Journal of Neuroimmunology 252 (2012) 16–23

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

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

The nucleotide-binding oligomerization domain-containing-2 ligand muramyl dipeptide enhances phagocytosis and intracellular killing of *Escherichia coli* K1 by Toll-like receptor agonists in microglial cells

Sandra Ribes [a,*,1], Nina Adam [a,1], Sandra Schütze [a], Tommy Regen [a], Sandra Redlich [a], Hana Janova [a], Angela Borisch [a], Uwe-Karsten Hanisch [a], Roland Nau [a,b]

[a] Institute of Neuropathology, University Medical Center Göttingen, Göttingen, Germany
[b] Department of Geriatrics, Ev. Krankenhaus Weende, Göttingen, Germany

ARTICLE INFO

Article history:
Received 3 April 2012
Received in revised form 15 July 2012
Accepted 18 July 2012

Keywords:
Toll-like receptors
Muramyl dipeptide
Microglia
Escherichia coli
Phagocytosis

ABSTRACT

Increasing the phagocytic activity of microglia could improve the resistance of immunocompromised patients to CNS infections. We studied the microglial responses upon stimulation with the Nod2 ligand muramyl dipeptide (MDP) alone or in combination with a TLR1/2, 3 or 4 agonist. MDP caused a mild release of NO, but induced neither a significant release of pro-inflammatory cytokines nor an expression of molecules associated with professional antigen presentation. Using the *Escherichia coli* K1 model, microglial pre-stimulation with MDP enhanced bacterial phagocytosis which was strengthened on TLR-pre-stimulated cells. Dual pre-stimulation of Nod2 and TLR1/2 or 4 caused maximal phagocytosis and intracellular killing.

1. Introduction

Escherichia coli is the leading cause of Gram-negative neonatal bacterial meningitis with a high fatality rate and permanent neurological dysfunction in more than half of the survivors (Dawson et al., 1999; Mittal et al., 2011). *E. coli* also causes meningitis in older (Cabellos et al., 2009) and immunocompromised patients (Roos, 2009). The presence of the polysaccharide capsule K1 allows *E. coli* strains to survive in the bloodstream in order to ultimately cross the blood–brain barrier by penetrating the brain micro-vascular endothelial cell layer and to enter the CNS (Kim, 2002).

In mammals, the immune system plays a pivotal role in protecting the organism from invading pathogens, such as bacteria, fungi or viruses. Microglial cells are the resident immune cells of the CNS and act as the first active defense against microorganisms (Mariani and Kielian, 2009; Kettenmann et al., 2011). They express different members of the Toll-like receptor (TLR) family which detect pathogen-associated molecular patterns (Kielian, 2006; Hanisch and Kettenmann, 2007; Mariani and Kielian, 2009). Upon recognition, microglia mediate and induce an immune response by releasing chemokines, cytokines and free radicals (Olson and Miller, 2004; Sterka and Marriott, 2006; Sivagnanam et al., 2010). An excessive and long-lasting pro-inflammatory response may lead to neurotoxicity (Aloisi, 2001). On the other hand, microglia activation can exert a protective effect by increasing phagocytosis and subsequent killing of invading pathogens. Activation by TLR increases the bacterial uptake as well as intracellular killing by microglial cells (Ribes et al., 2009).

In recent years, nucleotide-binding oligomerization domain (NOD) proteins are gaining interest since NOD2 receptor mutations have been identified in patients suffering from Crohn's disease (Hugot et al., 2001). NOD2 is a member of the nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family (Ting et al., 2008). NOD2 recognizes peptidoglycan (PG)-derived fragments, including muramyl dipeptide (MDP), the minimal bioactive PG motif common to all bacteria that is also used as a synthetic ligand (Girardin et al., 2003). After recruitment of the serine/threonine kinase RIP2 (receptor-interacting protein 2; also known as RICK), it triggers the production of pro-inflammatory mediators through nuclear factor κB (NF-κB) signaling (Strober et al., 2006).

