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Protein kinase-regulated expression and immune function of thioredoxin reductase 1 in mouse macrophages

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Abstract

Macrophages exposed to lipopolysaccharide (LPS) exhibit radical changes in mRNA and protein profiles. This shift in gene expression is geared not only to activate immune effector and regulatory mechanisms, but also to adjust the immune cell's metabolism to new physiological demands. However, it remains largely unknown whether immune function and metabolic state are mutually regulatory and, if so, how they are mechanistically interrelated in macrophages. Selenium, a dietary trace element exerting pleiotropic effects on immune homeostasis, and selenium-containing proteins (selenoproteins) may play a role in such coordination. We examined the incorporation of radiolabeled selenium into protein during LPS stimulation, and identified thioredoxin reductase 1 (TR1) as the only LPS-inducible selenoprotein in macrophages. TR1 induction occurred at the transcriptional level and depended on the intracellular signaling pathways mediated by p38 MAP kinase and I κ B kinase. Macrophage-specific ablation of TR1 in mice resulted in a drastic decrease in the expression of VSIG4, a B7 family protein known to suppress T cell activation. These results reveal TR1 as both a regulator and a regulated target in the macrophage gene expression network, and suggest a link between selenium metabolism and immune signaling.

Keywords

Thioredoxin reductase; Macrophage; Lipopolysaccharide; VSIG4; Selenoprotein

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1. Introduction

Recognition of microbial products by germline-encoded receptors and resultant leukocyte activation represent key early events in the immune response to pathogen infection. Lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram-negative bacteria, is the best-characterized example of microbial-derived immunostimulatory molecules. LPS triggers an intracellular signaling cascade and transcriptional responses in cells expressing its receptor, toll-like receptor 4 (TLR4) (Poltorak et al., 1998). The signaling pathways linking TLR4 engagement to gene regulation include those mediated by I κ B kinase (IKK), which signals activation of the transcription factor NF- κ B, and the MAP kinases ERK, JNK and p38 (Kawai and Akira, 2007).

Macrophages are tissue-resident phagocytes of myeloid origin, and the centerpiece of the immune surveillance system (Mosser and Edwards, 2008). Primary mouse macrophages have been the experimental model of choice for genetic analyses of LPS-induced signaling and gene regulatory mechanisms. LPS-treated macrophages display globally altered gene expression with accompanying changes in protein abundance and function (Ricciardi-Castagnoli and Granucci, 2002). Most studies of LPS-inducible gene products have been more or less confined to investigating the proteins whose structure and activity readily suggest roles in antimicrobial defence, leukocyte trafficking, or the restructuring of infected and inflamed tissue. There are other LPS-inducible proteins that display no discernible functional properties related to immunity, and their importance in macrophage biology is not as immediately apparent.

Either dietary excess or insufficiency of the trace element selenium can perturb immune function and homeostasis (Hatfield et al., 2006a; Hoffmann and Berry, 2008). Selenium exerts its physiological effects, at least in part, and perhaps principally in the form of selenium-containing proteins (selenoproteins). Selenium incorporation into protein depends on a unique tRNA, designated tRNA^{[Ser]Sec}, which plays crucial roles in both assimilation of selenium into selenocysteine (Sec) and translational insertion of Sec into selenoproteins in response to UGA codons (Hatfield et al., 2006b; Xu et al., 2006). In the study reported here, we examined selenoprotein abundance in mouse macrophages, and found that LPS-exposed macrophages produced higher amounts of the selenoprotein thioredoxin reductase 1 (TR1). We also identified the signaling pathways crucial for the induction of TR1 expression, and determined the effect of TR1 gene ablation in macrophages. Our results present TR1 as an LPS-inducible selenoprotein with a potential role in regulation of immune responses.

2. Materials and Methods

2.1. Mice and primary macrophages

A C57BL/6 mouse line carrying floxed (fl) *Txnrd1* was described previously (Jakupoglu et al., 2005). A transgenic C57BL/6 line carrying the Lysozyme M-Cre transgene (Clausen et al., 1999) was from the Jackson Laboratory. These lines were mated to obtain macrophage-specific *Txnrd1* knockout (Δ TR1^M) mice. The maintenance and care of mice were in accordance with the National Institutes of Health institutional guidelines under the expert direction of Dr. John Dennis (NCI, NIH, Bethesda, MD, U.S.A.). Bone marrow-derived macrophages were prepared and cultured as described (Park et al., 2002) and used in all experiments that involved cultured macrophages. The extent of *Txnrd1* deletion was determined by qPCR analysis of the fl region of the genes. Macrophages lacking the genes encoding tRNA^{[Ser]Sec}, p38 α , IKK β , and RelA were described previously (Park et al., 2002; Kim et al., 2008; Carlson et al., 2009).

