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The Tec Kinase–Regulated Phosphoproteome Reveals a Mechanism for the Regulation of Inhibitory Signals in Murine Macrophages

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Previous work has shown conflicting roles for Tec family kinases in regulation of TLR-dependent signaling in myeloid cells. In the present study, we performed a detailed investigation of the role of the Tec kinases Btk and Tec kinases in regulating TLR signaling in several types of primary murine macrophages. We demonstrate that primary resident peritoneal macrophages deficient for Btk and Tec secrete less proinflammatory cytokines in response to TLR stimulation than do wild-type cells. In contrast, we found that bone marrow–derived and thioglycollate-elicited peritoneal macrophages deficient for Btk and Tec secrete more proinflammatory cytokines than do wild-type cells. We then compared the phosphoproteome regulated by Tec kinases and LPS in primary peritoneal and bone marrow–derived macrophages. From this analysis we determined that Tec kinases regulate different signaling programs in these cell types. In additional studies using bone marrow–derived macrophages, we found that Tec and Btk promote phosphorylation events necessary for immunoreceptor-mediated inhibition of TLR signaling. Taken together, our results are consistent with a model where Tec kinases (Btk, Tec, Bmx) are required for TLR-dependent signaling in many types of myeloid cells. However, our data also support a cell type–specific TLR inhibitory role for Btk and Tec that is mediated by immunoreceptor activation and signaling via PI3K. *The Journal of Immunology*, **2015, 195: 246–256.**

The TLR signaling pathways can be activated by a variety of ligands commonly found in viruses and bacteria. Upon activation, TLRs transduce their signals via interaction with distinct combinations of adaptor molecules, including Mal (also known as Tirap), Myd88, Trif (Ticam1), and Tram (Ticam2), resulting in activation of a common pathway that culminates in signaling via the MAPKs (Mapk family members), NF-κB, and IFN regulatory factor transcription factors. Following activation of these proteins by the TLR pathways, the cell produces inflammatory cytokines, such as TNF, IL-12, and IL6. These cytokines promote pathogen clearance by the innate and adaptive immune systems (1).

The Tec (tyrosine kinase expressed in hepatocellular carcinoma) family kinases have critical roles regulating immunoreceptor and TLR signaling in immune cells. Three members of the Tec kinase family (Btk, Tec, and Bmx) are expressed in monocytes and macrophages (2–5), and their expression levels vary in the subsets of these cells (6). Recent research has demonstrated a variable role for Btk in TLR-dependent cytokine secretion and signaling in murine macrophages (reviewed in Refs. 7, 8). In several studies, resident peritoneal macrophages (9) and bone marrow–derived macrophages (BMMϕ) (10, 11) isolated from mice deficient for Btk were found to secrete lower levels of the proinflammatory cytokines TNF, IL-6, or IL-12 in response to activation of the TLR pathways. In contrast, another group reported that the same cell types isolated from Btk-deficient mice secrete higher levels of IL-6 (12) in response to TLR activation. Finally, one study reported that Btk deficiency led to increased TLR-dependent IL-12, but decreased TNF secretion in both thioglycollate-elicited peritoneal macrophages and BMMϕ (13). Similar to the data in mice, human monocytes derived from patients lacking functional Btk have been shown to exhibit decreases (2, 14), increases (15, 16), and no change (17) in TLR-dependent proinflammatory cytokine secretion. Taken together, these results demonstrate that Tec kinases can positively and negatively regulate secretion of proinflammatory cytokines in response to TLR activation in macrophages; however, the reasons for the observed differences in polarity of their effect has not been clearly established.

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Abbreviation used in this article: BMMϕ, bone marrow–derived macrophage.

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The positive role for Btk in TLR signaling has been proposed to involve a direct requirement for Btk via interaction with receptor, coreceptor, and/or the TLR-associated kinase Irak1 (7, 8, 10, 18). One possible mechanistic explanation for the inhibitory role observed for Tec kinases is that they promote immunoreceptor signaling, which blocks signaling downstream of TLRs in certain macrophage populations. Immunoreceptors have a ligand-binding receptor subunit and an adapter protein that contains an intracellular signaling domain, such as an ITAM. One important inhibitory immunoreceptor complex in macrophages is that composed of the ITAM-containing protein Dap12 (19) and the Trem2 receptor (20, 21). Based on a series of proteomics-based signaling analyses described in the present study, we hypothesize that Tec kinases can play two opposing roles during myeloid TLR signaling: promoting TLR signals downstream of the TLR receptor, and inhibiting TLR signals in cell types regulated by TREM2/DAP12. We test this hypothesis by investigating the role of Tec kinases in TLR signaling in several primary mouse populations. Our combined results help to clarify the role for Tec kinases in TLR signaling.

Materials and Methods

Mice

Wild-type C57BL/6, Trem2-deficient, Btk-deficient, and Tec/Btk-deficient mice from both genders were bred and maintained in a specific pathogen-free facility. For simplicity sake, in our figures we have labeled Btk knockout animals $Btk^{-/-}$ regardless of gender despite its location on the X chromosome. Animal studies were carried out according to the guidelines of Seattle Children's Research Institute or the Benaroya Research Institute Institutional Animal Care and Use Committee.

Generation of BMMϕ

Bone marrow cells from 6- to 15-wk-old mice were flushed from femurs and tibias. Following RBC lysis, the remaining cells were filtered and plated at 7.5×10^5 cells/ml on 10-cm petri dishes (Fisherbrand). Cells were grown in complete media: DMEM high-glucose medium (Thermo Scientific) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), penicillin/streptomycin solution (100 U/ml penicillin, 100 μg/ml streptomycin; Thermo Scientific), 1× GlutaMAX (Life Technologies), 10 mM HEPES (Thermo Scientific), 1 mM sodium pyruvate (Mediatech), and 10% conditioned medium isolated from CMG14-12 cells (22). The culture medium was changed on days 3 and 5, when the cells were counted and replated for further experiments.

Isolation of peritoneal and thioglycollate-elicited peritoneal macrophages

Mice were injected i.p. with 1 ml sterile thioglycollate medium (BD Biosciences). Peritoneal macrophages were harvested by peritoneal lavage with sterile PBS (Thermo Scientific) supplemented with penicillin/streptomycin solution (100 U/ml penicillin, 100 μg/ml streptomycin; Thermo Scientific) and 5% heat-inactivated FBS (Sigma-Aldrich). The cells from the peritoneal exudate were blocked with anti-CD16/CD32 (BD Biosciences) for 5 min at 4°C and then bound with biotin anti-F4/80 (eBioscience) for 15 min at 4°C. Macrophages were purified by positive selection using avidin paramagnetic beads (Miltenyi Biotec, Auburn, CA) and purity was determined by flow cytometry analysis.

Cytokine measurement and apoptosis assays

For cytokine secretion, 5×10^4 cells were plated per well of 96-well plates in 200 μl complete media and allowed to adhere 3 h overnight. TLR stimuli were added to the wells, and after 16 h the levels of TNF, IL-6, IL-12 p40, and IL-10 were measured by ELISA (ELISA Ready-SET-Go!; eBioscience). For intracellular cytokine staining, 1×10^5 cells were plated per well of 48-well non–tissue culture–treated plates and stimulated in the presence of the protein transport inhibitor BD GolgiStop (BD Biosciences) for 6 h. For IL-10 neutralization experiments, cells were pretreated with indicated dilutions of anti–IL-10 (clone JES5-2A5, eBioscience) or 1000 ng/ml rat IgG2b isotype control (eBioscience) for 30 min prior to addition of stimuli. After stimulation, cells were lifted using enzyme-free Hank's cell dissociation buffer (Life Technologies), blocked with anti-CD16/CD32 (BD Biosciences), fixed, permeabilized, and stained with eFluor 450 anti-F4/80 (eBioscience), FITC anti–TNF-α (eBioscience), and PE anti–IL-6 Abs (eBioscience). Apoptotic cells were identified by staining with annexin V and 7-aminoactinomycin D (BD Biosciences). For each experiment, cells were analyzed by flow cytometry using a BD LSR II running FACSDiva software (BD Biosciences) and with FlowJo (Tree Star).

