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The Tec kinase-regulated phosphoproteome reveals a mechanism for the regulation of inhibitory signals in murine macrophages

Giacomo Tampella[*,‖,¶], **Hannah M. Kerns**[*,‖], **Deqiang Niu**[‡‡], **Swati Singh**[‖], **Socheath Khim**[‖], **Katherine A. Bosch**[‖], **Meghan E. Garrett**[‖], **Albanus Moguche**[‡,‖], **Erica Evans**[‡‡], **Beth Browning**[‡‡], **Tahmina A. Jahan**[§], **Mariana Nacht**[‡‡], **Alejandro Wolf-Yadlin**[§], **Alessandro Plebani**[††], **Jessica A. Hamerman**[‡,#,¶], **David J. Rawlings**[**,†,‡,‖,¶], and **Richard G. James**[**,†,‖,¶]

[†]Department of Pediatrics, University of Washington School of Medicine, Seattle WA 98195

[‡]Department of Immunology, University of Washington School of Medicine, Seattle WA 98195

[§]Department of Genome Sciences, University of Washington School of Medicine, Seattle WA 98195

[‖]Seattle Children's Research Institute, Seattle WA 98101

[#]Benaroya Research Institute, Seattle WA 98101

[††]Experimental Sciences, Pediatrics Clinic and Institute for Molecular Medicine A. Nocivelli, University of Brescia, Spedali Civili of Brescia, Italy

[‡‡]Celgene Avilomics Research, Bedford, MA

Abstract

Previous work has shown conflicting roles for Tec family kinases in regulation of Toll-like receptor (TLR)-dependent signalling in myeloid cells. In the present study, we performed a detailed investigation of the role of Btk and Tec kinases in regulating TLR signalling in several types of primary murine macrophages. We demonstrate that primary resident peritoneal macrophages deficient for Btk and Tec secrete less pro-inflammatory cytokines in response to TLR stimulation than wild type cells. In contrast, we found that bone marrow-derived and thioglycollate-elicited peritoneal macrophages deficient for Btk and Tec secrete more pro-inflammatory cytokines than wild type cells. We then compared the phosphoproteome regulated by Tec kinases and lipopolysaccharide in primary peritoneal and bone marrow derived macrophages. From this analysis we determined that Tec kinases regulate different signalling programs in these cell types. In additional studies using bone marrow-derived macrophages, we find that Tec and Btk promote phosphorylation events necessary for immunoreceptor-mediated

[**]Correspondence: David J. Rawlings, M.D., Center for Immunity and Immunotherapies, Seattle Children's Research Institute, 1900 9th Ave, Seattle, WA 98101. Tel. 206.987.7450, drawling@uw.edu; Richard G. James, Ph.D., Center for Immunity and Immunotherapies, Seattle Children's Research Institute, 1900 9th Ave, Seattle, WA 98101, Tel. 206.884.3290, rickerj@uw.edu.

[*]These authors contributed equally to this work

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inhibition of TLR signalling. Taken together, our results are consistent with a model where Tec kinases (Btk, Tec, Bmx) are required for TLR-dependent signalling 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 signalling via PI3K.

Introduction

The toll-like receptor (TLR) signalling 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, and Tram, resulting in activation of a common pathway that culminates in signalling via the mitogen activated protein kinases MAPKs (Mapk family members), nuclear factor-kappa B (NF-κB) and interferon regulatory factor (Irf) transcription factors. Following activation of these proteins by the TLR pathways, the cell produces inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-12 (IL12), 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 signalling 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 signalling in murine macrophages (reviewed in (7, 8)). In several studies, resident peritoneal(9) and bone marrow derived(10, 11) macrophages isolated from mice deficient for Btk were found to secrete lower levels of the pro-inflammatory cytokines TNF, IL6 or IL12 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 IL6(12) in response to TLR activation. Finally, one study reported that Btk deficiency led to increased TLR-dependent IL12, but decreased TNF secretion in both thioglycollate-elicited peritoneal and bone marrow derived macrophages(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 pro-inflammatory cytokine secretion. Taken together, these results demonstrate that Tec kinases can positively and negatively regulate secretion of pro-inflammatory 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.

The positive role for Btk in TLR signalling has been proposed to involve a direct requirement for Btk via interaction with receptor, co-receptor 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 signalling, which blocks signalling downstream of TLRs in certain macrophage populations. Immunoreceptors have a ligand-binding receptor subunit and an adapter protein that contains an intracellular signalling domain, such as an immunoreceptor tyrosine-based activation motif (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 proteomics-based signalling studies described here, we hypothesize that Tec kinases can play two opposing roles during myeloid TLR signalling: 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 signalling in several primary mouse populations. Our combined results help to clarify the role for Tec kinases in TLR signalling.

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 labelled Btk knock-out 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 BM-derived macrophages

Bone marrow cells from six to fifteen week old mice were flushed from femurs and tibias. Following red blood cell lysis, the remaining cells were filtered and plated at 7.5×10^5 cells/ml on 10cm petri dishes (Fisherbrand). Cells were grown in complete media: DMEM high glucose medium (Thermo Scientific), supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 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 Inc.), and 10% CMG 12-14 cell conditioned medium as a source of M-CSF(22). The culture medium was changed on day 3 and day 5, when the cells were counted and re-plated for further experiments.

