Journal of Neuroimmunology 323 (2018) 49–52

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

High levels of antibodies against PtpA and PknG secreted by *Mycobacterium avium* ssp. *paratuberculosis* are present in neuromyelitis optica spectrum disorder and multiple sclerosis patients

Yael N. Slavin[a,1], Marco Bo[b,1], Elisa Caggiu[b], GianPietro Sechi[c], Giannina Arru[c], Horacio Bach[a,**], Leonardo A. Sechi[b,*]

[a] Division of Infectious Diseases, Faculty of Medicine, The University of British Columbia, Vancouver, BC V6H 3Z6, Canada
[b] Dipartimento di Scienze Biomediche, Università di Sassari, Viale San Pietro 43 b, 07100 Sassari, Italy
[c] Dipartimento di Scienze Cliniche, Chirurgiche e Sperimentali, Viale San Pietro 8, Università di Sassari, Sassari 07100, Italy

ARTICLE INFO

Keywords:
Mycobacterium avium ssp. *paratuberculosis*
Signal transduction
Virulence factors
Neuromyelitis optica spectrum disorder
Multiple sclerosis
Astrocyte

ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (Map) is the etiological agent of Paratuberculosis in ruminants. Protein tyrosine phosphatase A (PtpA) and protein kinase G (PknG) are secreted proteins necessary for the survival of the pathogen within macrophages. In this study we analyzed if Map was able to grow within astrocytes and investigated on the presence of antibodies against PtpA and PknG proteins in MS and NMOSD patients by ELISA. Map was unable to proliferate in astrocytes after of 72 h post-infection, but we observed a high level of antibodies against both virulence factors, suggesting that these patients have been exposed/infected with Map.

1. Introduction

Mycobacterium avium ssp. *paratuberculosis* (Map) is the etiological agent of Johne's disease (JD), a long-standing inflammatory bowel disease in ruminants (Chacon et al., 2004). Concerns have been raised about the potential transmission of Map to humans through dairy products leading to the development of Crohn's disease (Feller et al., 2007; Bach, 2015; Kuenstner et al., 2017). Many reports support this fact because Map has been successfully cultured from different human fluids, such as breast milk and blood, and resist pasteurization (Naser et al., 2000; Naser et al., 2004; Grant, 2003; Juste et al., 2008).

Similar to other pathogenic mycobacteria such as *M. tuberculosis*, Map is able to infect and proliferate in macrophages. In order to survive in the harsh environment presented by the macrophage, Map has to secrete virulence factors within the host cell to orchestrate the immune response and survive.

Two virulence factors interfering with signal transduction in the host are the protein tyrosine phosphatase A (PtpA) and the protein kinase G (PknG), which are secreted proteins necessary for the survival of the pathogen (Bach et al., 2006; Zulauf et al., 2018; Bach et al., 2018b). Previous studies have reported the finding of high levels of antibodies against both PtpA and PknG proteins from Map in sera of patients with Crohn's disease (Bach et al., 2010; Xia et al., 2010). Moreover, recent studies showed an increase of interferon gamma release when the blood of animals infected with Map was exposed to PknG, suggesting that the immune system reacts to previously exposed antigens (Bach et al., 2018b; Nakase et al., 2011).

Other studies also reported the finding of antibodies against other Map antigens, such as in diabetes, multiple sclerosis (MS), rheumatoid arthritis, Hashimoto's thyroiditis, and recently in neuromyelitis optica spectrum disorder (NMOSD) (Sechi and Dow, 2015; Masala et al., 2014; Bo et al., 2018a; Bo et al., 2018b; Niegowska et al., 2016; Cossu et al., 2011).

During progressive MS when exposed to specific triggers (Rossi et al., 2014) is been observed that the astrocytes are able to secrete the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) (Chung and Benveniste, 1990). Thus, we hypothesize that a continuous secretion of TNF-α from Map-infected astrocytes will contribute to the progress of MS with a concomitant secretion of PtpA and PknG from Map-infected astrocytes. This secretion will increase the levels of serum antibodies against both virulence factors.

