and two critical transcription factors—Stat3 and Stat5a. As shown in Fig. 10C, 10 μM of PCI-32765 suppressed TSLP-induced phosphorylation of Y551 in Btk and Y705 in Stat3 but not Y694 in Stat5, suggesting that Btk may function upstream of Stat3 but not Stat5. Taken together, our data suggest that Btk may be an upstream kinase for Stat3 and plays an important role in the TSLP biology.

Kinase Inhibitor Assays Reveal Potential Therapeutic Targets in TSLP-associated Diseases—Hyperactivation of TSLP/TSLPR signaling has been reported in a number of diseases such as asthma and leukemias (14, 15, 21–28, 93). We propose that some kinase(s) in the TSLP signaling pathway could be potential targets for TSLP-induced diseases and that pharmacological inhibition of these kinases could serve as novel therapeutic strategies for treatment of these diseases. We compiled commercially available kinase inhibitors, some of which target kinases with TSLP-up-regulated phosphorylation, to study their effects on TSLP-dependent cellular proliferation (Table III). These inhibitors were placed into a plate at seven graded concentration that bracket the reported EC50 of the inhibition of cell growth. After wash and starvation, exponentially growing Ba/F3-IT cells were cultured in the plate in TSLP-containing media. Colorimetric MTS assays were used to examine TSLP-dependent cell growth in the presence of these kinase inhibitors (supplemental Table S5). Interestingly, as shown in the Fig. 11, not all inhibitors that inhibit the activities of the kinases in TSLP signaling can suppress TSLP-dependent cellular proliferation. When activity of PI-3 kinase, Jak family kinases, Src family kinases or Btk was suppressed, TSLP was unable to support the growth of Ba/F3-IT cells. In contrast, the inhibition of Akt, JNKs, or p38 MAPKs could not suppress TSLP-dependent cellular proliferation in Ba/F3-IT cells suggesting that these kinases are not required for TSLP-driven growth of Ba/F3-IT cells. Table III also shows that TSLP-dependent cellular proliferation cannot be suppressed by the inhibitors targeting the kinases, phosphorylation of which was not up-regulated by TSLP, including Abl, Alk/Igf1r, Braf, Csf1r, Egfr, Erbb2, Flt3, Met, and Vegfr2. Overall, our data provides potential therapeutic targets for hyperactive TSLP/TSLPR signaling-associated diseases.

**DISCUSSION**

TSLP signaling plays an important role in a number of biological processes. Dysregulated TSLP signaling contributes to the development of a number of diseases, including asthma and leukemia. Current understanding of molecular mechanisms by which TSLP transmits its signals in cells remains limited. Here, we present the first systematic study of the TSLP signal transduction pathway mediated by phosphorylation. Phosphoproteomic analysis is generally carried out by enriching phosphopeptides away from unphosphorylated proteins.
involved. Significantly, our data demonstrates that the phosphorylation sites that were determined of the exact phosphorylation sites that were determined to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides (50). Our group has previously dissected the function of tyrosine residues in the TSLP receptor complex and demonstrated that tyrosine phosphorylation plays a critical role in TSLP-dependent cellular proliferation (57). Here, we combined two enrichment methods—TiO₂ and antiphosphotyrosine antibodies—to study TSLP-induced phosphoproteomic changes, which not only provided a global view of TSLP signaling but also significantly increased the coverage of TSLP-mediated tyrosine phosphorylation events. The SILAC-based quantitation revealed that a significant portion of tyrosine residues on proteins are inducibly phosphorylated upon TSLP stimulation. Indeed, our study revealed the activation of many tyrosine kinases by TSLP for the first time.