NOD proteins have been identified in immune and non-immune cell types (Ting and Davis, 2005; Strober et al., 2006). MDP derivatives enhanced phagocytic and microbicidal activities of monocytes and macrophages (Cummings et al., 1980). In human dendritic cells, MDP treatment augmented the expression of major histocompatibility complex class II (MHC class II) antigens (Cooney et al., 2010) and resulted in a weak but consistent up-regulation of CD80 and CD86 (Kramer et al., 2006). More

* Corresponding author. Tel.: +49 551 / 39 12889; fax: +49 551 / 39 10800.
E-mail addresses: sribes@med.uni-goettingen.de, sandragottingen@gmail.com (S. Ribes).
[1] These authors contributed equally to this work.

0165-5728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneuroim.2012.07.012

recently, Nod2 stimulation has been shown to induce autophagy (Travassos et al., 2010) and to drive antigen-specific $CD4^+$ T cell responses (Cooney et al., 2010).

The immunomodulatory effect of MDP has been scarcely explored in microglial cells. Microglia expressed Nod2 receptors as well as the downstream effector molecule RIP2, and Nod2 expression was up-regulated after exposure to Gram-negative bacteria, MDP as well as TLR4 and 5 ligands (Sterka and Marriott, 2006; Sivagnanam et al., 2010).

Here, we hypothesized that MDP could represent a mild stimulus of microglial cells by enhancing bacterial phagocytosis without an excessive associated inflammatory response. This mild immunostimulation may increase protection of the brain against *E. coli* infection without the hazard of collateral neuronal injury, and may be particularly useful in immunocompromised patients. Therefore, we determined the response of murine microglial cells after stimulation with MDP alone focusing on phagocytic and bactericidal activities, expression of MHC class II, CD80 and CD86 and induction of nitric oxide (NO) and chemo-/cytokine release. We also characterized the effect of stimulation with MDP in combination with one TLR1/2, 3 or 4 agonists on the release of selected chemo-/cytokines, bacterial phagocytosis and intracellular killing of ingested bacteria. We demonstrated that MDP increases the phagocytosis and intracellular killing of *E. coli* K1 without inducing the release of pro-inflammatory chemo-/cytokines. Most notably, the Nod2 system can enhance both the phagocytic and bactericidal activities of TLR-stimulated microglia. These findings further underline the cooperative action of innate immune receptors and their respective signaling systems in fighting infectious threats.

2. Material and methods

2.1. Primary mouse microglial cell cultures

Brains of newborn (1 to 3 days, P0 microglia) and adult (49 days, P49 cells) C57Bl6 mice were used to prepare primary microglial cell cultures (Ribes et al., 2009; Scheffel et al., unpublished data). After removing the meninges, cells were mechanically dissociated and suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany). Cells were plated at a density of two brains per T75 culture flask (Corning Costar, Wiesbaden, Germany) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. After 10 to 14 days of culture, microglial cells were isolated from the mixed glial (astrocytes and microglia) cultures by shaking 200 times/min for 30 min and plated in either 96-well plates (for nitrite, ELISA and phagocytosis assays), 24-well plates (for intracellular survival assay) or in 12-well plates (flow cytometry) and cultured in medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin until stimulation. To test for the purity of the cultures, *Griffonia simplicifolia* isolectin B4 (IL-B4) staining was performed. The staining showed that the cultures contained >98% microglia.

2.2. Microglial stimulation

Microglial cells were exposed to different concentrations of MDP, one TLR ligand or both in combination for 24 h. In flow cytometry experiments the stimulation time was 48 h. In NO assays with different concentrations of MDP, the stimulation times were 24 and 48 h. N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP; InvivoGen, San Diego, USA) was used as ligand of NOD2. Tripalmitoyl-*S*-glyceryl-cysteine (Pam_3CSK_4; 910.5 Da; EMC Microcollections, Tübingen, Germany), polyinosine–polycytidylic acid [poly(I:C), 1.5–8 kb, InvivoGen, San Diego, USA], and lipopolysaccharide (LPS) from *E. coli* serotype 026:B6 (Sigma, Taufkirchen, Germany) were used as agonists of TLR1/2, TLR3 and TLR4. A group of microglial cells incubated with medium (DMEM supplemented with 10% FBS and antibiotics) was included in all experiments as unstimulated control.

