Toll-like receptors stimulate regulated intramembrane proteolysis of the CSF-1 receptor through Erk activation

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Abstract The CSF-1 receptor is a protein-tyrosine kinase that regulates the renewal, differentiation and activation of monocytes and macrophages. We have recently shown that the CSF-1 receptor undergoes regulated intramembrane proteolysis, or RIPping. Here, we report that RIPping can be observed in response to pathogen-associated molecules, which act through Toll-like receptors (TLRs). TLR-induced CSF-1 receptor RIPping is largely independent of protein kinase C, while maximal RIPping depends on Erk activation. Our studies show that CSF-1 receptor RIPping can be activated by various intracellular signal transduction pathways and that RIPping is likely to play an important role during macrophage activation.

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1. Introduction

CSF-1 regulates the proliferation and survival of cells of the monocyte/macrophage lineage, their differentiation into macrophages, and macrophage activation [1,2]. The receptor for CSF-1 is a ligand activated protein-tyrosine kinase that utilizes autophosphorylation sites to regulate kinase activity and to control interactions with signaling proteins [1,2]. We previously reported that the CSF-1 receptor also undergoes regulated intramembrane proteolysis, releasing the intracellular domain from the plasma membrane, enabling it to move to other compartments within the cell to participate in signaling [3].

Regulated intramembrane proteolysis, or RIPping, is a conserved process that consists of two consecutive proteolytic cleavage events [4]. Integral membrane proteins are cleaved within the extracellular region close to the plasma membrane, resulting in ectodomain shedding, followed by cleavage within the transmembrane region, yielding a soluble intracellular domain (ICD). Several metalloproteases, including TACE, have been implicated in the first cleavage [5]. Intramembrane cleavage is often carried out by γ-secretase [6]. In the case of Notch, the ICD translocates to the nucleus where it alters gene expression [6,7]. Thus far, two protein-tyrosine kinases, ErbB4 and the CSF-1 receptor, have been shown to undergo RIPping [3,8]. RIPping is essential for the biological activity of Notch and it may provide an alternate and still largely unexplored signaling mechanism for receptor protein-tyrosine kinases.

Toll-like receptors (TLR), which are expressed on macrophages, recognize various pathogen-associated molecules, including lipopolysaccharide, bacterial lipoproteins, double stranded RNA, and bacterial DNA [9–11]. Upon ligand binding, TLRs initiate signaling pathways leading to the phosphorylation of a variety of proteins, including the transcription factors NF-κB, IRF3, and IRF5, culminating in increased production of inflammatory cytokines. Thus, TLRs recognize molecular patterns that are associated with pathogenic intruders and that function as essential activators of the innate immune response [12].

We previously found that the CSF-1 receptor undergoes RIPping induced by CSF-1, TPA, and LPS [3,13]. Here we report that ligands for other TLRs also induce CSF-1 receptor RIPping. RIPping in response to TLR activation is largely independent of protein kinase C (PKC) or oxygen radical production. Inhibition of MEK1/2 reduces CSF-1 receptor RIPping, suggesting that stimulation of CSF-1 receptor RIPping by TLRs involves Erk activation.

2. Materials and methods

2.1. Reagents

Recombinant human CSF-1 was obtained from R&D Systems. A polyclonal antiserum was raised against the kinase insert domain of the murine CSF-1 receptor [14]. Lipopolysaccharide (Escherichia coli O111:B4), lipid A (Salmonella minnesota Re 595), and E. coli O102 were from EMD Biosciences (San Diego, CA). Lipoteichoic acid (Staphylococcus aureus), and polyinosinic-polycytidylic acid (pI:pC) were obtained from Sigma (St. Louis, MO). DNA was isolated from E. coli DH5α using the Easy-DNA kit according to the manufacturer’s directions (Invitrogen, Carlsbad, CA). Phospho-specific antibody directed against Erk1/2 (Thr202/Tyr204) was obtained from Cell Signaling (Danvers, MA). An antibody directed against Erk1/2 was obtained from Promega (Madison, WI).

2.2. Cell culture and treatments

P388D1 mouse macrophages were grown, stimulated, and lysed as described previously [3]. Cells were seeded at 2–3 × 10⁶ cells per 10 cm dish two days prior to stimulation.

