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In vitro activation of murine peritoneal macrophages by recombinant YopJ: Production of nitric oxide, proinflammatory cytokines and chemokines

Ajit Sodhi*, Ashok Kumar Pandey

School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi 221005, India

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ABSTRACT

Recently it was reported that 3 μg/ml of recombinant YopJ induced apoptosis in murine peritoneal macrophages *in vitro*. However, in this study, we report the activation of murine peritoneal macrophages *in vitro* on treatment with sub-apoptotic dose of recombinant YopJ protein (1 μg/ml). The activation involves enhanced production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), IL-12, and IL-6. Production of NO and IL-6 was found to peak at 24 h of rYopJ treatment, whereas IL-12 and IFN-γ production peaked at 18 h of rYopJ treatment. Increased mRNAs expression of nitric oxide, IL-12, IL-6 and IFN-γ molecules, was also observed in rYopJ-treated macrophages by RT-PCR. rYopJ induced the enhanced activity of protein tyrosine kinases which was inhibited by pharmacological inhibitor genestein, wortmanin and H-7 suggesting the role of tyrosine kinases, PI3K and PKC in the above process. rYopJ also induced increased enhanced production chemokines MIP-1α, MCP-1, and RANTES in macrophages. Significantly, increased expression of TLR-2, TLR-6, MyD 88 and IRAK-1 was also observed by immunoblotting in rYopJ-treated macrophages. rYopJ induced production of NO, TNF-α and IL-6 was significantly inhibited in macrophages pretreated with pharmacological inhibitor wortmanin, genestein and H-7 demonstrating the probable involvement of protein tyrosine kinases in the above process.

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Introduction

Yersinia species has been responsible for the maximum number of death caused by any infectious disease in the history (Kelly, 2006). *Yersinia pestis* causes the bubonic plague whereas *Y. enterocolitica* and *Y. pseudotuberculosis* are responsible for the gastro diseases in humans (Viboud and Bliska, 2005). All the three Yersinia species harbor a 70 kb virulence plasmid that encodes Type III Secretion System (TTSS) and effector proteins (Hao et al., 2008). The virulence of *Yersinia pestis* is due to its ability to paralyze the host innate immune cells through its effector proteins also known as Yops (Yersinia outer proteins) (Cornelis and Wolf-Watz, 1997). The TTSS functions like a syringe, whereby it injects or translocates effector molecules from the bacterial cell into the infected host cell. The injected Yops perturb the dynamics of the cytoskeleton, disrupting phagocytosis, and blocking the production of proinflammatory cytokines, thus favoring the survival of the invading *Yersinia* (Buttner and Bonas, 2002; Cornelis, 2002). Among Yops, one of the effector proteins, YopJ, is a 32 kDa protein which inhibits the host immune response by preventing activation of the MAP Kinase and the NFκB pathway, preventing the production of cytokines and activation of the host immune response and antiapoptotic factors (Mills et al., 1997; Monack et al., 1997; Orth et al., 1999; Ruckdeschel et al., 1997; Schesser et al., 1998; Zhang et al., 2005). Recent studies suggested that instead of acting as proteases, YopJ acts as an acetyltransferase to prevent phosphorylation and subsequent activation by kinases (Mittal et al., 2006; Mukherjee et al., 2006). Macrophages on activation play key role in host defense by recognizing, engulfing, and killing microorganisms (Nau et al., 2002). The activation program is induced by different bacterial components that are Toll-like receptor agonists, including lipopolysaccharide, lipoteichoic acid, muramyl dipeptide, heat shock proteins and other functional proteins (Girardin et al., 2003; Akira and Takeda, 2004). Upon activation macrophages produces reactive oxygen species, nitrogen intermediates, proinflammatory cytokines and chemokines (Giacomini et al., 2001; Haidaris and Bonventre, 1982; Kelk et al., 2005; Murtaugh and Foss, 2002). Toll-like receptors (TLRs) are the principal signaling molecules through which mammals sense infection and activate the immune cells against them. Individual TLRs recognize distinct pathogen-associated molecular patterns (PAMPs) and direct the activation of immune cells of the host to confine and defeat an invasive organism before it becomes

