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Involvement of TLR6/1 in rLcrV-mediated immunomodulation of murine peritoneal macrophages in vitro

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Abstract

LcrV of *Yersinia pestis* is an enigmatic antigenic protein having multiple functions such as effector, translocator and regulator in Type III secretion system. In present study, it is reported that rLcrV causes subversion of macrophage-mediated immune functions. rLcrV treatment down regulated the transcription of IL-12, IRAK-1, MHC-II, phosho-STAT1 and adhesion molecule CD18 in LPS stimulated macrophages. rLcrV induced up regulation of phospho-STAT3 expression, while had no effect on expression of phospho-STAT6. Neutralization and immunoprecipitation experiments suggest the probable involvement of TLR2 and TLR6 heterodimer in rLcrV-mediated immunomodulation of macrophages. Adaptor molecule MyD88, CD11b, and MHC-I expression did not modulate upon treatment with rLcrV.

Keywords: Macrophages; rLcrV; IL-12; MHC; LFA-1; STATs; Toll-like receptors

1. Introduction

The interplay between pathogen-encoded virulence factors and host cell signaling networks is critical for both the establishment and clearance of microbial infections. LcrV is a secreted protein encoded in the lcrGVHYopBD operon located on pYV (Cornelis et al., 1998; Nilles et al., 1997) and, thus, belongs to the common virulence-associated antigen of the three *Yersinia* species. *Yersinia pestis*, the etiological agent of bubonic and pneumonic plague; the enteropathogenic species *Yersinia psudotuberculosis* and *Yersinia enterocolitica*. *Y. psudotuberculosis* and *enterocolitica* cause adenitis, septicemia and gastrointestinal syndromes. All pathogenic *yersinia* employ strategies to disarm the macrophages and to disrupt their response to infection (Beuscher et al., 1995; Boland and Cornelis, 1998). LcrV is a key player in the pathogenecity mechanism by which *yersinia* exploits host Pattern Recognition Receptors for subverting the host immune system (Sing et al., 2002a, 2002b).

LcrV a multifunctional protein with strong immunomodulatory effects is associated with full virulence of *Y. pestis* (Brubaker, 1991; Sing et al., 2003). Translocation of Yersinia outer proteins (Yops) into eukaryotic cells requires LcrV (Fields et al., 1993; Nilles et al., 1998). Previous reports indicate that LcrV, a 327-residue soluble protein is exposed at the bacterial surface prior to contact with host cells (Straley, 1988). Within *Y. pestis*, LcrV is required for induction of the LCR (Nilles et al., 1997, 1998). The observation that LcrV-specific antibodies confer resistance to experimental plague (Brown and Cooper, 1996; Une and Brubaker, 1987) prompted the hypothesis that LcrV also promotes virulence outside the bacteria, and it has been proposed that LcrV acts directly to compromise innate host immune responses (Sing et al., 2002a, 2002b). Recently, LcrV has been shown to form pores in eukaryotic membranes (Holmstrom et al., 2001). This complex array of functions has made the elucidation of LcrV's mechanisms of action difficult during infection.

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The profound suppression of proinflammatory cytokine during *Y. pestis* infection was first attributed to LcrV (Nakajima et al., 1995). It was, further, reported that LcrV inhibits zymosan-induced production of tumor necrosis factor (TNF)-α by inducing IL-10 (Sing et al., 2002a, 2002b). LPS is known to mediate its response through TLR4 and CD14, and it has been suggested that LcrV also uses CD14 (Sing et al., 2002a, 2002b). The role of LcrV and other Yops in inhibition of innate immunity has been also reported (Brubaker, 2003). More recently, Sing et al. (2003) have suggested a crucial role of LcrV in evasion of *Y. enterocolitica*. rLcrV-mediated inhibition of TNF-α and enhanced expression of IL-10 by macrophages, and involvement of TLR2 in this immunomodulation has been demonstrated by us (Sharma et al., 2004). It is also reported that rLcrV significantly inhibited Mitogen activated protein kinases (MAPKs) (Sodhi et al., 2004). However, the possible role of STATs, adhesion molecules, MHCs, TLRs and its down stream adaptor signaling molecules MyD88 and IRAK-1 in rLcrV-mediated immunosuppession of macrophages is not known.