Isolation of peritoneal and thioglycollate-elicited peritoneal macrophages

Mice were injected intraperitoneally with 1 ml of sterile thioglycollate medium (BD, MD, USA). Peritoneal macrophages were harvested by peritoneal lavage with sterile phosphate-buffered saline (PBS, Thermo Scientific) supplemented with penicillin/streptomycin solution (100 U/ml penicillin, 100 μg/ml streptomycin, Thermo Scientific) and 5% heat-inactivated fetal bovine serum (Sigma). 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 hours - overnight. TLR stimuli were added to the wells and after 16 hours the levels of TNF, IL6,IL12 p40 and IL-10 were measured by enzyme-linked

immunosorbent assay (ELISA Ready-SET-Go eBioscience). For intracellular cytokine staining, 1×10^5 cells were plated per well of 48-well non-TC 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 pre-treated with indicated dilutions anti-IL-10 (clone JES5-2A5, eBioscience) or 1000 ng/ml rat IgG2b isotype control (eBioscience) for 30 minutes 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 eFluor450 anti-F4/80 (eBioscience), FITC anti-TNF alpha (eBioscience) and PE anti-IL6 antibodies (eBioscience). Apoptotic cells were identified by staining with annexin V and 7-amino-actinomycin D (BD Biosciences). For each experiment, cells were analyzed by flow cytometry using a BD LSR II running FACSDiva software (BD) and with FlowJo (TreeStar).

Reagents

The following primers were used for quantitative PCR (Eurofins MWG Operon): *Actb* (5′-ctaaggccaaccgtgaaaag, 5′-accagaggcatacagggaca). *Tnf* (5′-tcttctcattcctgcttgtgg 5′-ggtctgggccatagaactga), *Il6* (5′-gctaccaaactggatataatcagga, 5′-ccaggtagctatggtactccagaa), *Il12* (5′-ccatcagcagatcattctagacaa, 5′-cgccattatgattcagagactg), and *Bmx* (5′-gagcagcttcgcttcacc, 5′-gatttactctccatattgtcgtcca). The following compounds were used: CC-292(23) and Compound1. The following antibodies were used: Trem2(24), pY-100 (Cell Signaling Technologies, 9411), Mapk1/3 (Cell Signaling Technologies, 4695), pMapk1/3 (Cell Signaling Technologies, 4377), PT66 (Sigma, P3300), 4G10 (Millipore, 05-321), Tec (Millipore, 05-551), Bmx (BD Biosciences, 610792), Btk (BD Biosciences, 558528), and IRDye (LI-COR, 800CW and 680RD). The following additives and TLR agonists were used: Lipopolysaccharide (List Biological Labs, 434), CpG DNA (Invitrogen, tlrl-1826), Pam3CSK4 (Invitrogen, tlrl-pms), Gardiquimod (Invitrogen, tlrl-gdgs) and Polymyxin B (Sigma, P4932).

Western blotting and PCR

Whole cell protein extracts were prepared by cell lysis with buffer containing 50mM Tris pH 7.4, 150mM sodium chloride, 1mM EDTA, 1% Triton X-100, 1mM 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 (Licor Biosciences, Lincoln, NE) (Li-Cor). 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 Compound1

Synthesis of tert-butyl (3-((2-chloro-5-fluoropyrimidin-4-yl)amino)phenyl)carbamate (2)—2,4-dichloro-5-fluoropyrimidine (800mg, 4.8mmoL), tert-butyl (3-aminophenyl)carbamate (996mg, 4.8mmoL) and diisopropylethylamine (DIPEA, 948uL, 5.75mmoL) were dissolved in THF (20mL). 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%). LCMS: *m/e* 339.1 (M+1) (Figure S1A).

Synthesis of tert-butyl (3-((5-fluoro-2-((3-fluoro-4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl)carbamate (4)—To a solution of compound **2** (600mg, 1.77mmol) and 3-fluoro-4-(2-methoxyethoxy)aniline (**3**, 390 mg, 2.12 mmol) in 10 ml ethanol was added TFA (5 drops). The mixture was stirred at reflux for 4 hours. 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 subject to chromatography on silica gel (hexane:EtOAc = 1:1). 730 mg of compound **4** was obtained (85%) (Figure S1A).

Synthesis of N-(3-((5-fluoro-2-((3-fluoro-4-(2-methoxyethoxy)phenyl)amino)pyrimidin-4-yl)amino)phenyl)acrylamide (1)—To a solution of **4** (600 mg, 1.2 mmol) in DCM (20 mL) was added TFA (2 mL). The solution was stirred at room temperature for 4 hours. 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 (Figure S1A).

A solution of the compound obtained above in DCM (20 mL) was cooled to -70°C. To this solution was added acryloyl chloride (96 μL, 1.2 mmol) followed by diisopropylethylamine (DIPEA) (200 uL, 1.2 mmol). The reaction was stirred for 10 minutes at -70°C and was quenched by $NaHCO_3$ aqueous solution. The organic layer was separated, dried over Na_2SO_4. After removal of solvent, the crude product was subject to chromatography on silica gel (hexane: EtOAc = 1:1) to give 335 mg of compound**1**. LCMS: *m/e* 442.0 (M +1).^{1}HNMR (DMSO, 400 MHz) δ10.13 (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, 3H) (Figure S1A).