Abbreviations: rYopJ, recombinant yersinia outer protein J; MAPK, mitogen-activated protein kinases; PTK, protein tyrosine kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinases; TNF-α, tumor necrosis factor-α; IFN, interferon; IL-12, interleukin-12; NO, nitric oxide; H-7, PKC inhibitor; PD98059, ERK1/2 inhibitor; SP600125, JNK inhibitor; Wortmannin, PI3K inhibitor; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; PMSF, phenyl methyl sulfonyl fluoride.

* Corresponding author. Tel.: +91 542 2307314/2368331; fax: +91 542 2368693.
E-mail address: ajit.sodhi@lycos.com (A. Sodhi).

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widespread (Akira, 2003; Akira and Sato, 2003; Beutler et al., 2003). They also play crucial role in dendritic cell (DCs) maturation and differentiation of T helper (Th) cells (Agrawal et al., 2003). The bacterial species with T3SS (including yersinia) circumvent the host defense by inhibiting the macrophages activation. This is achieved by the direct deployment of their effector proteins in to the host cytosol, thus preventing their exposure to the cell surface receptors primarily the TLRs (Hornef et al., 2002). In yersinia, YopJ is also such an effector molecule (Trosky et al., 2008). Recombinant proteins of yersinia, mycobacterium, chlamydia species, etc., have been shown to activate the macrophages (Gaddis et al., 2009; Ilangumaran et al., 1994; Maguire et al., 2005; Sodhi et al., 2004). We have recently reported that 3 μg/ml of rYopJ protein when provided extracellularly induced apoptotic cell death of murine peritoneal macrophages *in vitro* (Pandey and Sodhi, 2009). While studying the response of macrophages to the lower doses of rYopJ protein it was observed that at concentration of 1 μg/ml, rYopJ induced activation of macrophage *in vitro* instead of apoptosis. In the present studies we have investigated the mechanism and functional consequences of rYopJ induced macrophage activation, namely, the increased expression of TLRs, production of NO, proinflammatory cytokines and chemokines. Based on the data presented, it is proposed that sub-apoptotic dose (1 μg/ml) of rYopJ protein can act as a potential biological response modifier (BRM) activating macrophages *in vitro*.

Materials and methods

Mice

Inbred strains of Balb/c mice of either sex at 8–10 weeks of age were used for obtaining peritoneal macrophages.

Cell cultures and reagents

Macrophage were cultured in RPMI 1640 medium supplemented with heat-inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamycin (20 μg/ml) at 37 °C in humidified air containing 5% CO_2. Medium RPMI 1640, TRI-reagent, MTT, L-NMMA, L-arginine, proteinase K, polymixin B, PTK Assay Kit, lipopolysaccharides (LPS), wortmanin, genestein and most of the other reagents were obtained from Sigma–Aldrich Chemicals, St Louis, MO, USA. Fetal calf serum was purchased from Biological Industries, Haemek, Israel. PD98059, SB202190, SP600125, and H-7 were purchased from Calbiochem, La Jolla, CA, USA. Polyclonal antibodies against iNOS, TLR-2, TLR-6, MyD88, IRAK-1, actin, HRP-conjugated anti-rabbit and anti-goat IgGs were obtained from Santa Cruz Biotechnology Inc., California, USA. TNF-α, IL-12, IFN-γ and IL-6 ELISA kits were purchased from BD Pharmingen, San Diego, CA, USA. RANTES, MIP1-α and MCP-1 ELISA kits were purchased from R&D System, Inc., Minneapolis, MN, USA. One-step RT-PCR kit was from Qiagen, Hilden, Germany. Mouse RT-PCR primers for iNOS, cytokines, chemokines, TLRs and GAPDH were purchased from Eurofins MWG Operon, Ebersberg, Germany. Purified, recombinant YopJ protein (32.5 kDa) of *Y. pestis* was provided by Dr. H.V. Batra, Head, Division of Microbiology, DRDE, Gwalior, M.P., India. The protein has been cloned and over expressed in *Escherichia coli* by induction to IPTG. Recombinant protein was purified using nickel–NTA column chromatography and was observed as single band on SDS-PAGE (Khushiramani et al., 2006; Pandey and Sodhi, 2009). All the reagents were endotoxin-free as determined by the Limulus amoebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

