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Institut Pasteur

Microbes and Infection xx (2016) 1–5

www.elsevier.com/locate/micinf

Short communication

Signalling through MyD88 drives surface expression of the mycobacterial receptors MCL (Clecsf8, Clec4d) and Mincle (Clec4e) following microbial stimulation

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Received 19 January 2016; accepted 13 March 2016
Available online ■ ■ ■

Abstract

The heterodimeric mycobacterial receptors, macrophage C-type lectin (MCL) and macrophage inducible C-type lectin (Mincle), are upregulated at the cell surface following microbial challenge, but the mechanisms underlying this response are unclear. Here we report that microbial stimulation triggers Mincle expression through the myeloid differentiation primary response gene 88 (MyD88) pathway; a process that does not require MCL. Conversely, we show that MCL is constitutively expressed but retained intracellularly until Mincle is induced, whereupon the receptors form heterodimers which are translocated to the cell surface. Thus this "two-step" model for induction of these key receptors provides new insights into the underlying mechanisms of anti-mycobacterial immunity.

Keywords: C-type lectin receptor; Clec4d; Dectin-3; Clec4e; MyD88; TLR signalling

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the most prevalent infectious diseases with an estimated two billion individuals infected worldwide [1]. Interestingly, only a fraction of these carriers develop active disease, a process that is not yet fully understood [2]. Human genetic association studies have demonstrated an important role for innate immune receptors and their signalling pathways in TB susceptibility and disease progression [1]. Pattern recognition receptors on innate immune cells play a crucial role in both homoeostasis and host defence against pathogens. Toll-like receptors and C-type lectin receptors (CTLRs) are the major families of surface expressed PRRs that detect pathogen associated molecular patterns (PAMPs), triggering complex signalling cascades to initiate host defences such as the release of cytokines and chemokines, which are key for the activation and recruitment of leukocytes during TB [1,3,4]. Toll-like receptors (TLRs) directly sense a variety of mycobacterial components such as glycolipids and glycoproteins (TLR2/1), diacylated lipoproteins (TLR2/6), heat shock protein 60/65 (TLR4), and DNA motifs (TLR9) [1,5,6]. More recently, several members of the Dectin-2 family of CTLRs were shown to play a role in anti-mycobacterial immunity through recognition of trehalose dimycolate (MCL, Mincle) or mannose-capped lipoarabinomannan (Dectin-2) [7–10]. These receptors associate with the signalling adaptor FcRγ for surface expression and the transduction of an activating signal [11]. Of particular importance here is MCL, which was shown to play a non-redundant role in a murine pulmonary TB model [10]. The current model for mycobacterial trehalose

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http://dx.doi.org/10.1016/j.micinf.2016.03.007

dimycolate (TDM) recognition by MCL and Mincle suggests a constitutive expression of MCL, which recognises the lipid moiety of TDM [12]. This induces an activation signal, transduced through the CARD9/Bcl10/MALT1 axis, leading to NF-κB p65 initiation of Mincle expression [13,14].

Although MCL was described as a constitutively expressed receptor and is highly expressed on resident peritoneal macrophages, we and others have recently demonstrated that its surface expression on myeloid cells can be significantly upregulated by microbial stimuli *in vitro* and pulmonary infection with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) *in vivo* [15,16]. MCL and Mincle were reported to form functional heterodimers [15,16]. Notably, each receptor was essential for surface expression of its heterodimeric partner under naive conditions as well as during upregulation of expression on BCG-infected bone marrow-derived macrophages (BMM) [15]. In this study, we investigated the mechanism underlying the induction of MCL and Mincle surface expression following microbial stimulation.

2. Materials and methods

2.1. Cells, bacterial strains, mice and reagents

C57BL/6 wildtype and matching Mincle$^{-/-}$, MCL$^{-/-}$ and MyD88$^{-/-}$ mice (on a C57BL/6 background) were housed with access to water and food *ad libitum* in the specific pathogen free animal facilities at the University of Aberdeen (UK). Procedures were carried out in accordance with approved protocols from the UK Home Office under project licences 60/4007 and 70/8073. Bone marrow-derived macrophages (BMM) were generated in the presence of conditioned L929 supernatant in complete RPMI medium (Gibco), as described previously [17]. *M. bovis* BCG strain Pasteur was grown on Middlebrook 7H10 agar or in Middlebrook 7H9 broth (BD) [10]. BMM were plated at 2.5×10^5 cells/well in 24 well plates (Thermo Scientific) for flow cytometry, or 1×10^6 cells/well in 6 well plates (Sigma) for total protein. Stimulations with BCG (multiplicity of infection: 1), TLR-4 agonist LPS (100 ng ml^{-1}, Sigma) and TLR-2/1 agonist Pam_3CSK_4 (100 ng ml^{-1}, Invivogen) were carried out as described previously [15,18].