Reagents

The following primers were used for quantitative PCR (Eurofins MWG Operon): *Actb* (5′-CTAAGGCCAACCGTGAAAAG-3′, 5′-ACCAGAGGCATACAGGGACA-3′). *Tnf* (5′-TCTTCTCATTCCTGCTTGTGG-3′, 5′-GGTCTGGGCCATAGAACTGA-3′), *Il6* (5′-GCTACCAAACTGGATATAATCAGGA-3′, 5′-CCAGGTAGCTATGGTACTCCAGAA-3′), *Il12* (5′-CCATCAGCAGATCATTCTAGACAA-3′, 5′-CGCCATTATGATTCAGAGACTG-3′), and *Bmx* (5′-GAGCAGCTTCGCTTCACC-3′, 5′-GATTTACTCTCCATATTGTCGTCCA-3′). The following compounds were used: CC-292 (23) and compound 1 (see below). The following Abs were used: Trem2 (24), pY-100 (Cell Signaling Technology, 9411), Mapk1/3 (Cell Signaling Technology, 4695), pMapk1/3 (Cell Signaling Technology, 4377), PT66 (Sigma-Aldrich, P3300), 4G10 (Millipore, 05-321), Tec (Millipore, 05-551), Bmx (BD Biosciences, 610792), Btk (BD Biosciences, 558528), and IRDye (LI-COR Biosciences, Lincoln, NE, 800CW and 680RD). The following additives and TLR agonists were used: LPS (List Biological Laboratoties, 434), CpG DNA (Invitrogen, tlrl-1826), Pam3CSK4 (Invitrogen, tlrl-pms), Gardiquimod (Invitrogen, tlrl-gdgs), and polymyxin B (Sigma-Aldrich, P4932).

Western blotting and PCR

Whole-cell protein extracts were prepared by cell lysis with buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 0.25% sodium deoxycholate, and protease inhibitors. Cell lysates were cleared by centrifugation and were separated by SDS-PAGE under reducing conditions. Following electrophoretic transfer, nitrocellulose membranes were analyzed and quantified using the Odyssey infrared imaging system software (LI-COR Biosciences). Total RNA prepared by using RNeasy mini kit (Qiagen) was reversed transcribed with iScript reverse transcription (Bio-Rad) using oligo(dT) primer, and quantitative PCR was performed using iQ SYBR Green Supermix and CFX96 Touch (Bio-Rad).

Synthesis of compound 1

Synthesis of tert-*butyl(3-((2-chloro-5-fluoropyrimidin-4-yl)amino)phenyl) carbamate (compound 2).* 2,4-Dichloro-5-fluoropyrimidine (800 mg, 4.8 mmol), *tert*-butyl(3-aminophenyl)carbamate (996 mg, 4.8 mmol), and diisopropylethylamine (948 μl, 5.75 mmol) were dissolved in tetrahydrofuran (20 ml). The reaction mixture was heated at reflux overnight. After cooling, brine (10 ml) was added to the reaction mixture followed by ethyl acetate, the organic layer was separated and dried over sodium sulfate, and the solvent was removed via rotary evaporation. Titration with EtOAc and heptane gave compound 2 as a white solid after filtration (1.0 g, 65%) [liquid chromatography–mass spectrometry: *m/e* 339.1 (M+1)] (Supplemental Fig. 1A).

Synthesis of tert-*butyl(3-((5-fluoro-2-((3-fluoro-4-(2-methoxyethoxy) phenyl)amino)pyrimidin-4-yl)amino)phenyl)carbamate (compound 4).* To a solution of compound 2 (600 mg, 1.77 mmol) and 3-fluoro-4-(2-methoxyethoxy)aniline (compound 3, 390 mg, 2.12 mmol) in 10 ml ethanol was added trifluoroacetic acid (5 drops). The mixture was stirred at reflux for 4 h. After cooling, the solvent was removed via rotary evaporation. The residue was dissolved in ethyl acetate and washed with $NaHCO_3$ aqueous solution, water, and brine. The organic layer was separated, dried over Na_2SO_4, and the solvent was removed. The crude was subjected to chromatography on silica gel (hexane/EtOAc of 1:1) and 730 mg of compound 4 was obtained (85%) (Supplemental Fig. 1A).

Synthesis of N-*(3-((5-fluoro-2-((3-fluoro-4-(2-methoxyethoxy)phenyl) amino)pyrimidin-4-yl)amino)phenyl)acrylamide (compound 1).* To a solution of compound 4 (600 mg, 1.2 mmol) in dichloromethane (20 ml) was added trifluoroacetic acid (2 ml). The solution was stirred at room temperature for 4 h. The organic layer was washed with $NaHCO_3$ aqueous solution, separated, and dried over Na_2SO_4. After removal of the solvent, the crude product was used directly in the next step (Supplemental Fig. 1A).

A solution of the compound obtained above in dichloromethane (20 ml) was cooled to −70°C. To this solution was added acryloyl chloride (96 μl, 1.2 mmol) followed by diisopropylethylamine (200 μl, 1.2 mmol). The reaction was stirred for 10 min at −70°C and was quenched by $NaHCO_3$ aqueous solution. The organic layer was separated and dried over Na_2SO_4. After removal of solvent, the crude product was subject to chromatography on silica gel (hexane/EtOAc of 1:1) to give 335 mg compound 1. Liquid

chromatography–mass spectrometry: *m/e* 442.0 (M+1). ^{1}HNMR (DMSO, 400 MHz) δ 10.13 (s, 1H), 9.43 (s, 1H), 9.18 (s, 1H), 8.09 (d, 1H, J = 3.68 Hz), 7.92 (s, 1H), 7.65 (dd, 1H, J = 2.3, 14.2 Hz), 7.47 (d, 1H, J = 8.24 Hz), 7.41 (d, 1H, J = 8.28 Hz), 7.27 (t, 2H, J = 8.0 Hz), 6.94 (t, 1H, J = 9.4 Hz), 6.44 (dd, 1H, J = 16.96, 10.1 Hz), 6.23 (dd, 1H, J = 1.84, 16.96 Hz), 5.73 (dd, 1H, J = 1.4, 10.1 Hz), 4.04 (m, 2H), 3.61 (m, 2H), 3.29 (s, 1H) (Supplemental Fig. 1A).

Kinase selectivity panel and occupancy analysis

Compound 1 was run in a kinase selectivity panel at Reaction Biology (Malvern, PA) using HotSpot technology and radioisotope-based P81 filtration. Compound 1 was dissolved in pure DMSO to the final 1 μM test concentration. Substrates for the various kinases tested against compound 1 were prepared fresh daily in reaction buffer. Any required cofactors were then added to substrate solution followed by kinase addition and preincubated for 30 min at room temperature. ^{33}P-ATP (10 μM) was delivered into the reaction mixture to initiate the reaction and continued for 2 h at room temperature. The reaction was terminated and any unreacted phosphate was washed away using 0.1% phosphoric acid prior to detection utilizing a proprietary technology (Reaction Biology). The study was performed in duplicate and 10 μM staurosporine, a nonselective, ATP-competitive kinase inhibitor, was used as the positive control. To determine IC_{50} values, compound 1 was tested in a 10-dose IC_{50} mode with 10-fold serial dilution starting at 10 μM. Staurosporine was tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 20 μM. Reactions were carried out at a Michaelis constant ATP or 10× a Michaelis constant ATP, according to the RBC binning structure. Btk occupancy analysis was performed on isolated spleens as previously described (23).

Quantitative Phosphoproteomics

BMMϕ were prepared, stimulated, and lysed on ice with 8 M urea supplemented with 1 mM Na_3VO_4. Following digestion of the proteins with trypsin (V5113, Promega), tryptic peptides isolated from individual samples were labeled with six-plex tandem mass tags reagent (Thermo Scientific). Phosphopeptide enrichment, chromatography, mass spectrometry, and quantification were performed as detailed previously (25–27). To assess the differences between the $Btk^{-/-}Tec^{-/-}$ LPS and wild-type LPS conditions, we calculated the fold effect caused by Tec deficiency and LPS using the following steps. First, we normalized the entire dataset for sample handling by calculating the median peptide quantification score among all serine, threonine, and tyrosine phosphorylated peptides for each condition. These median values were used to normalize all data to those in the jurkat-stimulated sample. The normalized ion intensities from the replicate experiment were then averaged for all conditions (wild-type, wild-type LPS, $Btk^{-/-}Tec^{-/-}$, and $Btk^{-/-}Tec^{-/-}$ LPS). Next, for each unique peptide, we calculated an intensity score in each condition by averaging the value across experimental replicates. Finally, for each peptide we calculated the ratio in each condition relative to that in unstimulated wild-type cells. To determine which peptides exhibited the largest effect size, we performed an interquartile range outlier test. Hierarchical clustering and heat map rendering were done using the GENE-E tool (http://www.broadinstitute.org/cancer/software/GENE-E). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) (28) via the PRIDE partner repository with the dataset identifier PXD002031.