Isolation and activation of macrophages

Peritoneal exudates cells were harvested from peritoneal cavity using chilled serum-free RPMI 1640 medium and added to wells of 24-well tissue culture plates (Nunc, Roskilde, Denmark). After 2 h incubation at 37 °C in an atmosphere of 5% CO_2 in air in a CO_2 incubator, the non-adherent cells were removed by vigorous washing with warm serum-free medium, and the adherent cells were incubated in complete medium overnight to form macrophage monolayers. Macrophage monolayers were incubated in fresh medium or the same volume of medium containing 10 μg/ml of LPS or different concentrations of rYopJ for various time intervals as indicated in the 'Results' section.

In another set the macrophage cultures were first incubated with PI3-K inhibitor, wortmannin (200 nM); JNK MAPK inhibitor, SP600125 (10 μM); protein tyrosine kinase inhibitor, genistein (10 μg/ml) and protein kinase C inhibitor H-7 (10 μM) for 1 h. Thereafter, medium was removed, macrophages washed with warm medium and further incubated in fresh medium with 1 μg/ml rYopJ for different time periods as indicated in the 'Results' section. For MAPK studies, the macrophage monolayers were incubated with 1 μg/ml of rYopJ for 15 min, for transcription factors up to 60 min. For MAPK experiments, macrophage monolayers were serum starved for 6 h before treatment.

Preparation of cell lysates and immunoblotting

The macrophage monolayers were washed with ice cold phosphate buffered saline containing 1 mM Na_3VO_4, then lysed in 50 μl of lysis buffer [20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin and 0.15 units/ml aprotinin] for 20 min at 4 °C. The lysates were centrifuged at 13000 × *g* for 15 min and the supernatants (containing Triton X-100 soluble proteins) were separated on 10% SDS-polyacrylamide gels at 20 mA. The separated proteins were transferred to nitrocellulose membrane (45 min at 200 mA) and immunoblotted with primary antibody, incubated with secondary antibody conjugated with horseradish peroxidase and visualized by the Chemiluminescence Western Blotting Kit (Santa Cruz Biotechnology, California, USA) on X-ray film. To monitor equal loading of protein, Western blotting using antibody against actin was done for each experiment.

PTK assay

PTK activity was measured using Protein Tyrosine Kinase Assay Kit (PTK-101) from Sigma–Aldrich Chemicals. Briefly, cell lysate was prepared using lysis buffer containing activated sodium vanadate solution (according to the kit instruction). Assay was performed in a 96-well microtiter plate. 125 μl of PTK substrate solution was added to each well and plate was incubated overnight at 37 °C. Coating solution was removed and each well was washed with 200 μl of washing buffer. Buffer was removed and wells were dried for 2 h at 37 °C. 90 μl of 13 tyrosine kinase buffer containing ATP was added to each well. 20 μl of cell lysates was added in each well. Plate was covered and incubated at room temperature for 30 min. Reaction mixture was removed and each well was washed with 200 μl of washing buffer 5 times. In each well, 100 μl conjugated antibody was added. Plate was covered and incubated at room temperature for 30 min. Antibody solution was removed and each well was washed with 200 μl of washing buffer 5 times. 100 μl of freshly prepared OPD substrate solution was added to each well and incubated for 7 min in dark at room temperature. 100 μl of 2.5 NH_2SO_4 was added to each well to stop the reaction. Plate was read in a microplate ELISA reader (Emax, Molecular

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