2.2. ⁷⁵Se-labeling and analysis of selenoproteins

Macrophages were incubated with 25 μ Ci/ml of ⁷⁵Se (University of Missouri Research Reactor) for 8 h, lysed, the resulting protein extractions electrophoresed on NuPage gels (Invitrogen), gels stained with Coomassie Blue R-250 (Sigma-Aldrich), vacuum dried and exposed to a PhosphorImager (GE Healthcare) as described (Gladyshev et al., 1999).

2.3. Kinase inhibitor treatment

Macrophages were treated with PD98059 (MEK1 inhibitor; Cell Signaling), SB203580 (p38 inhibitor; Cell Signaling), LY294002 (PI3 Kinase inhibitor; Cell Signaling), or JNK Inhibitor II (EMD Chemicals) 30 min prior to LPS stimulation. Dimethyl sulfoxide was used as a vehicle control in untreated cells.

2.4. mRNA and ROS analysis

Total RNA was isolated using Trizol (Invitrogen). cDNA was prepared from 1 μ g of RNA using an iScript cDNA synthesis kit (Bio-Rad). qPCR was performed as described (Park et al., 2002). The PCR primer sequences used were described previously (Carlson et al., 2009). ROS levels were measured by flow cytometry using oxidation sensitive dye DCFDA as described (Shrimali et al., 2008).

3. Results

3.1 LPS-treated macrophages produce higher amounts of TR1 protein and mRNA

The mouse genome encodes 24 selenoproteins, which include proteins with ubiquitous and cell type-specific expression patterns (Kryukov et al., 2003). Selenoproteins showing relatively high expression levels in mouse macrophages include glutathione peroxidase (GPx) 1 and 4, selenoprotein P, selenoprotein 15, selenophosphate synthetase 2, and TR1 (Carlson et al., 2009). To compare selenoprotein expression in unstimulated and LPS-stimulated macrophages, we performed metabolic labeling of mouse macrophages with ⁷⁵Se, and subjected whole cell protein extracts to gel electrophoresis and autoradiography. The identity of major ⁷⁵Se-labeled proteins was unambiguously established in previous studies (Gladyshev et al., 1999; Carlson et al., 2009). We found that the ⁷⁵Se-derived signal representing TR1 was markedly increased following LPS treatment (Fig. 1A). An immunoblot experiment with a TR1-specific antibody confirmed the LPS-induced change in TR1 amount (Fig. 1B). TR1 is a key component of the thioredoxin-based redox regulatory system (Sun et al., 1999). We examined LPS inducibility of TR1 and thioredoxin-related gene expression at the mRNA level. Among the genes encoding the TR isoenzymes (*Txnrd1*, *Txnrd2*, *Txnrd3*) and thioredoxin isoforms (*Txn1*, *Txn11*, *Txn15*, *Txn16*) tested in our analysis, only *Txnrd1*, the TR1 gene, exhibited strong and rapid mRNA induction in LPS-treated macrophages (Fig. 1C). Expression of all other detectable selenoproteins was not similarly induced by LPS. Of note, GPx1 expression was moderately decreased upon LPS exposure (Fig. 1A).

3.2 TR1 gene induction by LPS requires p38 MAP kinase and I κ B kinase signaling

We next sought to determine the signaling pathways that mediate LPS-induced TR1 expression in macrophages. To this end, we used chemical inhibitors of ERK, JNK, p38, and PI3K, which serve in distinct signaling modules downstream of TLR4. The p38 inhibitor, SB203580 (SB), but not the other inhibitors tested, blocked TR1 protein induction by LPS (Fig. 2A). SB did not affect basal TR1 expression in unstimulated macrophages (Fig. 2B). The effect of pharmacological p38 inhibition was also seen on TR1 mRNA induction (Fig. 2C). By contrast, SB had no effect on the LPS-induced decrease in GPx1 protein (Fig. 2A, B) and mRNA (Fig. 2C). There are four isoforms of mammalian p38 MAP kinase encoded

by distinct genes, of which p38 α is the most abundantly expressed in macrophages, and likely responsible for SB-sensitive p38 functions in this cell type (Kim et al., 2008). Genetic ablation of p38 α in macrophages indeed abolished LPS-induced TR1 gene expression (Fig. 2D, left panel). NF- κ B, which binds to many LPS-inducible gene promoters, and IKK, the NF- κ B-activating protein kinase, play a key role in transcriptional activation in response to LPS (Park et al., 2005). We found that macrophages lacking either IKK β , an IKK catalytic subunit, or NF- κ B RelA could not support TR1 induction by LPS (Fig. 2D, middle and right panels, respectively). These results indicate that the p38 MAP kinase and IKK pathways are the pivotal link between TLR4 activation and TR1 gene induction in macrophages.