Kinase selectivity panel and occupancy analysis

Compound1 was run in a kinase selectivity panel at Reaction Biology Corporation (Malvern, PA) using HotSpot^{SM} technology and radioisotope-based P81 filtration. Compound1 was dissolved in pure DMSO to the final 1μM test concentration. Substrates for the various kinases tested against Compound1 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 hr 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

Corp.; Malvern, PA, USA). The study was performed in duplicate and 10 μM staurosporine, a non-selective, ATP-competitive kinase inhibitor, was used as the positive control. To determine IC50 values, Compound1 was tested in a 10-dose IC_{50} mode with 10-fold serial dilution starting at 10μM. Staurosporine was tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 20 μM. Reactions were carried out at Km ATP or 10× Km ATP, according to the RBC binning structure. Btk-occupancy analysis was performed on isolated sleens as previously described(23).

Quantitative Phosphoproteomics

Bone marrow derived macrophages 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 labelled with six-plex TMT reagent (Thermo Scientific). Phosphopeptide enrichment, chromatography, mass spectrometry and quantification was performed as detailed previously(25-27). To assess the differences between the Btk-/-Tec-/- LPS and wild type LPS conditions, calculated the fold effect caused by Tec deficiency and LPS using the following steps: (1) 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 that in the jurkat-stimulated sample. (2) The normalized ion intensities from the replicate experiment were averaged for all conditions (wild type, wild type LPS, Btk-/-Tec-/-, and Btk-/-Tec-/- LPS), (2) Next for each unique peptide, we calculated and intensity score in each condition by averaging the value across experimental replicates. (3) 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 heatmap rendering was done using the GENE-E tool (www.broadinstitute.org/cancer/software/GENE-E). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (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 and bone marrow-derived (BMMΦ; (6)). To discover how Btk and Tec impact global TLR signalling 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 labelling reagent: tandem mass tags (TMT). We isolated BMMΦ and resident peritoneal macrophages from each genotype and stimulated them with the TLR ligand, Lipopolysaccharide (LPS) for 20 minutes (Figure 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; Figure 1B) and Mapk14 (P38; Figure 1B) behaved as predicted. Further, verification of the Mapk9 and Mapk14 results by

western blot (compare mass spectrometry-based quantification Figure 1B with western blot Figure 1C) confirmed that our method accurately quantifies phosphopeptide abundance.

In all experiments from both cell types, we quantified 6704 unique phosphopeptides (FDR ∼2.5%; Supplemental Table 1) derived from 2093 unique proteins, of which 1800 peptides were quantified in both macrophage types (Figure 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 (Figure S1B, Spearman's rank coefficient $r = .58$; $p < .0001$). 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 (Figure S1C, Supplemental Table 1). Global comparison of the outliers revealed several interesting differences between resident peritoneal macrophages and BMMΦ (Hierarchical clustering; Figure 1D). Despite the global reproducibility between phosphotyrosine peptide abundance, only a minority of the outlier peptides (Figure 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 (Figure 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 (Figure 1D; clusters 6, 7). 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 Lipopolysaccharide (LPS, Tlr4 ligand), CpG DNA (CpG, Tlr9 ligand) and Pam(3)Cys-Ser-(Lys)(4) (Pam3CSK4, Tlr1/2 ligand). To determine the degree of TLR pathway activation, we used flow cytometry to quantify expression of the pro-inflammatory cytokine TNF. We observed that in populations stimulated with LPS or Pam3CSK4, both the number cells expressing TNF and the intensity of expression per cell is decreased in Btk-/-Tec-/- macrophages relative to wild type controls (Figure 2A and 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 pro-inflammatory cytokines IL6 and TNF in response to different doses of LPS and Pam3CKS4 stimuli relative to cells isolated from wild type mice (Figure 2C). These data demonstrate that Tec kinases inhibit TLR-dependent cytokine secretion in resident peritoneal macrophages.

Increased TLR responses in bone marrow-derived 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 signalling in BMMΦ and thioglycollate-elicited

macrophages(29, 30). First, we investigated whether the kinetics of pro-inflammatory cytokine secretion were altered in Tec family kinase-deficient mice. Within 4-8 hours of stimulation, we observed a trend showing increase in TLR-dependent secretion of IL6, IL12 and TNF in BMMΦ isolated from both Btk-/- and Btk-/-Tec-/- mice relative to that in cells isolated from wild type mice (Figure S2A). 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 (Figure 3A) and secreted (Figure 3B)significantly higher concentrations of the pro-inflammatory cytokines IL6, IL12 and TNF than cells isolated from wild-type mice.

To further explore these results, we investigated whether Tec family kinases activate or inhibit TLR signalling in thioglycollate-elicited peritoneal macrophages. Similar to our findings in BMMΦ cells, we found that F4/80+ peritoneal macrophages isolated from Btk-/- and Btk-/-Tec-/- mice produced greater IL6 and TNF in response to simulation with LPS, CpG or Pam3CSK4 than cells isolated from wild type mice (Figure 4A, 4B). 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 down regulates transcription of the pro-inflammatory 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 pro-inflammatory cytokines, we observed no differences in IL-10 production from macrophages isolated from wild type, Btk-/- or Btk-/-Tec-/- mice (Figure 5A). To further investigate the role of IL-10 in TLR-dependent cytokine secretion in macrophages, we pre-treated BMMΦ with neutralizing antibodies to IL-10, and subsequently stimulated the cells with LPS, CpG or Pam3CSK4. Upon analysing 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 (Figure 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 bone marrow-derived (Figure 5C) and thioglycollate-elicited peritoneal (Figure 5D) macrophages 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 and BMMΦ macrophages. 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 (FDR < 10%; Supplemental Table 1) for proteins involved in FcGamma R-mediated phagocytosis (KEGG: mmu04666), phosphatidylinositol signalling system (KEGG: mmu04070) and several other categories related to lymphoid and myeloid signalling. Based on this analysis and on our integration of the data (Figure 6A, B), 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 (Figure 6A, B; Supplemental Table 1). Based on these results, we conclude that ITAM-mediated inhibitory signalling is decreased in Tec kinase-deficient BMMΦ.