Results

Quantitative phosphoproteomics reveals that Tec kinases and LPS regulate different phosphorylation events in different macrophage cell types

Btk and Tec are expressed in two populations of primary macrophages that can be isolated from animals: resident peritoneal macrophages and BMMϕ (6). To discover how Btk and Tec impact global TLR signaling in both of these cell populations, we systematically quantified phosphorylation in each population in the presence or absence of Btk/Tec and of LPS using mass spectrometry coupled with the isobaric labeling reagent, tandem mass tags. We isolated BMMϕ and resident peritoneal macrophages from each genotype and stimulated them with the TLR ligand, LPS, for 20 min (Fig. 1A). Following tryptic digestion, phosphopeptide enrichment, and quantitative mass spectrometry we evaluated the effect of genotype and stimulus in each cell population. We found that the TLR targets Mapk9 (Jnk2; Fig. 1B) and Mapk14 (P38; Fig.1B) behaved as predicted. Furthermore, verification of the Mapk9 and Mapk14 results by Western blot (compare mass spectrometry–based quantification in Fig. 1B with Western blot in Fig. 1C) confirmed that our method accurately quantifies phosphopeptide abundance.

In all experiments from both cell types, we quantified 6704 unique phosphopeptides (false discovery rate of ∼2.5%; Supplemental Table I) derived from 2093 unique proteins, of which 1800 peptides were quantified in both macrophage types (Fig. 1A). Global comparison of the effect of Tec kinase deficiency on phosphotyrosine-containing peptides, many of which could be Tec kinase substrates, demonstrated strong overlap between the cell types (Supplemental Fig. 1B, Spearman's rank coefficient $r = 0.58$; $p < 0.0001$). To identify phosphorylation changes caused by Tec deficiency and LPS stimulation, we applied an interquartile range outlier test and found 224 peptides that were responsive to either treatment (Supplemental Fig. 1C, Supplemental Table I). Global comparison of the outliers revealed several interesting differences between resident peritoneal macrophages and BMMϕ (hierarchical clustering; Fig. 1D). Despite the global reproducibility between phosphotyrosine peptide abundance, only a minority of the outlier peptides (Fig. 1D, cluster 3) responded the same way to Tec kinase deficiency in the two cell types. In fact, our data show that the effect of Tec kinase deficiency on phosphorylation is in some cases more pronounced in resident peritoneal macrophages (Fig. 1D, clusters 4, 5), in some cases more pronounced in BMMϕ (cluster 2), and in some cases exhibit opposite polarity (cluster 1). Finally, these data show a differential response to LPS on several proteins in the two macrophage populations (Fig. 1D, clusters 6, 7). Taken together, these data indicate substantial differences between macrophage cell types in their response to Tec deficiency and LPS stimulation.

Decreased TLR-dependent cytokine production by Btk and Btk/Tec-deficient resident peritoneal macrophages

To assess how Tec kinases impact TLR-dependent cytokine secretion ex vivo, we isolated resident peritoneal macrophages from wild-type, $Btk^{-/-}$, and $Btk^{-/-}Tec^{-/-}$ mice and stimulated them with the TLR agonists LPS (Tlr4 ligand), CpG DNA (Tlr9 ligand), and Pam3CSK4 (Tlr1/2 ligand). To determine the degree of TLR pathway activation, we used flow cytometry to quantify expression of the proinflammatory cytokine TNF. We observed that in populations stimulated with LPS or Pam3CSK4, both the number of cells expressing TNF and the intensity of expression per cell are decreased in $Btk^{-/-}Tec^{-/-}$ macrophages relative to wild-type controls (Fig. 2A, 2B). Next, to determine whether Btk and Tec inhibit secretion of TLR-induced cytokines in resident peritoneal macrophages, we investigated cytokine secretion via ELISA. Similar to our flow cytometry–based findings for TNF-α, Btk- and Btk/Tec-deficient resident peritoneal macrophages secreted lower concentrations of proinflammatory cytokines IL-6 and TNF in response to different doses of LPS and Pam3CKS4 stimuli relative to cells isolated from wild-type mice (Fig. 2C). These data demonstrate that Tec kinases inhibit TLR-dependent cytokine secretion in resident peritoneal macrophages.

Increased TLR responses in BMMϕ and thioglycollate-elicited peritoneal macrophages in the absence of Btk and Tec kinases

To assess the role of Tec kinases in additional macrophage populations, we also examined the effect of Btk and Tec knockout on TLR signaling in BMMϕ and thioglycollate-elicited macrophages (29, 30). First, we investigated whether the kinetics of proinflammatory cytokine secretion were altered in Tec family kinase-

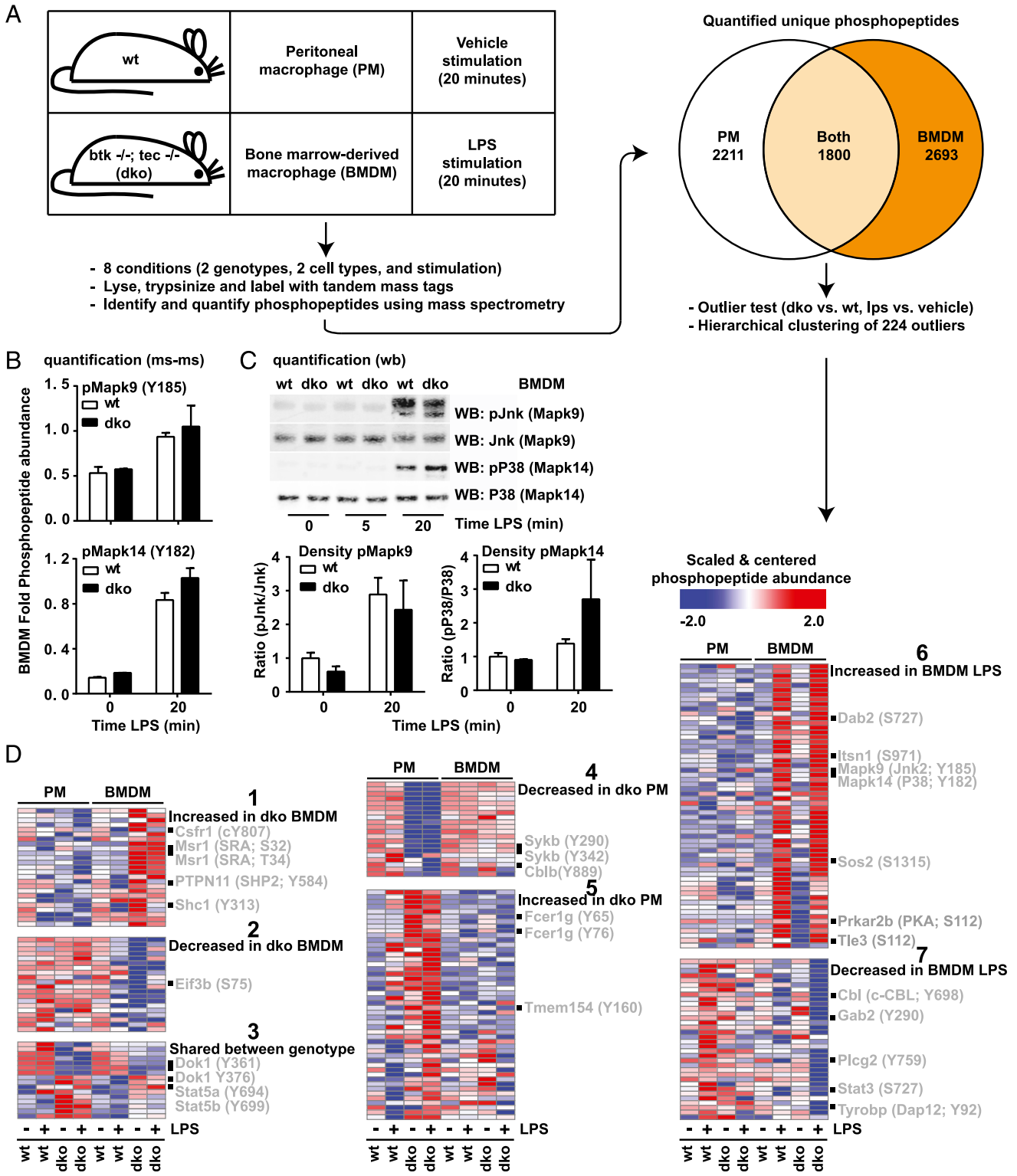

FIGURE 1. Differences in Tec kinase–deficient phosphorylation BMMϕ and resident peritoneal macrophages. (**A**) A flowchart showing the conditions and methods used to analyze Tec kinase–dependent phosphorylation in BMMϕ and resident peritoneal macrophages. Briefly, cell extracts and peptides were prepared from unstimulated wild-type and $Btk^{-/-}Tec^{-/-}$ macrophage cell populations stimulated with LPS (1 ng/ml) for 20 min. After phosphopeptide enrichment, phosphorylated peptides were identified and quantified by liquid chromatography–tandem mass spectrometry. (**B**) The mean of three biological replicates of the quantification of the indicated BMMϕ phosphopeptides by mass spectrometry ± SD. (**C**) Cytoplasmic extracts from BMMϕ from wild-type or $Btk^{-/-}Tec^{-/-}$ mice stimulated with LPS (1 ng/ml) for indicated time (min) were analyzed using Abs specific the indicated proteins and their phosphorylated versions. Immunoblots from three independent experiments were visualized, quantified, and the mean ratio between the abundance of the phosphorylated and nonphosphorylated forms was plotted ± SD. (**D**) A heat map plot of all 224 quantified outlier phosphopeptides identified in both the BMMϕ or resident peritoneal macrophage experiment grouped (1–7) using hierarchical clustering.

