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Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways ☆

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Abstract

Lactoferrin (LF) is a component of innate immunity and is known to interact with accessory molecules involved in the TLR4 pathway, including CD14 and LPS binding protein, suggesting that LF may activate components of the TLR4 pathway. In the present study, we have asked whether bovine LF (bLF)-induced macrophage activation is TLR4-dependent. Both bLF and LPS stimulated IL-6 production and CD40 expression in RAW 264.7 macrophages and in BALB/cJ peritoneal exudate macrophages. However, in macrophages from congenic TLR4$^{-/-}$ C.C3-*Tlr4*$^{lps\text{-}d}$ mice, CD40 was not expressed while IL-6 secretion was increased relative to wild-type cells. The signaling components NF-κB, p38, ERK and JNK were activated in RAW 264.7 cells and BALB/cJ macrophages after bLF or LPS stimulation, demonstrating that the TLR4-dependent bLF activation pathway utilizes signaling components common to LPS activation. In TLR4 deficient macrophages, bLF-induced activation of NF-κB, p38, ERK and JNK whereas LPS-induced cell signaling was absent. We conclude from these studies that bLF induces limited and defined macrophage activation and cell signaling events via TLR4-dependent and -independent mechanisms. bLF-induced CD40 expression was TLR4-dependent whereas bLF-induced IL-6 secretion was TLR4-independent, indicating potentially separate pathways for bLF mediated macrophage activation events in innate immunity.
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1. Introduction

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein that is a component of the innate immune system and found in many body fluids and secretions [1–7]. LF exhibits diverse biological activities ranging from activation of innate immunity [8,9] to direct microbicidal [10] and anti-cancer cell [11] effects. Although the mechanistic bases for these differential effects are not entirely clear, LF has been reported to bind to membranes of platelets [12], monocytes/macrophages [13], eosinophils [14], and all major lymphocyte subsets including α/β T cells (CD4 and CD8), γ/δ T cells, B cells and Natural Killer (NK) cells [15]. Exposure of these cells to LF modulates subsequent cellular functions such as cytokine gene activation [16], cytotoxicity [17], and B [18] and T [19] cell maturation, and a number of immunopotentiating activities have been ascribed to LF [20–33]. Further, direct receptor mediated interactions have been demonstrated via LF exposure to bacteria [34], parasites [35], and viruses [36], generally resulting in microbicidal effects. Thus LF may play a broad but key role in resistance to many types of disease.

The role of LF in activating cells of the innate immune system is of considerable interest. Toll-like receptor (TLR) pathways are major regulators of the innate immune response [37]. Perhaps the most widely studied TLR interaction involves bacterial lipopolysaccharide (LPS) through the TLR4 complex in which LPS initiates gene activation via distinct signaling pathways [38,39]. Co-expression of

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glycosylphosphatidylinositol-anchored proteins MD-2, CD14 [40] and CD11b/CD18 (Mac-1, CR3) [41] with TLR4 are essential to the functional integrity of the LPS receptor complex. In addition, LPS binding protein (LBP) mediates the interaction of LPS with CD14 [42] and CD11b/CD18 [43]. This event induces activation of the toll/IL-1 receptor (TIR) domains associated with either Toll/IL-1 receptor domain-containing adapter protein (TIRAP) and myeloid differentiation marker 88 (MyD88), or TIR domain-containing adapter inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), which operates independently via interferon regulatory factor-3 (IRF-3) [44,45]. Because toll receptors have no intrinsic kinase activity, IL-1 receptor associated kinase (IRAK) is recruited by an adaptor protein called Toll-interacting protein (Tollip). As a serine/threonine kinase, IRAK interacts directly with TIRAP (IRAK2) or with MyD88 death domains (IRAK1) [46]. Phosphorylation of tumor necrosis factor associated factor 6 (TRAF6) induces activation of a mitogen-activated protein kinase kinase kinase (MAP3K). MAP kinase kinase kinase 1 (MEKK1) and TGFβ-activated kinase 1 (TAK1) are common forms of MAP3K. Subsequent activation of IkB kinase (IKK) via MEKK1 or TAK1 encourages IKK degradation of IκB-α for NF-κB release and translocation into the nucleus [37,47]. MEKK1 also induces transcription via MAP kinase kinase 4 (MKK4) activation of c-Jun NH2-terminal protein kinase (JNK), MKK6 activation of p38, and mitogen-activated protein kinase kinase 1/extracellular signal-regulated protein kinase kinase (MEK1) activation of extracellular signal-regulated protein kinases (ERKs) [48]. Subsequent gene transcription includes inflammatory cytokines such as TNF-α, IL-6 and IL-12 which may be regulated by an adenylate/uridylate-rich element (ARE) [49–51].

