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Bruton's tyrosine kinase together with PI 3-kinase are part of Toll-like receptor 2 multiprotein complex and mediate LTA induced Toll-like receptor 2 responses in macrophages

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Abstract

Lipoteichoic acid (LTA) of Gram-positive bacteria initiates innate immune responses via Toll-like receptor-2 (TLR2), resulting in the activation of intracellular signaling and production of inflammatory cytokines in macrophages. Although Bruton's tyrosine kinase (Btk) is biologically important molecule implicated in immune regulation and recently in TLR signaling its importance for LTA-TLR2 mediated responses has not been evaluated. In this study, we detected Btk in the LTA signaling complex with TLR2 and PI 3-kinase (PI3K). The constitutive interaction of these proteins was mediated via PI3K Src homology (SH3) -domain. Both Btk and PI3K were activated by LTA stimulation and the LTA induced cytokine expression was differentially modulated by these kinases. LTA induced the activation of nuclear factor κB (NFκB), however, only Btk inhibition affected the LTA induced Ser536 phosphorylation and DNA-binding of NFκB. In conclusion, our results demonstrate that Btk and PI3K occupy important roles in TLR2-induced activation of macrophages, resulting in selective regulation of cytokines.

Keywords: Bruton's tyrosine kinase; PI 3-kinase; Toll-like receptor-2; Lipoteichoic acid

1. Introduction

Toll-like receptors (TLRs) are principal innate immune signaling receptors that recognize conserved microbial structures. Each TLR has its own pattern of recognition repertoire that varies from single molecule to the restricted subset of microbial components [1]. TLR2 has been shown to be the main receptor in recognizing components of gram-positive bacteria such as lipoteichoic acid (LTA) and peptidoglycan [2], as well as yeast and mycoplasma [3], thus having the most wide-ranging spectrum of ligand recognition among all TLRs.

LTA, a cell wall component of gram-positive bacteria is an amphiphilic, negatively charged glycolipid that shares many inflammatory properties of LPS even though there have been controversial results of its immune potency. The reason for this discrepancy is most likely the decomposition and LPS contamination of commercial LTA preparations, that have been produced by hot phenol extraction method [4]. Recently, a more gentle method for extraction of LTA by butanol has been developed [5]. Butanol extraction preserves LTA in an active form and devoid of LPS contamination and has now been shown to be a biologically active component of *Staphylococcus aureus*.

Recognition of LTA by TLR2 leads to induction of innate immune response through activation of many protein kinases, transcription factors, inflammatory cytokines, and chemokines. After ligand binding, TLRs undergo conformational change resulting in recruitment of common and specific downstream signaling molecules. For signal transduction, TLR2 utilizes adapter proteins MyD88 and Mal [6] to activate IL-1 receptor associated kinase, TNF receptor associated factor 6, TNF receptor associated kinase, and IκB kinase. IκB kinase phosphorylates the NFκB binding protein, IκB, which is then targeted for ubiquitination and proteosomal degradation [7]. This process allows NFκB to

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translocate to the nucleus where it mediates transcriptional gene activation through interaction with κ-elements. In addition, phosphorylation of p65 subunit of the NFκB is required for transactivation of gene expression [8].

PI3Ks play important roles in many cellular responses like cell growth, survival and cytoskeletal remodeling. Class IA PI3Ks, that are especially important in immune signaling pathways, comprise of regulatory p85 and of catalytic p110 subunits. p85 subunit contains a SH3- and two SH2-domains that bind to proline rich regions and to phosphorylated tyrosine residues of various proteins, respectively [9]. Phosphotyrosine binding of PI3Ks releases the p110 activity and causes translocation from cytosol to the membranes where PI3K phosphorylates its lipid substrates. Phosphorylated PI3K lipid substrates act as second messengers to activate the downstream protein kinases such as protein kinase B (PKB, also known as Akt) by binding to their pleckstrin homology (PH) domains [10].

Btk is a member of the Tec family of cytoplasmic protein tyrosine kinases that participate in signaling pathways of hematopoetic cells [11]. Btk has mainly been studied as a part of adaptive immunity, however, recent studies suggest that Btk has important implications also for the innate immunity. The biological importance of Btk is underlined by the finding that naturally occurring mutations in the Btk PH-domain cause X-linked agammaglobulinemia (XLA) in humans and xid-phenotype in mice. Patients with XLA are more susceptible to bacterial infections, and macrophages from xid-mice produce significantly lower amounts of pro-inflammatory cytokines compared to their wild type controls. Interestingly, gene disruption of p85α subunit of PI3K results in phenotype similar to xid-mice [12].

Btk activity has been shown to increase following the stimulation of several receptors for immune regulation [13–15]. In addition, direct interaction of Btk with TLR receptors TLR4, 6, 8, and 9 and their adaptors has been reported [16]. Also, the role of Btk in transactivation of NFκB has been suggested [17,18]. We and others have previously shown that PI3K plays an important role in intracellular signaling pathways activated by TLR2 [19,20] and TLR4 [21]. There is also evidence that PI3K and Btk are functionally linked [22]. On this background, we have studied the role of Btk for innate immune receptor TLR2 and PI3K signaling in mouse macrophages. We demonstrate evidence that both Btk and PI3K have important roles in modulating TLR2-induced inflammatory responses in macrophages.

