Veterinary Immunology and Immunopathology 155 (2013) 67–75

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Macrophage migration is controlled by Tribbles 1 through the interaction between C/EBPβ and TNF-α

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ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form 23 May 2013
Accepted 3 June 2013

Keywords:
Inflammation
Innate immunity
Tribbles
Pro-inflammatory cytokines
Migration

ABSTRACT

In mammals, three Tribbles gene family members have been identified, Tribbles 1, 2 and 3 (Trib1, Trib2 and Trib3). All family members are considered to be pseudokinases in that they contain domains homologous to serine/threonine kinase catalytic cores, but they lack several conserved residues in the ATP-binding pocket. Trib1 is implicated in the inflammatory response pathway through its ability to regulate mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB) and CCAAT Enhancer Binding Protein (C/EBP). However, its role in macrophages function is unknown. Here, we investigated the functional role of Trib1 in Toll-like receptor-mediated inflammatory responses to IFN-γ in RAW264.7 cells. In gene knock-down experiments in macrophages using small interfering RNAs targeted to Trib1, it was observed that TNF-α production was increased following treatment with IFN-γ and/or TLR2 ligands. Finally, Trib1-silenced macrophages failed to show MCP-1 induced chemokinesis and indicating involvement of Trib1 in controlling of macrophage migration.

This work demonstrates that Trib1 contributes to the pro-inflammatory response caused by TLR2 ligands and controls macrophage migration as well as being a biomarker in macrophage-related diseases in both human and veterinary medicine.

1. Introduction

Tribbles (Trib), the only member of the Trib homologue family in *Drosophila*, regulates *String/CDC25* activity and affects cell cycle progression, cell proliferation, and morphogenesis (Mata et al., 2000; Seher and Leptin, 2000). *Drosophila* Trib protein negatively regulates the level of *Drosophila* Slbo, which controls border cell migration and is a fly orthologue of the mammalian CCAAT Enhancer Binding Protein (C/EBP) transcription factor (Rorth et al., 2000). Three Trib gene family members have been identified in mammalian systems: *Trib1*, *Trib2* and *Trib3* (Hegedus et al., 2006) and the amino acid sequences between human and mouse Trib sequences are highly conserved (Yokoyama and Nakamura, 2011). Trib1 and Trib3 are thought to be nuclear proteins, whereas Trib2 localises to the cytoplasm (Hegedus et al., 2007). Mammalian Trib family members have been implicated in regulating inflammation, cancer development and nutrient metabolism (Du et al., 2003; Iynedjian, 2005; Qi et al., 2006).

Human *Trib1* was first identified as a *Trib2* homologue based on a partial cDNA sequence (Wilkin et al., 1997). Trib1 has been demonstrated to play a role in both innate and adaptive immunity and inflammation. Trib1 was first evaluated as a regulator of the Toll/IL-1 receptor in innate immune cells and was found to mediate inflammatory

Abbreviations: C/EBP, CCAAT enhancer binding protein; ERK, extracellular signal-regulated kinase; MARK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; NF-κK, nuclear factor kappa B; TLR2L, Toll-like receptor 2 ligand; Trib, Tribbles; Trib1, Tribbles 1.

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http://dx.doi.org/10.1016/j.vetimm.2013.06.001

gene expression in HeLa cells (Kiss-Toth et al., 2004, 2005). Study of macrophages from Trib1-deficient mice have demonstrated that the loss of Trib1 causes up-regulation of pro-inflammatory mediators, including prostaglandin E synthase, Arginase II and plasminogen activator inhibitor type II. In the nematode (*Caenorhabditis elegans*) system, a kinase has been identified that resembles human Trib1 and protects against fungal infection, possibly by activating MAPK signalling (Pujol et al., 2008). In addition, it has been demonstrated Trib1 regulates the MAPK pathway in HeLa cells, indicating a possible role for Trib1 in the innate immune response against infection (Kiss-Toth et al., 2004).

Macrophages have important direct roles in innate responses because they recognise, ingest and destroy pathogens via migrating to the inflamed site (Gordon, 2003). Moreover, the innate immune system responds to infection by producing pro-inflammatory mediators such as TNF-α and IL-6 to activated macrophages kill intracellular pathogens by generating nitric oxide and reactive oxygen species (Gordon and Taylor, 2005; Matsusaka et al., 1993). Therefore, enhance macrophage functions may control infectious diseases in both human and veterinary medicine (Elmowalid et al., 2013; Wiege et al., 2012).