2.2. Flow cytometry

For analysis of receptor expression, cells were stained and analysed by flow cytometry as described previously [15], in the Iain Fraser Cytometry Centre at the University of Aberdeen. Briefly, cells were harvested, passed through a 40 μm cell strainer and red blood cells lysed in PharmLyse (BD), before staining in FACS block (PBS, 0.5% BSA, 5% rabbit serum, 5 mM EDTA, 1 mM azide) containing 4 μg/ml 2.4G2 Fc-receptor block. For intracellular staining, cells were fixed in 1% paraformaldehyde in PBS, followed by permeabilisation in 0.05% saponin (Sigma) in FACS Block. Antibodies used were CD45 (clone 104), CD11b (clone M1/70), F4/80 (clone Cl:A3-1), MCL-biotin (clone 3A4 [15]), Mincle-biotin (clone 4A9 [7]) and Dectin-2-biotin (clone 11E4 [19]). Biotinylated antibodies were detected with an appropriate streptavidin conjugate (BD, Invitrogen) and measured by flow cytometry on LSR Fortessa or Array instruments (BD) and data analysed using FlowJo v.10.0.8. Mean fluorescent intensity (MFI) data reported are MFI of anti-CTLR minus MFI of isotype control.

2.3. Western blot

Cells were plated and stimulated as indicated in the figures followed by lysis in ice cold RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing complete EDTA-free protease inhibitor cocktail (Roche). Western blots on equal amounts of protein quantitated by BCA assay (Pierce) were performed following conventional protocols using the NuPAGE system (Invitrogen) and probed with antibodies as indicated in the figures. Equal loading was demonstrated by stripping the blots (re-blot mild buffer, Millipore) and re-probing the membranes with mouse anti-mGAPDH (clone mAbcam 9484).

2.4. Data analysis

Data was compiled and analysed using FlowJo v10.0.8, Excel and Graphpad Prism v5.04, and analysed with ANOVA and Bonferroni post-test. Data was considered statistically significant if $p < 0.05$.

3. Results

3.1. Surface expression of MCL and Mincle is induced by TLR ligands

We recently reported that microbial challenge led to increased expression of MCL and Mincle in an interdependent fashion [15]. To confirm these findings, we stimulated wild-type, Mincle$^{-/-}$ and MCL$^{-/-}$ BMM with LPS, Pam_3CSK_4 or *M. bovis* BCG and assessed receptor surface expression by flow cytometry. Consistent with our previous observations, stimulation with microbial agonists strongly increased surface expression of both MCL (Fig. 1A) and Mincle (Fig. 1B) on wild-type cells [15]. Furthermore, the induced surface expression of each of these CTLRs was dependent on co-expression of its heterodimeric partner, since expression of MCL or Mincle was substantially reduced on Mincle$^{-/-}$ (Fig. 1A) or MCL$^{-/-}$ cells (Fig. 1B), respectively. Notably, MCL expression was completely absent on Mincle$^{-/-}$ cells, but Mincle could still be induced on MCL$^{-/-}$ cells, albeit at low levels. This suggests that expression of Mincle is not absolutely dependent on MCL, as we had previously observed in alveolar macrophages [15].

3.2. MCL is constitutively expressed but retained intracellularly

While Mincle expression has been suggested to be controlled by MCL on a transcriptional level, MCL is thought

Please cite this article in press as: Kerscher B, et al., Signalling through MyD88 drives surface expression of the mycobacterial receptors MCL (Clecsf8, Clec4d) and Mincle (Clec4e) following microbial stimulation, Microbes and Infection (2016), http://dx.doi.org/10.1016/j.micinf.2016.03.007