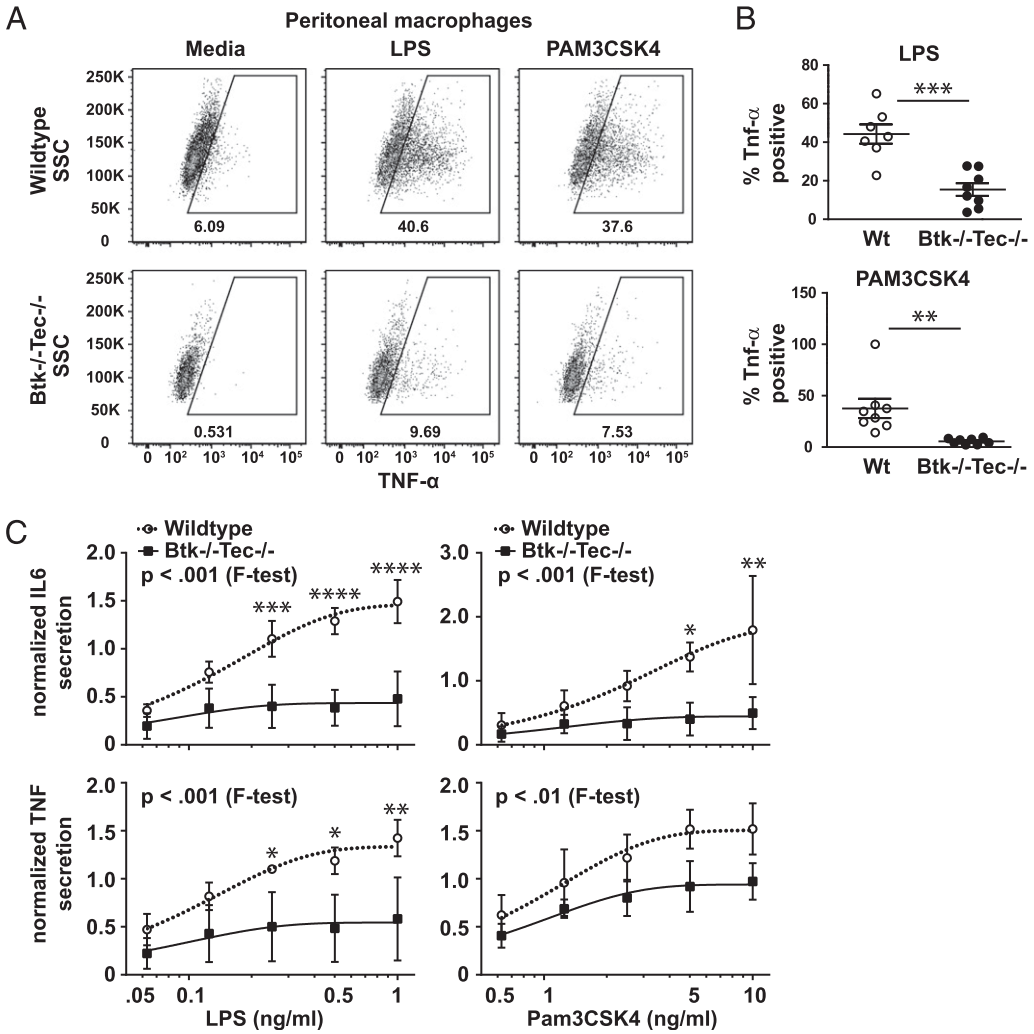

FIGURE 2. Decreased TRL-induced cytokine secretion from Btk$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ F4/80^{+} mouse-resident peritoneal macrophages. (**A**) Representative flow plots showing F4/80^{+} resident peritoneal macrophages cultured with LPS (0.5 ng/ml) or Pam3CSK4 (5 ng/ml) in the presence of GolgiStop protein transport inhibitor for 6 h, then stained for intracellular TNF and analyzed by flow cytometry (cells gated on F4/80^{+}). (**B**) Combined data from three independent experiments measuring intracellular TNF by flow cytometry. (**C**) Resident peritoneal macrophages were stimulated with indicated doses of TLR agonist (LPS and Pam3CSK4) for 16 h. The concentration of IL-6 and TNF in the cell culture supernatants was determined using ELISA. For each of three independent experiments, we normalized each ELISA measurement to the mean of all measurements in the wild-type condition on that day. The replicate data were then plotted (mean ± SD), and a line for each genotype was fitted using nonlinear regression (one-phase association) and we used and *F* test to assess the likelihood that the plateau of each curve was the same (indicated *p* values, *F* test). Additionally, *p* values for individual doses were calculated using two-way ANOVA coupled with a multiple comparison test using Bonferroni correction. **p* = 0.01–0.05, ***p* = 0.001–0.01, ****p* = 0.0001–0.001, *****p* < 0.0001.

deficient mice. Within 4–8 h of stimulation, we observed a trend showing increase in TLR-dependent secretion of IL-6, IL-12, and TNF in BMMϕ isolated from both Btk$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ mice relative to that in cells isolated from wild-type mice (Supplemental Fig. 2A). This kinetic analysis allowed us to pick time points for a detailed quantification of the effect of Tec deficiency on TLR-dependent cytokine secretion. Contrary to our observations in resident peritoneal macrophages, we found that when stimulated with LPS or CpG, BMMϕ isolated from Btk$^{-/-}$ Tec$^{-/-}$ mice produced (Fig. 3A) and secreted (Fig. 3B) significantly higher concentrations of the proinflammatory cytokines IL-6, IL-12, and TNF than did cells isolated from wild-type mice.

To further explore these results, we investigated whether Tec family kinases activate or inhibit TLR signaling in thioglycollate-elicited peritoneal macrophages. Similar to our findings in BMMϕ, we found that F4/80^{+} peritoneal macrophages isolated from Btk$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ mice produced greater IL-6 and TNF in response to simulation with LPS, CpG, or Pam3CSK4 than did cells isolated from wild-type mice (Fig. 4). In contrast to our findings in resident peritoneal macrophages, our data with BMMϕ and thioglycollate-elicited macrophages show that Tec kinases inhibit TLR-induced cytokine production in these specific macrophage populations.

Increased TLR-induced cytokine secretion in Btk$^{-/-}$ and Btk$^{-/-}$ Tec$^{-/-}$ macrophages is not due to differential IL-10 production or TREM-2 expression

After an inflammatory stimulus, monocytes/macrophages also secrete IL-10, an important immunoregulatory cytokine that downregulates transcription of the proinflammatory cytokines (31). We sought to determine whether the increased production of proinflammatory cytokines in Btk- and Btk/Tec-deficient BMMϕ might be explained by decreases in IL-10 secretion or function. Contrary to our observations with the proinflammatory cytokines, we observed no differences in IL-10 production from macrophages isolated from wild-type, Btk$^{-/-}$, or Btk$^{-/-}$Tec$^{-/-}$ mice (Fig. 5A). To further investigate the role of IL-10 in