In this paper we investigated the biological functions of Trib1 in the context of innate immunity and regulation of macrophage function, using RAW264.7 cells as a model system. We demonstrate that stimulation of TLR2 ligand on macrophages causes up-regulation of Trib1, and that inhibition of Trib1 causes changes in macrophage morphology and migration, while up-regulating TNF-α expression. Taken together, this data shows that Trib1 expression may have a direct effect on macrophage responses in innate immunity.

2. Materials and methods

2.1. Reagents and antibodies

TLR2 ligand Pam_3CSK_4 was purchased from Autogen Bioclear (InvivoGen, USA). Recombinant mouse IFN-γ was purchased from eBioscience (UK) and stored at −20 °C. The qPCR primers were purchased from Custom TaqMan Gene Expression Assay (Applied Biosystems, UK). The following antibodies were purchased from Cell Signalling USA: Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) Mouse mAb (cat. 9106), p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) Mouse mAb (cat. 9107). Anti-α/β tubulin (cat. 2148). The following antibody was purchased from Santa Cruz: C/EBPβ (C-19) sc-150 and Trib1 antibody was purchased from ProSci (USA). Secondary antibodies were purchased from DakoCytomation, UK: Anti-mouse HRP (P0260), Anti-rabbit HRP (P0217), Anti-goat/sheep HRP (P0449). MCP-1 was purchased from PeproTech (USA). The target siRNAs and negative control siRNA were ordered from Ambion (Applied Biosystems, UK, *Silencer*® Select siRNAs).

2.2. Cell culture

Passage 6 murine monocyte/macrophage RAW264.7 cells, generated from BALB/c mice (ATCC number TIB-71), were kindly provided by Professor Tony Nash (Royal Dick School of Veterinary Studies, University of Edinburgh). RAW264.7 cells were cultured in Dulbecco's modified essential medium (DMEM) (GIBCO, UK) supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin and 100 U/ml penicillin (GIBCO, UK) in a humidified incubator at 37 °C with 5% CO_2.

2.3. Cell stimulation procedure

RAW264.7 cell numbers were adjusted to 2×10^5 cells/well incubated overnight. Culture medium (0.5 ml) was removed from each well and replaced with 0.5 ml fresh medium containing cytokines (recombinant mouse IFN-γ 1.0 ng/ml or TLR2 ligands 1.0 ng/ml to the final concentrations). Cells were harvested 0 h, 0.5 h, 1 h, 3 h, 6 h, 8 h and 24 h post-stimulation for RNA extraction. Culture supernatants at 24 h time points were stored at −20 °C for ELISA analysis.

2.4. Transient siRNA transfection

For small interfering RNA (siRNA) knockdown experiments, 2×10^6 RAW264.7 cells were transfected with siRNA using the Amaxa Cell Line Nucleofector Kit V (cat.no. VCA-1003) and programme D-32 (Nucleofector II). Confluent RAW264.7 cells were split at a ratio of 1:4 into new 75 cm^2 flasks 24 h before transfection. The following day, cells were harvested and washed twice with PBS. Subsequently, 2×10^6 cells were resuspended in 90 μl solution V and mixed with 10 μl siRNA in a 1.5 ml microcentrifuge tube. Cells were transfected with either a scrambled siRNA as a negative control or the pmaxGFP green fluorescent protein (GFP) expression vector as a positive control. After transfection, 500 μl warmed culture medium was added to the microcentrifuge tube and transfected cells were transferred into pre-warmed 6-well plates for overnight incubation. The transfection efficiency was analysed by counting GFP-positive cells by FACS and knockdown efficiency was analysed at the mRNA level by qPCR. Trib1 and negative control *Silencer* Select siRNAs were purchased from Ambion, AppliedBiosystems.

2.5. Microscopic analysis

Cytocentrifuge smears were prepared from non-adherent cells as follows. Double cytofunnel cuvettes (Cytospin3, Shandon, Thermo Fisher Scientific Inc., USA) were mounted onto Superfrost microscope slides in metal holders. Cell suspension (100 μl) was place into each cuvette and cuvettes centrifuged at 350 rpm for 3 min in a Cytospin3 centrifuge. Slides were carefully extracted and air-dried and then cells were examined using an inverted microscope (Axiovert 40CFL, Carl Zeiss, MicroImaging GmbH, Germany). Photographs were taken using an AxioCam camera mounted on the inverted microscope.

2.6. Western blot analysis

Cells were lysed in 1% NP40 buffer containing 150 mM KCl, 25 mM Hepes (pH 7.4), 5 mM DTT, 50 mM