A number of groups have used traditional molecular biology techniques to study the TSLP signal transduction pathway. Stat5 was the first molecule that was shown to be phosphorylated by TSLP stimulation in both human and murine systems (2, 63, 95). Brown et al. showed that TSLP could induce the phosphorylation of ribosomal protein S6 and 4E-BP1 in leukemic cells (33). Rochman et al. reported the involvement of Akt in TSLP-mediated CD8+ homeostasis (12). More recently, three groups showed that two members of the Janus family kinases, Jak1 and Jak2 are activated in TSLP signaling (29–31). Erk1/2, JNK1/2, and p38 have also been reported as intermediates in TSLP signaling (29, 32). In agreement with these reports, our proteomic data indicates that Jak2, Erk1/2, JNK1/2, and p38 were all indeed inducibly phosphorylated by TSLP stimulation. Importantly, our proteomic analysis led to determination of the exact phosphorylation sites that were involved. Significantly, our data demonstrates that the phosphopeptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides. A number of groups have employed TiO₂-based phosphopeptide enrichment method to carry out global phosphoproteomic studies in several biological systems. However, this enrichment method only provides a low coverage of tyrosine phosphorylation events because of the lower abundance of tyrosine phosphorylation in cells. Our group has used phosphospecific antibodies to enrich phosphoproteins to study the subsets of the phosphoproteome—the tyrosine phosphorylated peptides.
An understanding of TSLP signaling has the potential to provide novel therapeutic strategies for treating TSLP/TSLPR-associated diseases including asthma and leukemia. Signaling molecules, especially tyrosine kinases, identified in our studies could serve as potential targets for treatment of these diseases. The results from our kinase inhibitor assays show that inhibition of some kinases could suppress TSLP-dependent cellular proliferation, suggesting that these kinase inhibitors could be novel therapeutic strategies for patients suffering from abnormal TSLP signaling-associated diseases. For example, PI-103 is a newly developed selective class I PI3K inhibitor, which can potently and competitively inhibit p110α, p110β, p110δ with IC50 values of 2, 3, and 3 nM, respectively (97) and has been shown to have antileukemic activity in acute myelogenous leukemia (98). AZD1480 (99), CP-690550 (100) and TG-101348 (101), three JAK family kinase inhibitors, have been shown to inhibit JAK2V617F-mediated myeloproliferation (101–103). SKI-606, a potent proliferative agent against chronic myelogenous leukemia mediated myeloproliferation (101–103). SKI-606, a potent JAK inhibitor (100) and TG-101348 (101), three JAK family inhibitors, have been shown to inhibit JAK2V617F-CP-690550 (100) and TG-101348 (101), three JAK family kinase inhibitors, have been shown to inhibit JAK2V617F-, two SH2-domain containing non-receptor protein tyrosine kinases. Furthermore, the validation of TSLP-induced phosphorylation of Shp2 revealed that TSLP also induced the binding of a number of tyrosine phosphorylated proteins to Shp2. We showed that Gab2, a known Shp2-interactor in other signaling pathways, binds to Shp2 after TSLP stimulation and that TSLP also induces tyrosine phosphorylation of Gab2. This is the first report of TSLP-induced formation of protein complex and it will be interesting to characterize the TSLP-induced Shp2 protein complex in the future. Further, our data shows that pharmacological inhibition of the Btk kinase activity suppresses TSLP-induced cellular proliferation and Stat3 phosphorylation. In addition, the identification of other signaling molecules such as PI-3 kinase, calmodulin and catenin delta-1 reveal the complexity of the TSLP signal transduction network (Fig. 5 and Table I and II). Taken together, our data greatly expand the understanding of the TSLP signaling pathway by discovering 226 TSLP-regulated phosphoproteins. These TSLP-regulated phosphoproteins have already been uploaded to a publicly available pathway resource NetPath (http://www.NetPath.org) (96). 