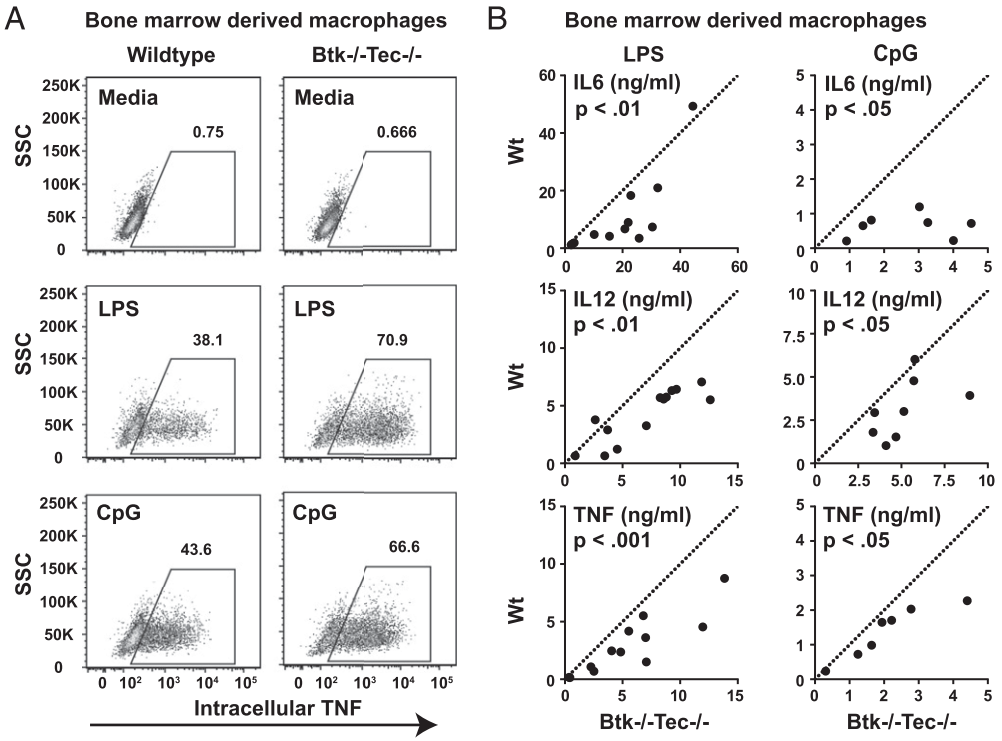

FIGURE 3. Increased TLR-induced cytokine secretion from Btk$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ BMMϕ. (**A**) Representative flow plots showing BMMϕ cultured with LPS (62.5 ng/ml) or CpG (12.5 nM) in the presence of GolgiStop for 6 h and then stained for intracellular TNF and analyzed by flow cytometry (cells gated on F4/80^{+}). (**B**) Combined ELISA data from at least seven independent paired experiments from wild-type and Btk$^{-/-}$Tec$^{-/-}$ cells stimulated with LPS (0.25 ng/ml) or CpG (12.5 nM) were plotted using an *x*–*y* scatter with the *y*-axis representing the quantification of the indicated cytokine (ng/ml) secreted by wild-type cells, and the *x*-axis representing that secreted by Btk$^{-/-}$Tec$^{-/-}$ cells. The dotted lines represent the value where cytokine secretion is equal in wild-type and Btk$^{-/-}$Tec$^{-/-}$ cells. The *p* values were calculated using the nonparametric Wilcoxon matched pairs test.

TLR-dependent cytokine secretion in macrophages, we pretreated BMMϕ with neutralizing Abs to IL-10 and subsequently stimulated the cells with LPS, CpG, or Pam3CSK4. Upon analyzing these cells using flow cytometry, we observed no alterations in TLR-dependent expression of TNF in macrophages isolated from either wild-type or Btk$^{-/-}$Tec$^{-/-}$ BMMϕ (Fig. 5B), suggesting that IL-10 does not regulate TNF expression in BMMϕ. A second possible explanation for our findings is that Btk and Tec regulate surface expression of Trem2, an immunoreceptor known to inhibit TLR signals that is specifically expressed in BMMϕ and thioglycollate-elicited macrophages, but not resident peritoneal macrophages (21). To test this idea, we examined the surface expression of Trem2 in BMMϕ (Fig. 5C) and thioglycollate-elicited peritoneal macrophages (Fig. 5D) using flow cytometry. We found that Trem2 is expressed in macrophages isolated from Btk$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ mice, suggesting that Tec kinases do not inhibit TLR-dependent cytokine secretion by modulating Trem2 surface expression.

Tec kinases promote inhibitory immunoreceptor signals in BMMϕ

The proteomics studies demonstrated that the global phosphorylation changes caused by Tec kinase deficiency are different in peritoneal macrophages and BMMϕ. To investigate how Tec deficiency increases TLR-dependent cytokine secretion, we performed pathway analysis on the proteins that had phosphorylation sites significantly increased or decreased in replicate experiments using Tec kinase–deficient and wild-type BMMϕ. This analysis revealed enrichment (false discovery rate of <10%; Supplemental Table I) for proteins involved in FcγR-mediated phagocytosis (KEGG: mmu04666), phosphatidylinositol signaling system (KEGG: mmu04070), and several other categories related to lymphoid and myeloid signaling. Based on this analysis and on our integration of the data (Fig. 6A, 6B), we found decreases in Tec kinase–deficient cells of many phosphorylation events that have been reported to promote TLR inhibitory signals in macrophages, including those on Dok1 (32), Pik3ap1 (Bcap) (33, 34), and Tyrobp (Dap12) (19). Conversely, we observed increases in several phosphorylation events associated with activation of the Csf receptor (Csf1r), including those on Ptpn11 (Shp2), Gab1, and Shc1 (Fig. 6A, 6B, Supplemental Table I). Based on these results, we conclude that ITAM-mediated inhibitory signaling is decreased in Tec kinase–deficient BMMϕ.

Dap12 inhibitory signaling blocks TLR-dependent cytokine secretion in part via increasing PI3K-dependent signals (35). Our proteomics data show that an activating phosphorylation event on Ship1 (Fig. 5A, 5B; Inpp5d; Y918), an event consistent with decreased PI3K signals (36). To further test whether PI3K signaling is altered in Tec kinase–deficient BMMϕ, we cultured BMMϕ isolated from three independent wild-type and Tec kinase–deficient mice. We found statistically significant decreases in phosphorylation of Akt at Ser^{473} in Tec-deficient BMMϕ with or without LPS stimulation (Fig. 6C). Taken together, our data indicate that a broad subset of inhibitory TLR signals including PI3K is blocked in Tec kinase–deficient BMMϕ, demonstrating that Tec kinases promote this inhibitory DAP12 cascade.

In vitro and in vivo effects of Tec kinase inhibitors in TLR-stimulated macrophages

Our previous data indicate that Btk and Tec are required for TLR signaling in resident peritoneal macrophages, but they inhibit the pathway in BMMϕ and thioglycollate-elicited macrophages. One

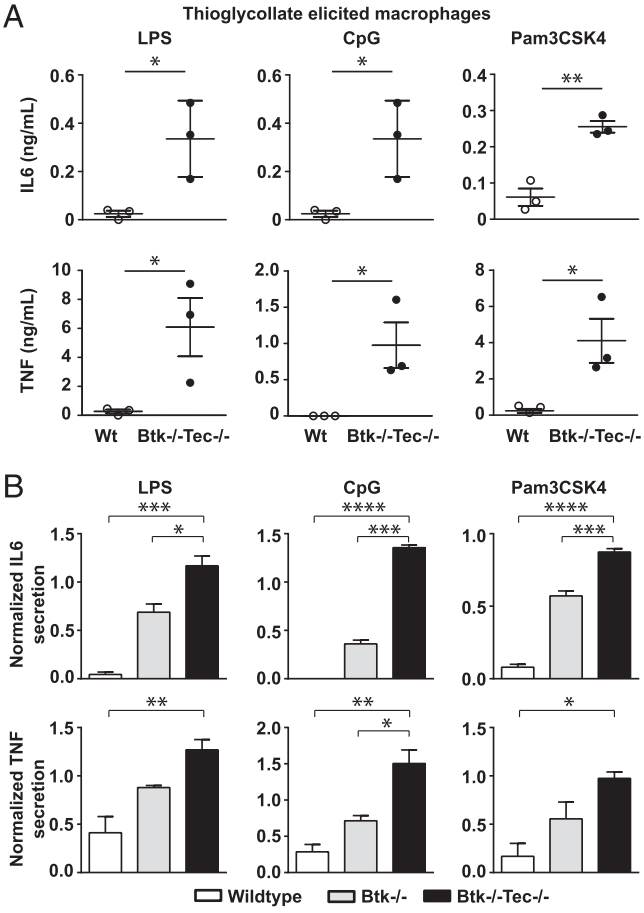

FIGURE 4. Increased TLR-induced cytokine secretion from $Btk^{-/-}$ and $Btk^{-/-}Tec^{-/-}$ thioglycollate-elicited peritoneal macrophages. (**A**) Thioglycollate-elicited peritoneal macrophages were stimulated with the TLR agonists LPS (2 ng/ml) and CpG (200 nM) for 16 h. IL-6 and TNF concentrations in the culture supernatant were determined by ELISA. Data show means ± SD of three independent experiments. The *p* values were calculated using an unpaired Student *t* test. (**B**) Combined ELISA data from three independent experiments of thioglycollate-elicited peritoneal macrophages stimulated with LPS (2 ng/ml), CpG (200 nM), or Pam3CSK4 (10 ng/ml). For each independent experiment, we normalized each ELISA measurement to the mean of all measurements in the $Btk^{-/-}Tec^{-/-}$ condition on that day. The replicate data were then plotted (mean ± SD). The *p* values were calculated using one-way ANOVA (one-factor: genotype) followed by a multiple comparisons test using Bonferroni correction. $*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p = 0.0001–0.001$, $****p < 0.0001$.