3.3 TR1 gene ablation preserves macrophage viability and LPS responsiveness

To investigate TR1 functions in macrophages, we generated mice in which TR1 ablation was specifically targeted to myeloid cells. In this mutant line, designated Δ TR1^M, floxed (fl) *Txnrd1* alleles were removed by Cre recombinase expressed from the lysozyme M promoter. Δ TR1^M mice were viable, and manifested no discernible abnormalities throughout development and adulthood (data not shown). In Δ TR1^M macrophages, ⁷⁵Se-labeled TR1 amounts were greatly decreased (Fig. 3A). Many selenoproteins serve as physiological antioxidants, and elimination of the entire selenoproteome, i.e., the whole selenoprotein set, was found to result in higher steady state levels of cellular reactive oxygen species (ROS) in macrophages (Carlson et al., 2009). This ROS dysregulation, however, did not occur in TR1-deficient macrophages (Fig. 3B), suggesting that loss of TR1 alone does not severely hamper the macrophage's ability to quench ROS, and that the antioxidant function of other proteins and/or selenoproteins likely compensates or overrides that of TR1.

We examined cytokine gene expression in control and Δ TR1^M macrophages. Both macrophage groups exhibited similar kinetics and intensities of LPS-induced expression of genes encoding tumor necrosis factor- α , interleukin-1 α , and interleukin-12 p40 (*Tnf*, *Il1a*, and *Il12b*, respectively) (Fig. 3C). Consistent with these in vitro responses, there was no difference between wild-type and Δ TR1^M mice in sensitivity to LPS-induced lethal shock (data not shown). Therefore, TR1 deficiency does not produce substantial effects on macrophage- and cytokine-mediated inflammatory responses in mice.

3.4 TR1 is indispensable for the maintenance of VSIG4 expression in macrophages

Our previous study targeted the removal of the tRNA^{[Ser]^{Sec}} gene, *Trsp*, in macrophages that in turn resulted in the total loss of selenoprotein expression (Carlson et al., 2009). We performed a DNA microarray experiment in that study and identified several genes overexpressed in tRNA^{[Ser]^{Sec}}-deficient macrophages. Among those genes, many encoded components or regulators of the extracellular matrix (ECM), namely *Bgn*, *Ctcf*, *Timp3*, *Tagln*, and *Serpinh1*. In the present study, however, TR1-deficient macrophages did not display aberrant expression of ECM-related genes (Fig. 4A). Other groups of genes were expressed at greatly reduced levels in tRNA^{[Ser]^{Sec}}-deficient macrophages (Carlson et al., 2009). Among those tRNA^{[Ser]^{Sec}}-dependent genes, *Vsig4* showed similarly reduced expression in Δ TR1^M macrophages (Fig. 4A, bottom right panel). The protein product of this gene, VSIG4, is structurally related to B7 family members, key regulators of antigen-presenting cell-T cell interactions. VSIG4 was found to inhibit T cell activation, and its expression was restricted to resting tissue macrophages (Vogt et al., 2006). Stimulation of wild-type macrophages with LPS indeed led to a precipitous decrease in VSIG1 gene expression; of note, VSIG1 mRNA amounts in resting Δ TR1^M macrophages were already low without LPS exposure, comparable to those seen in LPS-treated wild-type macrophages (Fig. 4B). This observation suggests that the VSIG4-dependent ability to control T cell activation is constitutively diminished or lost in TR1-deficient macrophages, a state

permissive for adaptive immune responses that is normally induced by LPS or other immunostimulatory signals.

4. Discussion

Global gene expression changes in macrophages following LPS treatment reflect not only the induction of immune effectors and regulators, but also the cell's attempt to cope with the new physiological demands posed by the activation of the immune system. Our results of LPS-induced TR1 expression implicate this enzyme and the thioredoxin-based antioxidant system as potential components of macrophage immune responses. Furthermore, the newly identified role of p38 MAP kinase and IKK-NF- κ B signaling in TR1 gene induction calls for a careful examination of whether TR1 ablation accounts for the therapeutic and the adverse effects of p38 MAP kinase and IKK inhibitors currently in clinical development and tests (Strnad and Burke, 2007; Coulthard et al., 2009).

Ablation of TR1 expression did not give rise to overt defects in the initiation and regulation of LPS responses. This may be due to functional redundancy of multiple cellular antioxidant mechanisms (Holmgren, 2000). However, even if such compensatory mechanisms offset the effects of TR1 deficiency in Δ TR1^M macrophages, there still seems to be nonredundant gene regulatory functions served by TR1. Our gene expression data show that macrophages lacking TR1 cannot maintain VSIG4 gene expression in resting macrophages. It remains to be determined whether this gene expression defect has a functional outcome in Δ TR1^M mice, particularly as related to T cell-mediated immunity and tolerance. It is known that VSIG4 expression is high in tissue-resident macrophages such as those of the liver, myocardium, and adipose tissue, but absent in the splenic white pulp in normal healthy mice (Vogt et al., 2006). Therefore, VSIG4 may play a more prominent role in, and its ablation in TR1-deficient macrophages have a major impact on, the regulation of T cell reactivity in non-lymphoid tissues.