Dap12 inhibitory signalling blocks TLR-dependent cytokine secretion in part via increasing PI3K-dependent signals (35). Our proteomics data show that an activating phosphorylation event on Ship1 (Figure 5A, B; Inpp5d; Y918), an event consistent with decreased PI3K signals (36). To further test whether PI3K signalling is altered in Tec kinase-deficient BMMΦ, we cultured BMMΦ isolated from 3 independent wild type and Tec kinase-deficient mice. We found statistically significant decreases in phosphorylation of Akt at Serine 473 in Tec deficient BMMΦ with or without LPS stimulation (Figure 6C). Together, our data indicate that a broad subset of inhibitory TLR signals including PI3K are 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 indicates that Btk and Tec are required for TLR signalling in resident peritoneal macrophages, but inhibits the pathway in BMMΦ and thioglycollate-elicited macrophages. One possible contributing factor to these cell type differences is that other Tec family members may promote downstream TLR signalling in BMMΦ and thiogylcollate-elicited macrophage populations. To assess this possibility, we analysed 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 (Figure S2B) and mRNA (Figure S2C). To determine whether Bmx might positively regulate TLR-dependent secretion of pro-inflammatory cytokines in Btk/Tec-null BMMΦ, we employed CC-292, a compound that potently inhibits the enzymatic activity of the Tec kinases Btk (IC50 = 5.9nM) Bmx (IC50 =.7nM) and Tec (IC50 = 6.2 nM)(23). We pretreated wild-type and Btk-/-Tec-/- BMMΦ cell cultures with several doses of CC-292 or DMSO (vehicle control) for 30 minutes, and then stimulated with LPS or CpG. First we verified that CC-292 did not alter the percentage of dead (7-AAD positive) or apoptotic (Annexin V positive) cells (Figure S2D). Next, we evaluated TLR-

dependent cytokine secretion and found that inhibition of Tec kinases with CC-292 resulted in decreased TLR-dependent secretion of IL6 and TNF (Figure 7A) regardless of the genetic background, implying that Bmx can promote TLR signalling 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 (Figure 7A), implying that some inhibitory signalling, 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 signalling in BMMΦ.

To further investigate the role that Tec kinases play in regulating TLR signalling in resident peritoneal macrophages, we queried whether pharmacological inhibition of Btk, Tec and Bmx would inhibit TLR signalling *in vivo*. Wild-type mice were given drinking water containing the novel Tec kinase inhibitor, Compound1, or vehicle for 40-90 hours. Compound1 has a similar structure (Figure 7B) to CC-292, is highly selective for Tec kinases (Supplemental Table 2) and potently inhibits Btk (IC_{50} = 12.5 nM), Tec (IC_{50} = 22 nM) and Bmx (IC_{50} = 2.1 nM), but not ITK (IC_{50} = 172 nM). To demonstrate the efficacy of *in vivo* delivery of Compound1 in our experiments, we processed spleens from untreated and treated animals and found that approximately 80% of Btk was bound by Compound1 (Figure 7D). To assess the response of macrophages from Compound1-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 hours, and subsequently assayed their secretion of IL6 and TNF by ELISA. We found that resident peritoneal macrophages isolated from Compound1 treated mice secreted lower concentrations of pro-inflammatory cytokines IL6 and TNF in response to different doses of LPS and Pam3CKS4 relative to cells isolated from vehicle treated control mice (Figure 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 signalling in resident peritoneal macrophages.

Discussion

We (19, 20, 37) and others(21) have investigated the mechanisms mediating crosstalk between the immunoreceptor and TLR signalling pathways in immune cells, including macrophages. Here, we have elucidated two roles that the Tec kinases, Btk and Tec, play in TLR signalling in different myeloid subsets: resident peritoneal, thyoglycollate-elicited and bone marrow-derived macrophages. 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 signalling: 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 signalling events mediated by the TLR1/2-, TLR4- and TLR9- receptors. Conversely, in bone marrow-derived or thioglycollate-elicited macrophages Btk and Tec inhibit TLR signalling. Our quantitative

phosphoproteomic data provides the first characterization of the Tec kinase-regulated phosphoproteome and surprisingly 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 signalling 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 signalling is physical interaction between Btk and the receptor (reviewed in (7, 8)). Consistent with this idea, yeast two-hybrid and co-precipitation 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 signalling. 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 IL6. Overexpression of Bmx in the same cells promotes TLR4-induced production of IL6 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 signalling, 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 signalling 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.

Our findings suggest that Btk and Tec inhibit TLR-dependent signalling in BMMΦ is via positive regulation of immunoreceptor signalling in macrophages. Similar to Btk and Tec, the immunoreceptor TREM2 and its signalling chain DAP12 inhibit TLR-dependent inflammatory cytokine secretion in BMMΦ and dendritic cells(19-21, 40). Tec kinases also regulate immunoreceptor signalling in osteoclasts, where DAP12 scaffolds Btk and Tec enabling them to promote RANKL signalling(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 that 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 signalling via the Trem2/Dap12-inhibitory pathway, proximal to the TLR receptor.