2. Materials and methods

2.1. Reagents

Mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was from Euroclone (West Yorks, UK). Penicillin, Streptomycin, L-glutamine, pyrrolidinedithiocarbamate (PDTC), Polymyxin B sulphate and Dulbecco's modified Eagle's medium were from Sigma (St. Louis, MO). Highly purified LTA was derived from *Staphylococcus aureus* [23]. The Btk and PI3K inhibitors LFM-A13 and LY294002 were from Calbiochem (Merck, Darmstat, Germany). The LIVE/DEAD Viability/Cytotoxicity kit used to measure viability of the RAW 264.7 cells after LTA, LFM-A13 or LY294002 incubations was from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-mouse TLR2 antibodies were obtained form R&D system inc. (Minneapolis, MN) and HyCult Biotechnology (Uden, Netherlands). Anti-Btk and NFκB specific anti-p65 and anti-p50 antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). PI3K and phosphotyrosine antibodies were purchased from BD Biosciences (San Diego, CA). Polyclonal anti-mouse Akt, phospho-Akt (Ser473) and phospho-NFκB p65 (Ser536) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-surfactant protein B antibody was from Chemicon International (Temcula, CA). The peroxidase conjugated affinity-purified secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Phosphatidylinositol (PI) was from Avanti Polar Lipids (Alabaster, AL). All radioactive labels were from Perkin Elmer (Boston, MA).

2.2. Protein analysis

The RAW 264.7 cells were grown to subconfluency and the stimulations with LTA were made in the presence or absence of Btk inhibitor LFM-A13 (100 μM) or PI3-kinase inhibitor LY294002 (50 μM) as described in the text. Treatments with inhibitors were started 1 h before LTA stimulations. The cells were lysed with modified radioimmune precipitation buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% DOC, 100 mM NaF, 0.5 mM Na_3VO_4, 5 mM EDTA, 10% glycerol, 0.1 U/μl aprotinin, 10 μg/ml leupeptin and 0.1 mM phenylmetylsulphonylfluoride. The lysates were centrifuged and the protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad Hercules, CA). Total protein samples (30 μg) were separated on SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (PVDF) (Millipore, MA). After blocking and washes, the membranes were incubated with indicated antibodies. The proteins were detected using the ECL-Plus Detection kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

For immunoprecipitations, 600 μg of total proteins were incubated with the certain antibodies and the antibody–antigen complexes were coupled to Gammabind–sepharose (Amersham Biosciences) at +4 °C for overnight. The immunoprecipitates were either separated by SDS-PAGE and further treated in the same way as in Western blotting gels or used for activity assays. Anti-surfactant protein B was used as an unspecific immunoprecipitation control.

2.3. GST fusion proteins

GST fusion proteins containing the SH2 or SH3 -domains of PI3K were expressed in *E. coli* and purified as described [21]. Briefly, 600 μg total protein lysates of untreated or LTA stimulated RAW 264.7 cells were incubated with 10 μg of GST alone or GST fusion proteins coupled with glutathione–sepharose beads (Amersham Biosciences) at +4 °C for overnight. Associated protein complexes were centrifuged and washed with protein lysis buffer. Bound proteins were separated by SDS-PAGE and analyzed by immunoblotting using TLR2, Btk and PI3K specific antibodies.

2.4. RNA isolation and Northern blotting

Total cellular RNAs from untreated or LTA stimulated RAW 264.7 cells were extracted using Tri-reagent (Sigma). RNAs were electrophoresed on 1% agarose/formaldehyde gel and transferred to nylon membrane (Millipore). The membranes were hybridized with probes of TLR2 cDNA and mouse TLR4 cDNA as described [24]. The membranes were stripped and re-hybridized with a ribosomal gene 28S RNA to ensure equal loading and even transfer. Intensities of the RNAs were measured by a PhosphorImager (BioRad) and Quantity one software (BioRad) was used for quantitative analysis.

2.5. Ribonuclease protection assay

Total cellular RNAs from untreated or LTA stimulated RAW 264.7 cells were isolated with Tri-reagent. The mRNA expression of cytokines and chemokines (INOS, RANTES, TNFα, IL-1α, IL-1β, MIP-2 and IL-6), and housekeeping gene L32 was measured by custom made Multi-Probe Ribonuclease Protection Assay, BD Biosciences (San Diego) according to manufacturer's instructions. Briefly, custom-made multi-probe set was hybridized in excess with RNA samples (5 μg of each). Free probe and other single-stranded RNAs were digested with RNAases. Phenol extracted and ethanol precipitated samples were suspended in loading buffer and samples were separated on a 5% denaturating polyacrylamide gel. The gel was vacuum dried and scanned with the PhosphorImager. The expression levels