2.3. Immunoprecipitation and Western analysis

Cleared lysates were incubated with a polyclonal anti-CSF-1 receptor serum and proteins were collected using Protein A-sepharose. Samples were fractionated by SDS-PAGE and analyzed by anti-CSF-1 receptor immunoblotting as described previously [3].
3. Results

3.1. TLR agonists induce CSF-1 receptor RIPping

LPS is a potent and physiologically relevant activator of macrophages. Our results confirm previous observations and show that LPS induces CSF-1 receptor RIPping in a concentration and time dependent fashion (Fig. 1). Proteins of 150, 130, and 55 kDa are seen on these blots. The 150 kDa protein represents the mature receptor, present on the cell surface; the 130 kDa protein corresponds to a precursor protein, present in the endoplasmic reticulum or Golgi apparatus; the 55 kDa protein is produced by RIPping and represents the CSF-1 receptor ICD. To test whether CSF-1 receptor RIPping is an integral part of macrophage activation, P388D1 cells were activated with lipid A, lipoteichoic acid, polyI:polyC, or bacterial DNA, agonists of TLR4, TLR2, TLR3, and TLR9, respectively [11]. Mammalian DNA was included as a negative control. Our results show that all TLR agonists that were tested strongly induce CSF-1 receptor RIPping (Fig. 2).

3.2. TACE inhibitors block TLR-induced CSF-1 receptor RIPping

The metalloprotease, TACE, is important for TNFα release in response to LPS [15,16]. Here we have shown that pretreatment of P388D1 cells with the TACE inhibitor, TAPI-0, abrogated both receptor downregulation and the appearance of the ICD in response to LPS or bacterial DNA (Fig. 3). Thus, TACE, or a related protease, is essential for CSF-1 receptor RIPping downstream of both TLR4 and TLR9.

3.3. LPS-induced CSF-1 receptor RIPping is independent of PKC

Activation of PKC leads to increased TACE activity [17]. To determine whether PKC plays a role in LPS-induced RIPping, P388D1 cells were treated for 24 h with TPA to downregulate PKC, and then stimulated with LPS. Both the disappearance of mature receptors and the generation of the ICD were unaffected by prolonged pretreatment with TPA (Fig. 4). As expected, downregulation of PKC abrogated subsequent...

Fig. 1. LPS induces CSF-1 receptor RIPping. P388D1 cells were stimulated with increasing amounts of LPS for 20 min (A) or with 100 ng/ml LPS for varying lengths of time (B) and CSF-1 receptor RIPping was analyzed by CSF-1 receptor immunoprecipitation followed by anti-CSF-1 receptor blotting.

Fig. 2. CSF-1 receptor RIPping in response to TLR activation. P388D1 cells were treated for the indicated amounts of time with 100 ng/ml lipid A (A), 10 μg/ml lipoteichoic acid (B), 25 μg/ml polyI:polyC (C), or 10 μg/ml bacterial DNA (D) and analyzed for CSF-1 receptor RIPping. P388D1 cells were treated for 20 min with increasing amounts of bacterial DNA or 10 μg/ml human DNA and analyzed for CSF-1 receptor RIPping (E).
TPA-induced CSF-1 receptor RIPping. These observations suggest that TLR-induced RIPping is independent of PKC.

3.4. MAP kinase dependent and independent pathways are important for LPS-induced CSF-1 receptor RIPping

We have confirmed that LPS activates several protein kinases in P388D1 cells, including IKK, p38 MAPK, JNK, and Erk1/2 (data not shown). Pharmacological inhibitors were used to assess the role of these kinases in LPS-induced CSF-1 receptor RIPping by pretreating P388D1 cells for 1 hour prior to stimulation. With the exception of the MEK1/2 inhibitor, U0126, none of the compounds tested had a significant effect on LPS-induced CSF-1 receptor RIPping (data not shown). In U0126-treated P388D1 cells there was a dose-dependent decrease in LPS-induced CSF-1 receptor RIPping which correlated with a dose-dependent inhibition of Erk1/2 activation (Fig. 5). Thus, Erk activation is one of several pathways that connect TLRs to CSF-1 receptor RIPping.