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

The effects of Tec kinase deficiency on TLR-dependent signalling likely also involve phosphorylation downstream of the M-CSF receptor (Figure 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 IL6. Our data support the idea that loss of Tec kinases cause activation of a M-CSF-Gab1 pathway that collaborates with decreased DAP12 signalling to promote pro-inflammatory cytokine secretion. However, both Tec kinase- (6) and Dap12- (48) deficient 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 hyper-activation of Csfr1 and Gab1/Shc1/Ptpn11 or by partial rescue of the diminished AKT program, or both signalling cascades.

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 like CC-292, repress TLR responses across macrophage subsets. These pan-Tec kinase inhibitors are also likely to inhibit TLR-dependent signalling in B cells, which can be activated by dual signals from the B cell receptor and TLRs(49). As B cell proliferative diseases and lymphoma can have simultaneous activating mutations in components B cell receptor 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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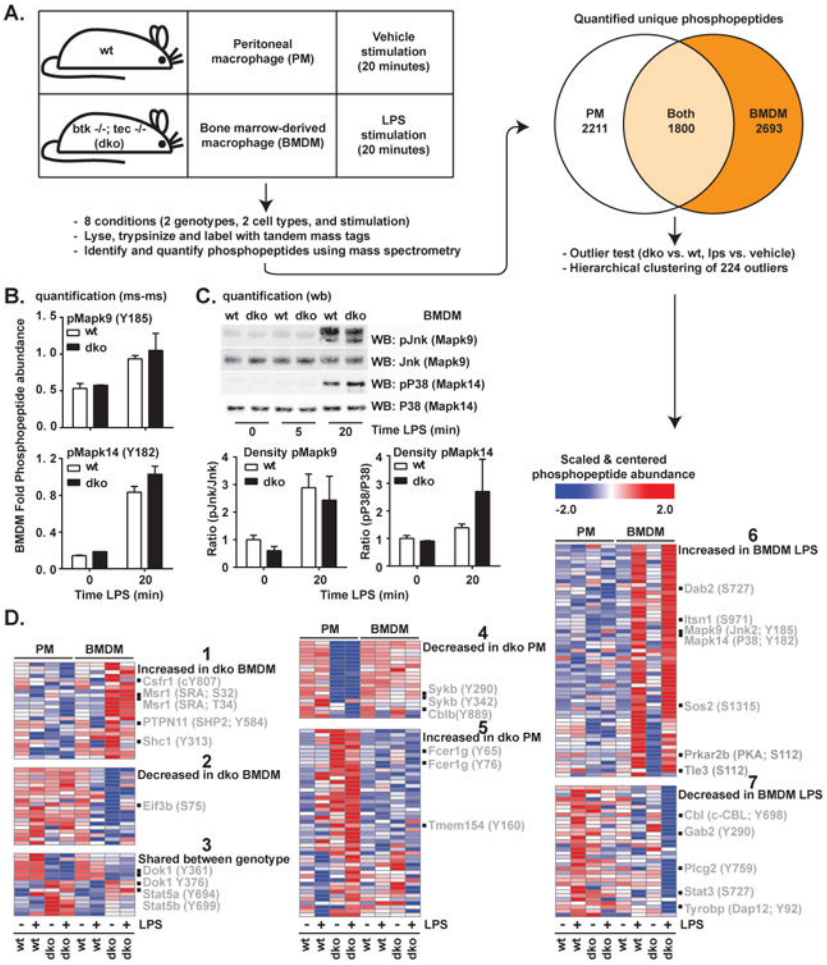

Figure 1. Differences in Tec-kinase-deficient phosphorylation between bone marrow-derived and resident peritoneal macrophages

(A) A flow chart showing the conditions and methods used to analyze Tec-kinase dependent phosphorylation in bone marrow-derived and resident peritoneal macrophages. Briefly, cell extracts and peptides were prepared from unstimulated wild-type and Btk-/-Tec-/- macrophage cell populations stimulated with LPS (1ng/ml) for 20 min. After phosphopeptide enrichment, phosphorylated peptides were identified and quantified by LC-MS/MS. (B) The mean of three biological replicates of the quantification of the indicated bone marrow-derived macrophage phosphopeptides by mass spectrometry, plus and minus the standard deviation (B). (C) Cytoplasmic extracts from bone marrow-derived macrophages from wild-type or Btk-/-Tec-/- mice stimulated with LPS (1 ng/ml) for indicated time (min) were analyzed using antibodies 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 non-phosphorylated forms was plotted ± the standard deviation. (D) A heatmap plot of all 224 quantified outlier phosphopeptides identified in both the bone marrow-derived or resident peritoneal macrophage experiment grouped (1-7) using hierarchical clustering.