possible contributing factor to these cell type differences is that other Tec family members may promote downstream TLR signaling in BMMϕ and thiogylcollate-elicited macrophage populations. To assess this possibility, we analyzed the mRNA and protein expression of Bmx in BMMϕ. In contrast to what has been previously reported (6), we found that BMMϕ derived from wild-type, $Btk^{-/-}$, and $Btk^{-/-}Tec^{-/-}$ mice expressed detectable levels of Bmx protein (Supplemental Fig. 2B) and mRNA (Supplemental Fig. 2C). To determine whether Bmx might positively regulate TLR-dependent secretion of proinflammatory cytokines in Btk/Tec-null BMMϕ, we employed CC-292, a compound that potently inhibits the enzymatic activity of the Tec kinases Btk (IC_{50} of 5.9 nM) Bmx (IC_{50} of 0.7 nM), and Tec (IC_{50} of 6.2 nM) (23). We pretreated wild-type and $Btk^{-/-}Tec^{-/-}$ BMMϕ cultures with several doses of CC-292 or DMSO (vehicle control) for 30 min and then stimulated them with LPS or CpG. First, we verified that CC-292 did not alter the percentage of dead (7-aminoactinomycin D^+) or apoptotic (annexin V^+) cells (Supplemental Fig. 2D). Next, we evaluated TLR-dependent cytokine secretion and found that inhibition of Tec kinases with CC-292 resulted in decreased TLR-dependent secretion of IL-6 and TNF (Fig. 7A) regardless of the genetic background, implying that Bmx can promote TLR signaling in BMMϕ even when Btk and Tec are absent. Additionally, we observed that CC-292 treatment results in less TLR-dependent cytokine production in BMMϕ isolated from wild-type mice relative to those isolated from $Btk^{-/-}Tec^{-/-}$ mice (Fig. 7A), implying that some inhibitory signaling, possibly via Tec, is maintained in wild-type cells at low doses of CC-292. Taken together, our data show evidence for a role for Tec kinases in both positive and negative regulation of TLR signaling in BMMϕ.

To further investigate the role that Tec kinases play in regulating TLR signaling in resident peritoneal macrophages, we queried whether pharmacological inhibition of Btk, Tec, and Bmx would inhibit TLR signaling in vivo. Wild-type mice were given drinking water containing the novel Tec kinase inhibitor, compound 1, or vehicle for 40–90 h. Compound 1 has a similar structure (Fig. 7B) to CC-292, is highly selective for Tec kinases (Supplemental Table II), and potently inhibits Btk (IC_{50} of 12.5 nM), Tec (IC_{50} of 22 nM), and Bmx (IC_{50} of 2.1 nM), but not ITK (IC_{50} of 172 nM) . To demonstrate the efficacy of in vivo delivery of compound 1 in our experiments, we processed spleens from untreated and treated animals and found that ~80% of Btk was bound by compound 1 (Fig. 7D). To assess the response of macrophages from compound 1–treated mice to TLR ligands, we isolated resident peritoneal macrophages and stimulated them with LPS (0, 0.25, and 0.5 ng/ml) or Pam3CSK4 (0, 2.5, and 5 ng/ml) for 16 h and subsequently assayed their secretion of IL-6 and TNF by ELISA. We found that resident peritoneal macrophages isolated from compound 1–treated mice secreted lower concentrations of proinflammatory cytokines IL-6 and TNF in response to different doses of LPS and Pam3CKS4 relative to cells isolated from vehicle-treated control mice (Fig. 7C). These in vivo pharmacological studies confirmed our findings with cells isolated from mutant mice, and collectively our data suggests that Tec kinases positively orchestrate TLR signaling in resident peritoneal macrophages.

Discussion

We (19, 20, 37) and others (21) have investigated the mechanisms mediating crosstalk between the immunoreceptor and TLR signaling pathways in immune cells, including macrophages. In the present study, we have elucidated two roles that the Tec kinases, Btk and Tec, play in TLR signaling in different myeloid subsets: resident peritoneal macrophages, thyoglycollate-elicited macrophages, and BMMϕ. We chose to study these signals in BMMϕ because this population is easy to generate the large numbers required for phosphoproteomic studies and because this population exhibits both TLR signals and ITAM-mediated immunoreceptor inhibitory signals. To expand upon these in vitro findings, we also performed studies in two primary populations that respond to TLR ligands but are different with respect to whether they express TREM2 and thus exhibit ITAM-mediated inhibitory signaling: thioglycollate-elicited macrophages do whereas residential peritoneal macrophages do not (21). First, we show that in resident peritoneal macrophages, Btk and Tec are required for signaling events mediated by the TLR1/2, TLR4, and TLR9 receptors. Conversely, in BMMϕ or thioglycollate-elicited macrophages, Btk and Tec inhibit TLR signaling. To our knowledge, our quantitative phosphoproteomic data provide the first characterization of the Tec kinase–regulated phosphoproteome and surprisingly

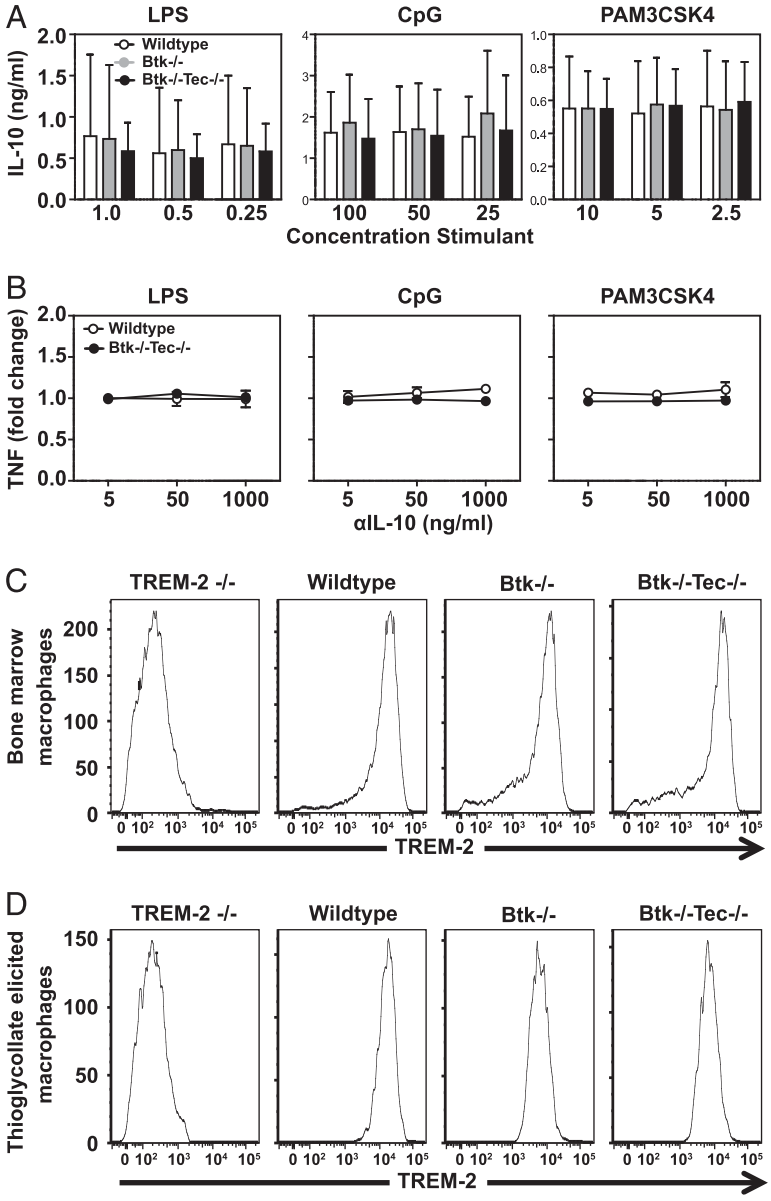

FIGURE 5. Role of IL-10 and Trem2 in Tec kinase–mediated inhibition of TLR-dependent cytokine secretion. (**A**) BMMϕ were stimulated with indicated doses of the TLR agonists (LPS, CpG, and Pam3CSK4) for 16 h. The concentration of IL-10 concentration present in the culture supernatants was determined using ELISA. The graph shows results from four independent experiments, and data are represented as means ± SD. (**B**) BMMϕ were cultured with increasing doses of IL-10–neutralizing Ab for 30 min or with an isotype control. Next, cells were stimulated with LPS (0.0625 ng/ml), CpG (12.5 nM), or Pam3CSK4 (2.5 ng/ml) in the presence of GolgiStop protein transport inhibitor for 6 h, stained for intracellular TNF-α, and analyzed by flow cytometry (cells gated on F4/80$^+$). The graph shows data from two independent experiments, expressed as mean fold change over isotype control ± SD. (**C**) BMMϕ were generated from TREM-2$^{-/-}$, wild-type, Btk$^{-/-}$, and Btk$^{-/-}$Tec$^{-/-}$ mice and stained for CD11b and TREM-2. (**D**) Wild-type, Btk$^{-/-}$, and Btk$^{-/-}$Tec$^{-/-}$ mice were injected i.p. with sterile thioglycollate medium. After 4 d, thioglycollate-elicited F4/80$^+$ peritoneal macrophages were collected and stained as described above for BMMϕ. TREM-2$^{-/-}$ F4/80$^+$ peritoneal macrophages were used as a negative control (*left panel*). Histograms are gated on CD11b$^+$ cells.

demonstrate that Btk and Tec act upstream of ITAM phosphorylation of Dap12. Therefore, deficiency of Btk and Tec lead to reduced phosphorylation of several proximal proteins critical for Trem2/Dap12-mediated immunoreceptor inhibitory signals. Finally, we show that in vivo treatment of mice with selective Tec kinase inhibitors reduces TLR signaling in resident peritoneal macrophages, a finding that has important implications for patients with autoimmunity or lymphoma being treated with such drugs.