In the experiments comparing NO release upon microglial activation with MDP, interferon-γ (IFNγ, 100 U/ml) was added as a co-stimulant to augment NO production. As a positive control, LPS was applied at 1 μg/ml, causing maximal NO release.

We previously demonstrated that Pam_3CSK_4, LPS and poly(I:C) stimulated microglial cells by inducing NO release in a dose-dependent manner. Here, Nod2 and TLR ligands were tested at the lowest concentration inducing maximum NO release (Ebert et al., 2005; Ribes et al., 2010). In addition, sub-maximal concentrations of Nod2 and TLR agonists were tested to allow better detection of the synergistic effects of MDP and the TLR agonists.

After stimulation, supernatants were stored at −20 °C until measurement of cyto-/chemokine levels and cells were used for flow cytometry, phagocytosis or intracellular survival assays In some experiments, supernatants were directly analyzed for NO production and cells were tested for viability to exclude a toxic effect of MDP.

2.3. Nitrite and cell viability assays

NO release was quantified as nitrite accumulation in the supernatants of cells stimulated with different concentrations of MDP (0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml) using the Griess reagent as previously described (Ebert et al., 2005). The presence of LPS as contaminant was excluded by co-incubation of cells with MDP and 10 μg/ml polymyxin B, a compound that neutralizes endotoxin activity.

Cell viability was determined using the WST-1 cell proliferation reagent (Roche Applied Science, Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt WST-1 by active mitochondria, producing a soluble formazan. This conversion occurs only in viable cells. Cells were incubated with WST-1 for 2 h. Then, the formazan dye formed was quantified by measuring the optical density at 490 nm using a Genios multiplate reader (Tecan, Crailsheim, Germany). The absorbance was directly correlated with the metabolic activity of the cells.

2.4. Cytokine and chemokine measurements in culture supernatants

DuoSet ELISA Development Kits (R&D Systems, Wiesbaden, Germany) were used to determine interleukin-6 (IL-6), IL-10, CCL2 (monocyte chemoattractant protein, MCP-1), CCL3 (macrophage inflammatory protein, MIP-1a), CCL5 (regulated upon activation normal T-cell expressed and presumably secreted, RANTES) CCL17 (thymus and activation regulated chemokine, TARC), CCL22 (macrophage derived chemokine, MDC) and CXCL1 (KC, the mouse equivalent of GROα) levels. TNFα concentrations were measured using an ELISA from BioLegend (San Diego, CA, USA) and total IL-12p40 (including monomeric p40) levels were determined with an ELISA kit from eBioscience (San Diego, CA, USA) (Ribes et al., 2009; Regen et al., 2011).

2.5. Flow cytometry

Primary microglia were seeded into 12-well plates at 2×10^5 cells per well and incubated overnight. After 48 h of stimulation, microglia were analyzed as previously described (Regen et al., 2011) for expression of the MHC class II and the co-stimulatory molecules CD80 and CD86 upon MDP stimulation (10 and 100 μg/ml). As positive control groups, cells were treated with 50 ng/ml IFNγ (MHC class II), 10 ng/ml LPS (CD80) and both in combination (CD86). To avoid nonspecific binding of primary antibodies, Fc receptors were blocked using a rat anti-mouse CD16/CD32 antibody (clone 2.4 G2, BD PharMingen, San Diego, CA). Cells were incubated with the appropriately diluted peridinin chlorophyll protein (PerCP)-conjugated rat anti-mouse CD11b (clone M1/70, Biolegend, San Diego, CA), allophycocyanin (APC)-conjugated rat anti-mouse CD80 (clone 1G10, Beckman Coulter, Fullerton, CA), phycoerythrin (PE)-conjugated rat anti-mouse CD86 (clone GL1, eBioscience, San Diego, CA) and fluorescein