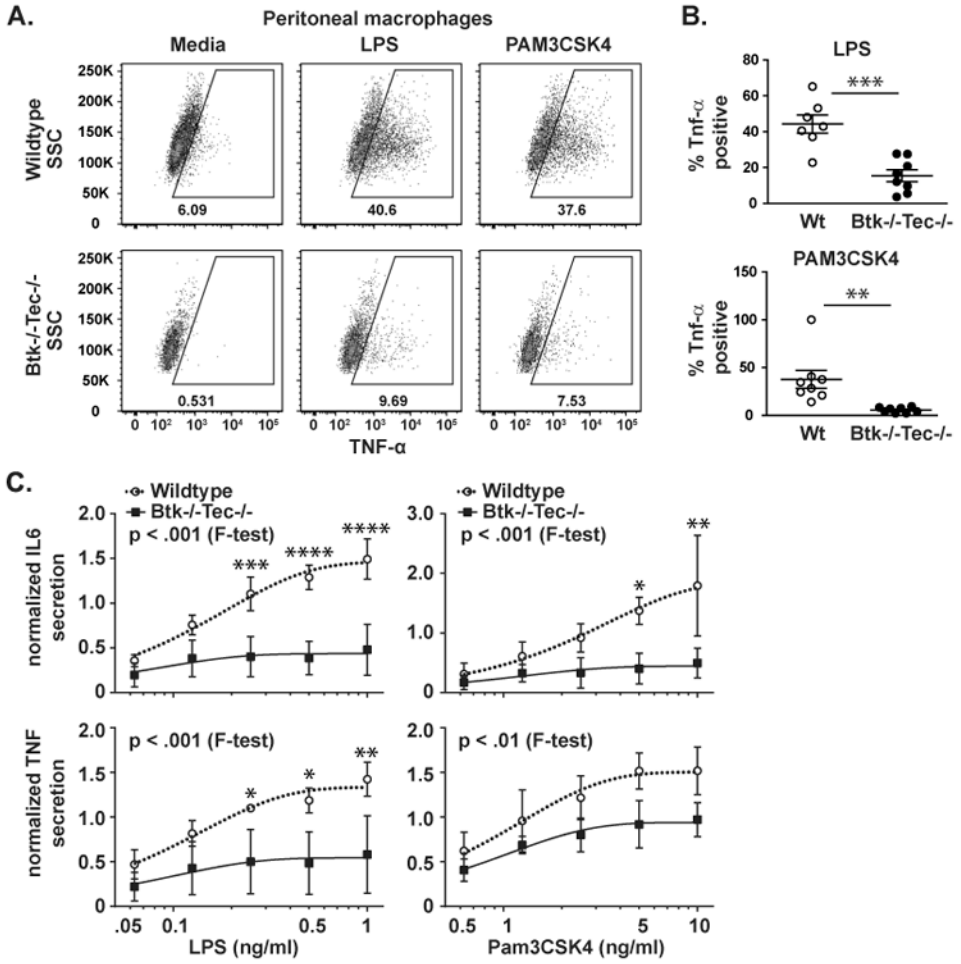

Figure 2. Decreased TRL-induced cytokine secretion from Btk-/- and Btk-/-Tec-/- F4/80+ mouse resident peritoneal macrophages

(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 hours, then stained for intracellular TNF and analysed by flow cytometry (cells gated on F4/80+). (B) Combined data from 3 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 IL6 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 was then plotted (mean ± standard deviation), and a line for each genotype was fitted using non-linear 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). In addition, p-values for individual doses were calculated using two-way analysis of variance coupled with a multiple comparison test using Bonferroni correction (* p = 0.01 - 0.05, ** p = 0.001 - 0.01, *** p = . 0001 - 0.001, **** p < 0.0001).

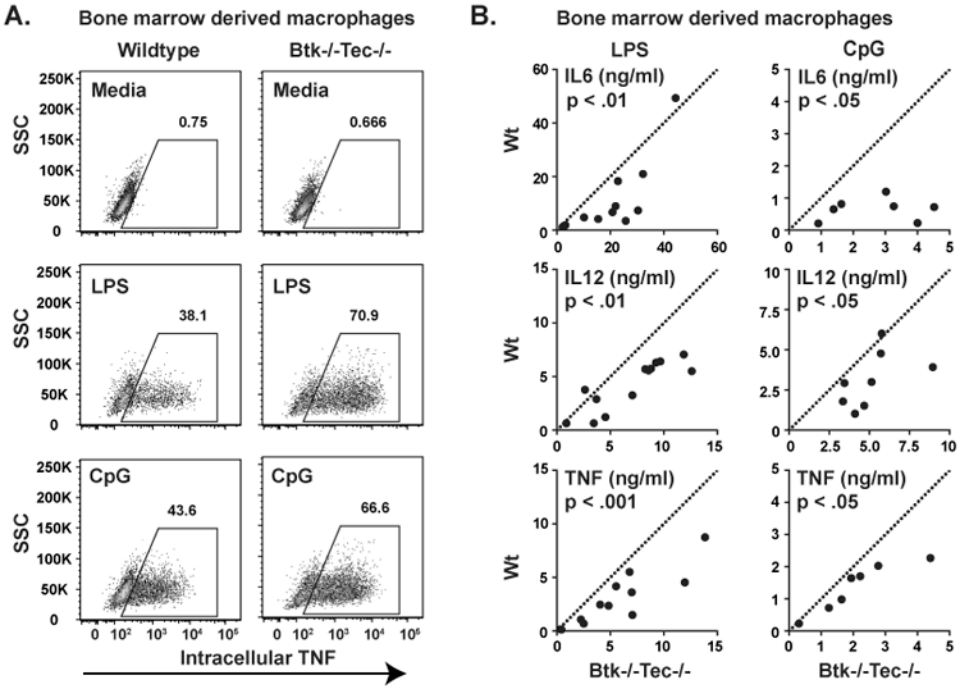

Figure 3. Increased TLR-induced cytokine secretion from Btk-/- and Btk-/-Tec-/- bone marrow-derived macrophages

(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 hours, 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. P-values were calculated using the non-parametric Wilcoxon matched pairs test.