In contrast to the more well known TLR activation pathways, LF induced cell signaling mechanisms are unknown. However, human LF (hLF) binds to *Escherichia coli* LPS [52], thereby affecting LPS-induced cytokine [53,54] and reactive oxygen species [55] production. hLF also binds to soluble CD14 and the CD14/LPS complex, reducing LPS-induced adhesion molecule expression, *E*-selectin and ICAM-1 [56]. In addition, hLF competes with LPS binding protein (LBP) [57] and up-regulates CD11b/CD18 [18], affirming an association with toll pathways. Therefore, the aim of the present work was to determine whether LF influences the host innate immune responses by interacting with the TLR4 receptor complex.

Purified bovine LF (bLF) was used in all experiments, and LPS was re-purified (pLPS) from commercial stocks via phenol extraction to remove contaminants known to activate other receptors, such as TLR2 [58–60]. Our studies showed that bLF and pLPS stimulated limited cytokine production and CD40 expression in RAW 264.7 macrophages. In addition, bLF and pLPS stimulated CD40 expression in BALB/cJ-*wt* but not in C.C3-*Tlr4*$^{lps\text{-}d}$ or C3H/HeJ peritoneal exudate macrophages. TNF-α secretion was not induced by bLF in any of the peritoneal exudate macrophage populations tested. pLPS-induced production of IL-6 was limited to wild-type cells whereas bLF-induced secretion occurred in both wild-type and TLR4 deficient cells. Thus, bLF-induced CD40 expression via a TLR4-dependent pathway while IL-6 production was mediated via a TLR4-independent pathway. bLF-induced BALB/cJ macrophage expression of CD40 and production of IL-6 were mediated by p38, NF-κB, ERK and JNK, signaling components common to LPS activation. In the absence of TLR4, activation of p38, NF-κB, ERK and JNK was enhanced, resulting in increased IL-6 production and enhanced cell signal activation in the absence of CD40 expression.

2. Materials and methods

2.1. Animals

Male and female C.C3-*Tlr4*$^{lps\text{-}d}$ (TLR4$^{-/-}$ congenic BALB/c), BALB/cJ-*wt* (wild-type) and C3H/HeJ mice (donor of mutant TLR4 allele for the congenic C.C3-*Tlr4*$^{lps\text{-}d}$ mice) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in the University of Wisconsin Food Research Institute animal care facilities. Animal care and housing were according to NIH and AAALAC guidelines.

2.2. Reagents

Highly purified bovine lactoferrin (bLF) was kindly provided by Morinaga Milk Industry Co., Ltd., (Tokyo, Japan). *E. coli* LPS 055:B5 was purchased from CALBIOCHEM (La Jolla, CA, USA). 5D4, F4/80 and M5/114 antibodies were kindly provided by Dr. Donna Paulnock, University of Wisconsin. IL-6, IL-12, IL-18 and TNF-α ELISA kits, tetramethylbenzidine (TMB) substrate reagent set, PE hamster IgG, group 2, κ, PE rat IgG2a, κ and anti-mouse CD40, CD80 and CD86 antibodies were purchased from BD Biosciences PharMingen (San Diego, CA, USA). Star73 anti-rat IgG was purchased from Serotec (Kidlington, Oxford, UK). Fetal bovine serum (FBS), Sodium dodecyl sulfate (SDS) and gentamicin were purchased from Life Technologies Inc. (Grand Island, NY, USA). Buffer saturated phenol was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). SYPRO Ruby protein gel stain, Colloidal Gold Total Protein Stain and goat anti-rabbit-HRP secondary antibodies were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Anti-p38 and anti-IκB-α antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-ACTIVE MAPK (ERK1/2), p38 and JNK were purchased from Promega (Madison, WI, USA). Anti-actin antibody, ethidium bromide monoazide, paraformaldehyde, thioglycollate, RPMI 1640 medium, sodium bicarbonate, triethylamine (TEA), sodium deoxycholate (DOC), serum-free and protein-free hybridoma medium were all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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