The Btk inhibitor and in vitro–resident peritoneal macrophage data are consistent with reports demonstrating that Tec kinases are required in murine macrophages subsets in vivo (11) and in vitro (9, 11–13) for TLR and bacteria-elicited inflammatory cytokine secretion. Furthermore, Btk$^{-/-}$ mice are less susceptible to sepsis-induced mortality (10), an event that is dependent on TLR-induced cytokine secretion. A mechanism proposed to explain the requirement for Btk in TLR-induced signaling is physical interaction between Btk and the receptor (reviewed in Refs. 7, 8). Consistent with this idea, yeast two-hybrid and coprecipitation experiments have elucidated interactions between Btk and several components of the TLR cascade, including Tlr3 (10), Tlr4 (18), Myd88 (18), Irak1 (18), and Tirap (also known as Mal) (18). Furthermore, Btk is required downstream of the TLR4 receptor for LPS-dependent phosphorylation of Rela (also known as p65) (38) and Tirap (39), which may explain how it promotes TLR signals in resident peritoneal macrophages.

Tec kinases play functionally redundant roles in the regulation of TLR signaling. We find that deficiency of both Btk and Tec caused marked alterations in TLR-dependent cytokine secretion, whereas deficiency of Btk alone produces intermediate phenotypes. In human monocytes, Btk mutation (14) or depletion (4) causes decreased TLR-dependent secretion of TNF, but not IL-6. Overexpression of Bmx in the same cells promotes TLR4-induced production of IL-6 and TNF (4), suggesting that Bmx and Btk collaborate to promote TLR-dependent cytokine secretion in monocytes. Despite reports to the contrary (6), we observed expression of Bmx in BMMϕ. We hypothesize that in BMMϕ Bmx alone is sufficient to promote TLR signaling, whereas Btk and Tec participate in a separate TLR inhibitory pathway. Our finding that CC-292, a small molecule that selectively targets both Bmx and Btk, can inhibit TLR signaling in BMMϕ and resident peritoneal macrophages strongly supports this hypothesis. Investigation of these phenomena using Btk$^{-/-}$Tec$^{-/-}$ Bmx$^{-/-}$ mice will be required to determine the precise role of Bmx.

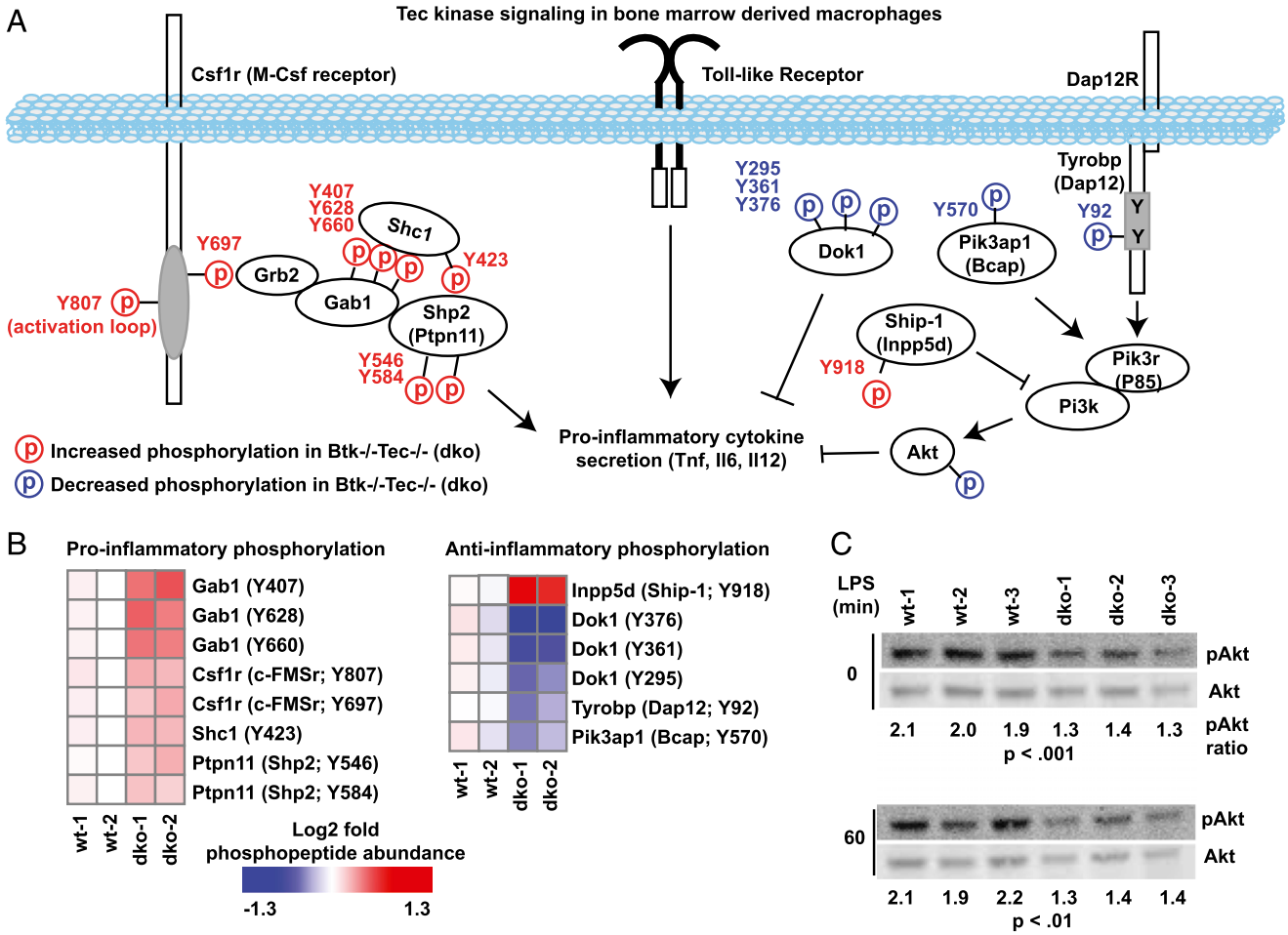

FIGURE 6. The effect of Tec kinase deficiency on BMMϕ. (**A**) BMMϕ from biological replicates isolated from wild-type or Btk$^{-/-}$Tec$^{-/-}$ mice were stimulated with LPS (1 ng/ml) for 20 min and analyzed using quantitative phosphoproteomics. Presented is a schematic model showing phosphorylation sites whose peptide abundance is increased (red) or decreased (blue) in Tec kinase–deficient cells. This schematic shows proteins important for regulation of myeloid TLR signaling (site is indicated by number and letter) with phosphorylation data supporting a model where Tec deficiency limits Dap12-mediated inhibitory signals and promotes TLR- and Csf1r-mediated proinflammatory signaling. (**B**) Heat map showing the normalized peptide abundance for each site schematized in (A). (**C**) Cytoplasmic extracts from cells isolated from three independent mice were analyzed using Abs specific for Akt or for its phosphorylated version. Immunoblots were visualized, quantified, and the ratio between the abundance of the phosphorylated form of Akt and total Akt is indicated below the blots. The *p* values indicated on the panel were calculated using a Student *t* test.