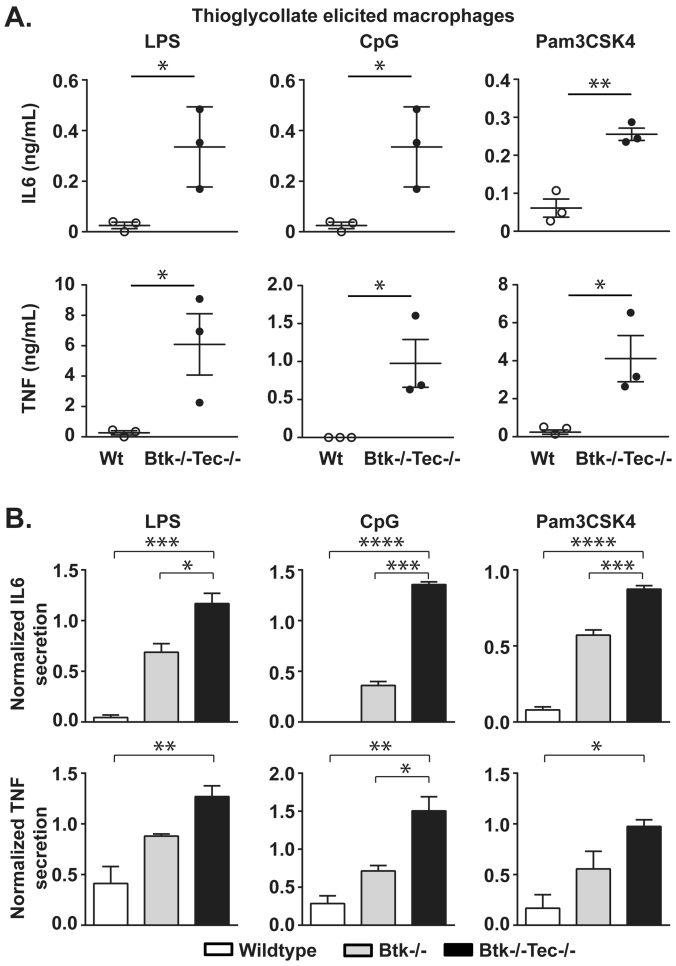

Figure 4. Increased TLR-induced cytokine secretion from Btk-/- and Btk-/-Tec-/- thioglycollate-elicited peritoneal macrophages

(A) Thioglycollate-elicited peritoneal macrophages were stimulated with the TLR agonists LPS (2ng/mL) and CpG (200nM) for 16 h. IL6 and TNF concentrations in the culture supernatant were determined by ELISA. Data show mean ± standard deviation of three independent experiments. P-values were calculated using unpaired student's t-test. (B) Combined ELISA data from 3 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 was then plotted (mean ± standard deviation). P-values were calculated using one-way analysis of variance (1-factor: genotype) followed by a multiple comparisons test using Bonferroni correction. (* p = 0.01 - 0.05, ** p = 0.001 - 0.01, *** p = .0001 - 0.001, **** p < .0001).

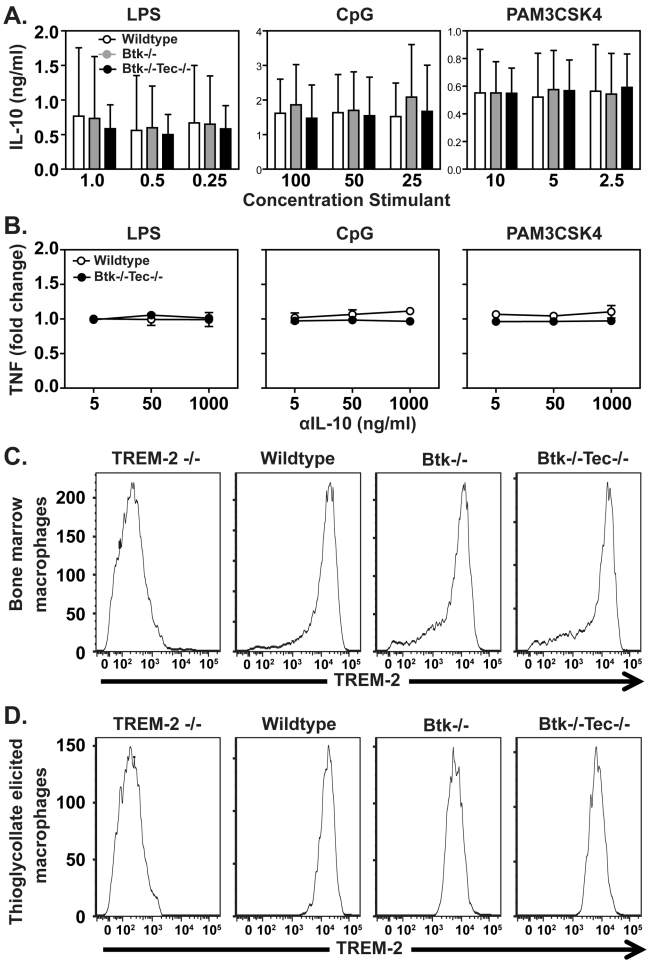

Figure 5. Role of IL-10 and Trem2 in Tec kinase-mediated inhibition of TLR-dependent cytokine secretion

(A) Bone marrow-derived macrophages 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 4 independent experiments and data are represented as mean ± SD. (B) BMMΦ were cultured with increasing doses of IL-10 neutralizing antibody for 30 minutes 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 hours, 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) Bone marrow-derived macrophages 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 intraperitoneally with sterile thioglycollate medium. After 4 days, 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.