Macrophages deficient in tRNA^{[Ser]Sec}, which lack TR1 as well as all other selenoproteins, displayed misregulation of a much broader set of genes (Carlson et al., 2009) than Δ TR1^M macrophages. Therefore, selenoproteins other than TR1 likely also possess gene regulatory functions in macrophages. Further investigation of the physiological roles of selenoproteins and the mechanisms of their contribution to macrophage biology will advance our understanding of the link between dietary selenium and the immune disorders that its excess or deficiency causes.

5. Conclusions

LPS-induced TR1 gene expression in macrophages depends on the signaling pathways mediated by p38 MAP kinase and IKK. TR1 in turn regulates the expression of other macrophage genes such as *Vsig4*. Our study establishes TR1 as a new player in the macrophage signaling and gene regulatory circuit.

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Abbreviations

ECM extracellular matrix

fl	floxed
GPx	glutathione peroxidase
IKK	I κ B kinase
LPS	lipopolysaccharide
ROS	reactive oxygen species
SB	SB203580
Sec	selenocysteine
TLR4	toll-like receptor 4
TR1	thioredoxin reductase 1

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Highlights

LPS induces thioredoxin reductase 1 (TR1) expression in macrophages.

LPS-induced TR1 expression depends on p38 MAP kinase and IKK-NF- κ B signaling.

TR1 is essential for VSIG4 gene expression in resting macrophages.

Fig. 1. LPS-induced expression of TR1 in macrophages. (A) ^{75}Se -labeled selenoproteins in macrophages are visualized by autoradiography following SDS-PAGE. Macrophages were left unstimulated (None) and stimulated with LPS (100 ng/ml; 8 h) prior to protein extraction. The identities of TR1 and GPx1 are designated on the left of the panel. (B) Western blot analyses of TR1 and SelT in unstimulated and LPS-stimulated macrophages are shown. (C) Macrophages were treated with LPS (100 ng/ml) for the indicated time points, and gene expression was analyzed by qPCR. Data are representative of two experiments.

Fig. 2.

Role of p38 MAP kinase and IKK-NF- κ B signaling in TR1 expression in LPS-treated macrophages. (A) Macrophages were treated with either 20 μ M PD98059 (PD), 5 μ M SB203580 (SB), 10 μ M JNK Inhibitor II (JNKi), or 5 μ M LY294002 (LY), stimulated with LPS (100 ng/ml) and 75 Se-labeled selenoproteins were visualized by autoradiography following SDS gel electrophoresis. Data are representative of two experiments. (B) Unstimulated or LPS-stimulated macrophages were treated with the indicated concentrations of SB and 75 Se-labeled selenoproteins visualized by autoradiography following SDS-PAGE. Data are representative of two experiments. (C) Macrophages were treated with LPS (100 ng/ml) and SB for the indicated time points, and *Txnrd1* and *Gpx1* gene expression was analyzed by qPCR. Data represent mean $\pm$ standard error (n=3) (D) Macrophages lacking p38 α , IKK β , and RelA were treated with LPS (100 ng/ml) for the indicated time points, and gene expression was analyzed by qPCR. Data are representative of two experiments.

Fig. 3.

Generation and characterization of mice lacking TR1 in macrophages. (A) ^{75}Se -labeled selenoproteins in wild-type and TR1 knockout (KO) macrophages are visualized by autoradiography following SDS-PAGE. (B) ROS production was analyzed by flow cytometry following DCFDA staining. H_2O_2 treatment (2 mM) was used as a positive control. Data are representative of two experiments. (C) Macrophages were treated with LPS (100 ng/ml) for the indicated durations, and cytokine gene expression, *Tnf* (upper graph), *Il1a* (middle graph) and *Il2b* (lower graph), was analyzed by qPCR. Data are representative of two experiments.

Fig. 4. Extracellular matrix (ECM)-related and *Vsig4* gene expression in TR1-deficient macrophages. (A) ECM-related gene expression (*Bgn*, *Ctgf*, *Timp3*, *Tagln*, *Serpinh1*) and *Vsig4* gene expression was measured in resting Trsp KO and TR1 KO macrophages by qPCR. Data are representative of two experiments. (B) Macrophages were treated with LPS (100 ng/ml) for the indicated time points, and *Vsig4* gene expression was analyzed by qPCR. Data are representative of two experiments.