### Table III

| Compound Name | Alternative Name | Primary Target | Reported IC50 (nM) | Reported EC50 (Proliferation, nM) | Reference | EC50 (Proliferation, nM) (this study) |
|---------------|------------------|----------------|-------------------|----------------------------------|-----------|-----------------------------------|
| 1 PI-103      |                  | Ptk3ca         | 2                 | 60–930                           | (97)      | 69                                |
| 2 CP-690550   | Tofacitinib      | Jak3           | 1                 | 250–2110                         | (100, 103)| 43                                |
| 3 TG-101348   |                  | Jak2           | 3                 | 300–420                          | (101)     | 317                               |
| 4 AZD1480     |                  | Jak2           | <0.4              | 300–100                          | (111, 112)| 1176                              |
| 5 SKI-606     | Bosutinib        | Fgr/Lyn        | 0.17/0.85         | 20–570                           | (106)     | 526                               |
| 6 PCI-32765   |                  | Btk            | 0.72              | ~10000                           | (89, 91)  | 3105                              |
| 7 A-674563    |                  | Akt1           | 11                | 400                              | (113)     | 7245                              |
| 8 GSK-690693  |                  | Akt1           | 2                 | 72                               | (114)     | >10000                            |
| 9 JNK Inhibitor II |        | JNK1/2         | 40                | 13000                           | (115, 116)| >10000                            |
| 10 BRIB-796   | Doramapimod      | p38 alpha      | 0.1               | NA                               | (117)     | >10000                            |
| 11 SB-203580  |                  | p38 alpha      | 40                | 2000–6000                        | (118, 119)| >10000                            |
| 12 VX-745     |                  | p38 alpha      | 0.8               | 10000                            | (120, 121)| >10000                            |
| 13 Imitinib   | STI571/CGP57148  | Abl1           | <1000             | 41                               | (122–126)| >10000                            |
| 14 Nilitinib  | AMN107           | Abl1           | 7                 | <12 nM                           | (127, 128)| >10000                            |
| 15 GSK-1838705A |                | Abl1           | 5/1.6             | 24–3971                          | (129)     | >10000                            |
| 16 CHIR-265   | RAF-265          | Braf           | 60                | 27–368                          | (130)     | 10000                             |
| 17 GDC-0879   |                  | Braf           | <1                | <500                             | (131)     | >10000                            |
| 18 Ki-20227   |                  | Csf1r          | 2                 | 11–700                           | (132)     | >10000                            |
| 19 Erlotinib  | CP-358774        | Egfr           | 2                 | 50–100                           | (133)     | >10000                            |
| 20 Gefitinib  | ZD1839/irressa   | Egfr           | 2                 | 54                               | (134)     | >10000                            |
| 21 HKI-272    | Neratinib        | Erbb2          | 59                | 2–81                             | (135)     | >10000                            |
| 22 Lapatinib  | GW572016         | Erbb2          | 13                | 1490                            | (136, 137)| >10000                            |
| 23 ABT-869    | Linilinib        | Fli3           | 4                 | 0.2                              | (138)     | >10000                            |
| 24 MLN-518    | CT53518/Tandutinib| Fli3          | 220               | 10–30                           | (139)     | >10000                            |
| 25 EXEL-2880  | XL-880/Foretinib | Met            | 3                 | 4–500                           | (140)     | >10000                            |
| 26 SGX-523    |                  | Met            | 2.7               | 20–113                          | (141)     | >10000                            |
| 27 PTK-787    | Vatalanib        | Vegfr2         | 37                | 7.1                              | (142)     | >10000                            |
diseases mediated by abnormal TSLP/TSLPR signaling, especially leukemia.

In conclusion, this study represents the first phosphoprotemic analysis of TSLP signaling and provides a rich resource for investigating the roles of signaling molecules in TSLP signaling under both normal and pathological conditions.

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Tables S1 to S5.

Fig. 11. Effects of inhibitors targeting TSLP-regulated kinases on TSLP-dependent cellular proliferation. Exponentially growing Ba/F3-IT cells were starved and resuspended in TSLP-containing RPMI 1640 medium. Kinase inhibitors were dissolved in DMSO with the stock concentration of 10–100 mM. Cells were incubated with graded concentrations of each kinase inhibitor (final concentrations were 10,000; 3333; 1111; 370; 123; 41; 14 mM) in RPMI 1640 media. Cells were seeded at a density of 1000 cells per well in a final volume of 50 μl into a 384 well plate. Three days after plating, cell viability was determined using a colorimetric MTS assay. All absorbance values were normalized to the mean of 45 wells containing no inhibitor. EC_{50} values on TSLP-dependent cellular proliferation were calculated using second-degree polynomial regression curve fit through eight data points (average of no inhibitor wells and seven serial dilution points).

| Inhibitors | PI3-Kinase | JAK family | Src family | Btk | Akt | JNKs | p38 MAPks |
|------------|------------|------------|------------|-----|-----|------|---------|
|            | PI-103     | CP-69050   | TO-101346  | AZD1480 | A-674563 | SKI-606 | PCH-2765 |
|            |            | BRD-796    | SB-203580  | VXR-745 |      |      |      |
|            |            |            |            |      |      |      |         |
| EC_{50} (Proliferation, nM) |            |            |            |      |      |      |         |
| 0           | 2000        | 3333        | 1111        | 370  | 123  | 41   | 14      |
| 1000        | 1000        | 1000        | 1000        | 1000 | 1000 | 1000 | 1000    |
| 5000        | 5000        | 5000        | 5000        | 5000 | 5000 | 5000 | 5000    |
| 10000       | 10000       | 10000       | 10000       | 10000| 10000| 10000| 10000   |

Conflict of interest: Albrecht Moritz and John Rush are employees of Cell Signaling Technology Inc. Other authors do not claim conflict of interest.

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