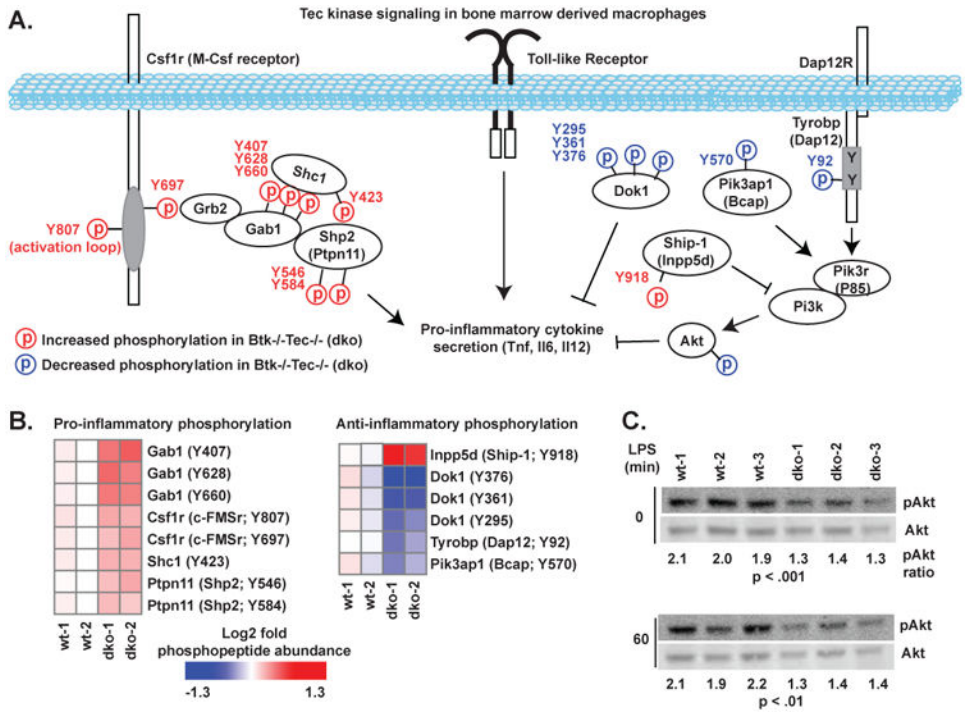

Figure 6. The effect of Tec kinase deficiency on bone marrow-derived macrophages

(A) Bone marrow-derived macrophages from biological replicates isolated from wild-type or Btk-/-Tec-/- mice were stimulated with LPS (1 ng/ml) for 20 minutes, and analyzed using quantitative phosphoproteomics. Here 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 signalling (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 pro-inflammatory signalling. (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 antibodies 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 was indicated below the blots. The p-values indicated on the panel were calculated using a Student's t-test.

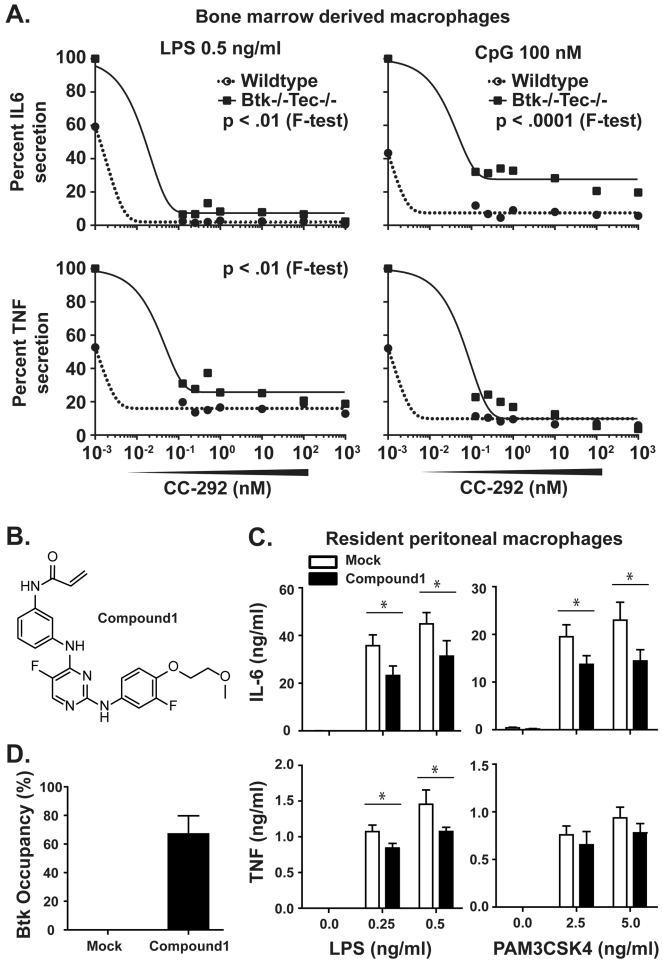

Figure 7. In vitro and in vivo effects of the Tec kinases inhibitor in TLR-stimulated macrophages

(A) Bone marrow-derived macrophages were pretreated for 30 min with the indicated concentrations of a specific inhibitor for Tec kinases (CC-292), or DMSO as vehicle control. After pre-treatment, indicated doses of TLR stimuli (LPS or CpG) were added directly to the cell culture media for 16 h. The concentration of IL6 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 non-linear regression to fit these dose response curves (one-phase decay), and used and 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, Compound1, or acidified water alone as a control for 40-90 hours. F4/80+ peritoneal macrophages were isolated from treated animals and stimulated with indicated doses of LPS or Pam3CSK4 for 16 hours and IL6 and TNFα concentrations in the culture supernatant were determined by ELISA. Data show mean ± standard deviation of 3 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 is normalized to water controls. (P-values were calculated using unpaired student's t-test (* $p < 0.05$).