4. Discussion

Macrophages are found in most tissues of the body. They are indispensable for our defense against foreign intruders and for tissue development. Macrophages engage in phagocytosis, the production of cytokines, and communicate with the adaptive immune system [18,19]. Macrophages have been implicated in various maladies, including autoimmunity, cancer, atherosclerosis, and obesity [19–21]. CSF-1 regulates the renewal and survival of macrophages, recruits macrophages to specific sites within the body, and helps regulate macrophage activation [1,2].

CSF-1 binds to a receptor protein-tyrosine kinase and utilizes autophosphorylation to initiate signal transduction [1,2]. In this classical paradigm, receptor autophosphorylation sites function as docking sites for cellular signaling proteins that are activated or inactivated as a consequence of their interaction with the receptor. To terminate signaling, receptors are internalized and degraded in the lysosomes [22]. Recently it has become clear that the CSF-1 receptor is also subject to RIPping, which results in ectodomain shedding and release of the ICD into the interior of the cell [3]. In other systems studied, the ICD moves to the nucleus where it interacts with various transcription factors to alter gene transcription [4,6]. Thus, while RIPping contributes to the removal of CSF-1 receptors from the cell surface, it also generates a soluble ICD capable of regulating gene expression (Fig. 6). The removal of receptors from the cell surface is referred to as downregulation. We now know that CSF-1 receptor
downregulation can be mediated either by internalization followed by lysosomal degradation, or by RIPping. The first process acts exclusively to terminate signaling. In contrast, RIPping results in the generation of a soluble ICD that is likely to function as a signaling intermediate.

Macrophage activation by LPS is mediated by TLR4, one of a family of cell surface receptors that recognize pathogen-associated molecular patterns [9–11]. It has become clear that TLRs are essential for activation of the immune response against microbial pathogens [23]. Previous work has shown that treatment of macrophages with LPS leads to the downregulation of the CSF-1 receptor from the cell surface [16,24]. Our work confirms and extends these observations, showing that the LPS-induced disappearance of the mature CSF-1 receptors is mediated by RIPping (Fig. 3). Moreover, we have shown that stimulation of other TLRs, including TLR2, TLR3, and TLR9, also induces CSF-1 receptor RIPping (Fig. 2). These observations reveal that physiologically relevant activators of TLRs stimulate CSF-1 receptor RIPping, resulting in the production of a soluble ICD. This is consistent with a model in which the CSF-1 receptor ICD functions in the signaling network that triggers the innate immune response upon detection of pathogenic microorganisms.

What pathways do macrophages use to turn on RIPping? We have shown previously that PKC activation can induce CSF-1 receptor RIPping (Fig. 6). However, our current study shows that PKC downregulation does not affect LPS-induced RIPping. There is also evidence linking the production of oxygen radicals during macrophage activation to TACE activation [25]. While we found that hydrogen peroxide can induce RIPping, our experiments show no effect of free radical scavengers on LPS-induced CSF-1 receptor RIPping (data not shown). We have used pharmacological inhibitors to show that activation of Erk1 and Erk2 downstream of TLR4 plays a role in the activation of RIPping (Fig. 5). This is consistent with the observation that TACE can bind to and is a substrate for Erk1 and Erk2 [26]. Together these observations support the premise that Erk activation is one of several processes that act in concert to stimulate maximal RIPping (Fig. 6). The other players in this seemingly complex network remain to be identified.

Currently two receptor protein-tyrosine kinases have been shown to undergo RIPping, ErbB4 and the CSF-1 receptor. ErbB4 RIPping is induced by its natural ligand, neuregulin. In contrast, CSF-1 appears to be a poor inducer of CSF-1 receptor RIPping [13]. Physiologically relevant inducers of CSF-1 receptor RIPping include ligands for TLRs, which are essential activators of the innate immune response. Preliminary results indicate that a stable version of the ICD localizes to the nucleus (unpublished results). Thus, our model holds that the CSF-1 receptor ICD travels to the nucleus to regulate gene transcription upon release from the plasma membrane, thereby contributing to the rapid changes in gene transcription that occur during the onset of the innate immune response. Our research efforts are currently aimed at further understanding the function of the CSF-1 receptor ICD in the nucleus.

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