Our findings suggest that Btk and Tec inhibit TLR-dependent signaling in BMMϕ via positive regulation of immunoreceptor signaling in macrophages. Similar to Btk and Tec, the immunoreceptor TREM2 and its signaling chain DAP12 inhibit TLR-dependent inflammatory cytokine secretion in BMMϕ and dendritic cells (19–21, 40). Tec kinases also regulate immunoreceptor signaling in osteoclasts, where DAP12 scaffolds Btk and Tec, enabling them to promote RANKL signaling (41). Our result that the surface expression of TREM2 is not affected by Tec kinase deletion in BMMϕ suggests that differences in TREM2 expression or localization cannot explain the increased TLR-dependent cytokine secretion we observe the Btk$^{-/-}$Tec$^{-/-}$ animals. Instead, we found that phosphorylation of the Dap12 ITAM and activating phosphorylation of other proteins that inhibit TLR-dependent cytokine secretion, including Dok1 and Pi3kap1, are decreased in Btk$^{-/-}$Tec$^{-/-}$ BMMϕ. Consistent with these findings, a subset of LPS-dependent phosphorylation, including that of Mapk14 (P38), is increased in Btk$^{-/-}$Tec$^{-/-}$ BMMϕ. These findings are similar to those in Dap12-deficient BMMϕ, which also have increased Mapk phosphorylation following LPS treatment (19). Furthermore, a large percentage of the LPS-dependent phosphorylation events that we and others (42) have identified in wild-type macrophages are enhanced in Btk$^{-/-}$Tec$^{-/-}$ macrophages. Finally, as in Dap12 (35) and Bcap (Pik3ap1 (11, 34)) deficiency, Pi3k signals are diminished in Tec kinase–deficient BMMϕ, thus providing a possible mechanism for how the TLR pathway is blocked by Tec kinases. Collectively, our findings strongly support the conclusion that Tec kinases are required for signaling via the Trem2/Dap12 inhibitory pathway, proximal to the TLR receptor.

A possible explanation for the divergent findings between resident peritoneal macrophages versus thyoglycollate-elicited macrophages or BMMϕ is that these cell types may differentiate or migrate differently in the context of Tec kinase deficiency, thus impacting signaling. In fact, Btk deficiency limits recruitment of M1 macrophages in response to LPS (43). Consistent with our findings, Btk deficiency results in upregulation of Ship1 protein expression in response to M1 polarizing signals, which likely contributes to diminished levels of Pi3k signaling (43). Further research will be necessary to determine how Tec kinases inhibit Trem2/Dap12 signaling, and whether this pathway impacts M1 polarizing signals and macrophage phenotype, or vice versa.

The effects of Tec kinase deficiency on TLR-dependent signaling likely also involve phosphorylation downstream of

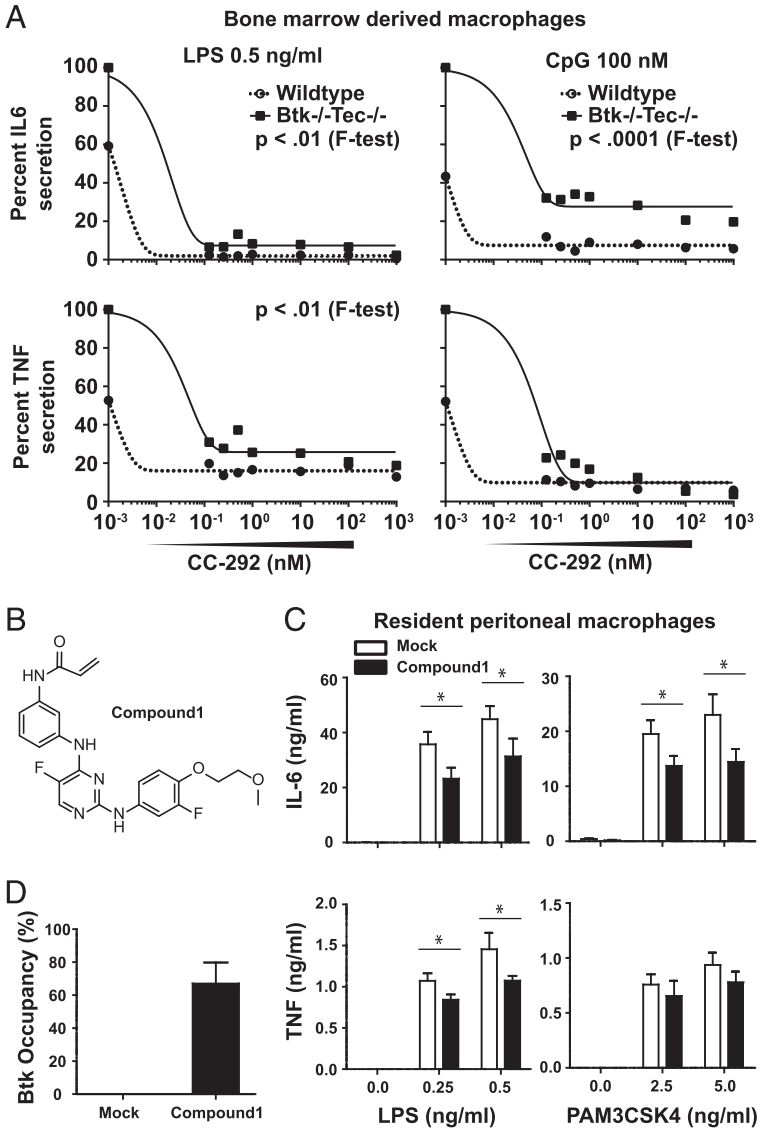

FIGURE 7. In vitro and in vivo effects of the Tec kinases inhibitor in TLR-stimulated macrophages. (**A**) BMMϕ were pretreated for 30 min with the indicated concentrations of a specific inhibitor for Tec kinases (CC-292) or DMSO as vehicle control. After pretreatment, indicated doses of TLR stimuli (LPS or CpG) were added directly to the cell culture media for 16 h. The concentration of IL-6 and TNF in culture supernatants was determined by ELISA from two independent experiments, and the values were normalized to those in the $Btk^{-/-}Tec^{-/-}$ condition (100%) and plotted. We used nonlinear regression to fit these dose response curves (one-phase decay) and used an *F* test to assess the likelihood that the plateau of each curve was the same (indicated *p* values). (**B**) Structure of compound 1. (**C**) Wild-type mice were given drinking water containing the Btk inhibitor, compound 1, or acidified water alone as a control for 40–90 h. $F4/80^{+}$ peritoneal macrophages were isolated from treated animals and stimulated with indicated doses of LPS or Pam3CSK4 for 16 h, and IL-6 and TNF-α concentrations in the culture supernatant were determined by ELISA. Data show means ± SD of three mice per group and are representative of two independent experiments. (**D**) Snap-frozen spleens from (C) were used to determine the percentage of total Btk that was occupied by the inhibitor. Data are normalized to water controls. The *p* values were calculated using an unpaired Student *t* test. $*p < 0.05$.

the M-CSF receptor (Fig. 6A). Some of the strongest increases in phosphorylation that we observed in Tec-deficient BMMϕ were found in Csfr1 (tyrosines 697 and 807) and a protein complex that binds tyrosine 697 of this receptor that includes Grb2, Gab1, Shc1, and Ptpn11 (Shp2) (44). Both Csfr1 (45, 46) and Gab1 (47) are required for TLR-driven secretion of TNF and IL-6. Our data support the idea that loss of Tec kinases cause activation of an M-CSF–Gab1 pathway that collaborates with decreased DAP12 signaling to promote proinflammatory cytokine secretion. However, both Tec kinase–deficient (6) and Dap12-deficient (48) BMMϕ exhibit increased reliance on soluble M-CSF for viability in culture. Although these data are consistent with our finding that AKT phosphorylation is diminished in Tec kinase–deficient BMMϕ, future studies are necessary to determine whether the reliance on M-CSF for survival of BMMϕ lacking the DAP12 pathway is driven by hyperactivation of Csfr1 and Gab1/Shc1/Ptpn11, by partial rescue of the diminished AKT program, or by the combined action of both of these signaling changes.

Because Btk and Tec inhibit TLR responses in BMMϕ and thioglycollate-elicited peritoneal macrophages, we predict that inhibitors that specifically target Btk, and not Bmx or Tec, will elevate TLR responses in some macrophage subsets in vivo. These potentially inflammatory effects should be carefully evaluated in clinical trials involving selective Btk inhibitors. We have also found that pan-Tec kinase inhibitors such as CC-292 repress TLR responses across macrophage subsets. These pan-Tec kinase inhibitors are also likely to inhibit TLR-dependent signaling in B cells, which can be activated by dual signals from the BCR and TLRs (49). As B cell proliferative diseases and lymphoma can have simultaneous activating mutations in component BCR and the TLR receptor pathways (50, 51), our findings imply that targeting Btk may be an especially attractive therapeutic option in these cases, because it enables attenuation of both pathways simultaneously.

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Disclosures

D.N., E.E., B.B., and M.N. are employees of and hold public stock in Celgene Avilomics Research. The remaining authors have no financial conflicts of interest.

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