Cells of the monocyte/macrophage lineage play an important role in the host's defense against various microbial infections and tumors either by direct cytotoxic activity and/or by their ability to regulate the activity of other cells in the immune system and antigen presentation (Adams and Hamilton, 1984; Adams and Hamilton, 1992; Germain and Margulies, 1993). Macrophage-mediated regulation of immune response is manifested by a variety of mechanisms involving secretion of bioactive molecules like nitric oxide (NO), tumor necrosis factor (TNF) by activated macrophages (Unanue and Allen, 1987). Toll-like receptors have a crucial role in the detection of microbial infection in mammals and insects (Medzhitov, 2001). We have demonstrated the involvement of TLR2 and IL-10 in rLcrV-mediated inhibition of proinflammatory cytokines in macrophages (Sharma et al., 2004). TLR2 works as heterodimeric complex either with TLR1 or TLR6 (Medzhitov, 2001). The data presented in this paper support the involvement of TLR2/TLR6 heterodimeric complex but not TLR2/TLR1 complex signaling pathway in rLcrV-mediated immunomodulation of murine peritoneal macrophages in vitro.

2. Materials and methods

2.1. Mice

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

2.2. Cell cultures and reagents

L929 (murine fibroblast cell line) and macrophage cultures were maintained 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, LPS and most of the other reagents were purchased from Sigma Chemicals USA. Fetal calf serum was purchased from Biological Industries (Israel). HRP-conjugated anti-rabbit and anti-goat IgG were from Santa Cruz Biotechnologies, CA, USA. Anti-TNF-α, anti-TLR1, anti-TLR2 and anti-TLR6, IRAK1, CD11b, CD18, phospho-STAT1, phospho-STAT3 and phospho-STAT6 antibodies were obtained from Santa Cruz Biotechnology, CA, USA. Anti MyD88 antibody was from Chemicon International, USA. Anti-MHC-I and MHC-II antibodies were from Becton Dickinson Immunocytmetry System, CA, USA and Pharmingen Ltd., USA, respectively. TRIzol reagent and one-step RT-PCR kit were bought from Life Technologies Inc., GibcoBRL, USA and Qiagen, Germany. Mouse primers for IL-12, and GAPDH were purchased from GENSET Singapore Biotech. Pte. Ltd. (Singapore). Purified recombinant proteins of Yersinia rLcrV (M.wt. = 31 kDa) were obtained from Dr. H.V. Batra, Division of Microbiology, DRDE, Gwalior. The proteins have been cloned and over expressed in *E. coli* by induction with IPTG. The proteins have been purified using nickel-NTA column chromatography and identified by single band on SDS-PAGE. All the reagents were endotoxin-free as determined by the Limulus amoebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

2.3. Isolation and activation of macrophages

Macrophage monolayers were prepared as described previously (Sodhi et al., 1992). Peritoneal exudates cells were harvested using chilled serum-free RPMI 1640 medium and added to wells of 24-well tissue culture plates (Nunc, 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 (three times) with warm serum-free medium and the adherent cells were incubated in complete medium overnight to form macrophage monolayers. More than 95% of the adherent cell population was macrophages as determined by morphology and non-specific esterase staining.

After preparation and overnight culture, the macrophage monolayers were preincubated in fresh medium containing 10 μg/ml of rLcrV for 2 h. Thereafter, macrophage monolayers were washed and treated with 10 μg/ml of LPS for various time intervals as indicated in results. The supernatants were collected for ELISA assay. The macrophage monolayers were used for MTT assay to ascertain cell viability. The macrophage monolayers were lysed and the cell lysates were used for immunoblotting or used for RNA extraction and RT-PCR.

2.4. Estimation of TNF-α and IL-12 production by ELISA

Murine TNF-α and IL-12 levels were measured by a commercial ELISA kits for TNF-α and IL-12 (BD Pharmingen) according to manufacturers recommendations.