In the present study, we tested the level of antibodies against both

* Corresponding authors at: L. Sechi, Università di Sassari, Dipartimento di Scienze Biomediche, Viale San Pietro 43 b 07100, Sassari, Italy.
** Corresponding authors at: H. Bach, 410-2660 Oak Street, Vancouver, BC V6H 3Z6, Canada.
E-mail addresses: hbach@mail.ubc.ca (H. Bach), sechila@uniss.it (L.A. Sechi).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.jneuroim.2018.07.007
Received 2 July 2018; Received in revised form 12 July 2018; Accepted 12 July 2018

PtpA and PknG in MS and NMOSD patients. Results showed that a high level of antibodies against both virulence factors was measured, suggesting that these patients encountered Map and probably have a persistent infection. Moreover, astrocytes were infected with Map and the proliferation of the pathogen was assessed.

2. Materials and methods

2.1. Strains, cell line, culture conditions, and infection

Map K10 (ATCC BAA-968) was cultured in 7H9 broth (BD) supplemented with OADC (10%, BD) and mycobactin J (2 mg/L, Allied Monitor) at 37 °C. Solidified 7H9 broth with the respective supplements was prepared after the addition of 1.5% agar (Difco). Normal human astrocytes (NHA, Lonza CC-2565) were cultured according to the manufacturer's instructions. Cells were cultured at 37 °C in an atmosphere supplemented with 5% CO2.

2.2. Astrocyte infection

Astrocytes were plated on 35 mm dishes at a final density of 1×10^6 cells/dish. The next day, cells were infected with Map using a multiplicity of infection (MOI) of 10 for 4 h. Cells were washed to eliminate non-internalized Map and amikacin at 50 μg/mL was added. Samples were processed at different times (0, 24, 48, 72 h) and serial dilutions were plated on 7H10 plates as described above. Plates were sealed and incubated at 37 °C until colonies were observed. All the experiments were performed in triplicate.

2.3. Immunofluorescence

Immunofluorescence of infected astrocytes was performed according to published protocols (Bach H et al., 2006 a) Briefly, astrocytes were dispensed on cover slips and infected at a MOI of 10 with Map prelabeled with rhodamine B. After infection, astrocytes were prepared for immunostaining using antibodies against PtpA and PknG and FITC-goat-anti-rabbit for both antibodies and processed as reported (Bach et al., 2006; Zulauf et al., 2018; Bach et al., 2018b).

2.4. Recombinant protein production and plate preparation

Recombinant proteins were produced according to published protocols. In brief, PtpA (UniProtKB Accession number: A0A200GPK8) was produced in *M. smegmatis* harboring the *ptpA* gene cloned into the vector pALACE (hygromycin resistance), whereas PknG (UniProtKB Accession number: A0A202FS53) was produced in *E. coli* harboring the plasmid *pknG*-pET-30b (kanamycin resistance). Both proteins were purified by affinity chromatography using Ni-NTA resin as published (Bach et al., 2006). Produced proteins were stored at −20 °C until used.

ELISA plates were coated with 50 μg/mL of each antigen in PBS overnight at 4 °C. The next day, plates were washed with PBS supplemented with Tween-20 (PBS-T) × 3 and blocked with BSA 3% in PBS overnight at 4 °C. The concentration of antigen used in this study was already determined in previous studies as the concentration necessary to obtain a differential change in the readout (Xia et al., 2010; Gurung et al., 2014; Bach et al., 2018a). The next day, the blocking solution was discarded and the plates were dried at room temperature. Plates were then shipped to Italy to perform the ELISA. Previous studies performed in our laboratory indicated that the shipping of dried plates did not affect the antigen conformation (Gurung et al., 2014).

2.5. Subjects

Archived serum samples of 34 NMOSD patients (5 males, 29 females; mean age 51.32) and 72 MS at onset patients (18 males, 54 females; mean age 39.88 years) were collected at the University of Sassari. We matched the patients with two different group of healthy control, one of 79 healthy controls to compare with MS patients (HCs; 25 males, 54 females; mean age 46.78), whereas a group of 38 subjects was enrolled for NMOSD patients (HCs; 10 males, 28 females; mean age 54.37). The samples were collected in Vacutainer tubes for the separation of serum. The clinical characteristics of NMOSD patients have been described previously (Bo et al., 2018b). MS patients (10 Clinically Isolated Syndrome - CIS and 62 Relapsing-Remitting - RRMS) diagnosed according to the revised McDonald diagnostic criteria (Polman et al., 2011) were enrolled at the MS Centre for Diagnosis and Treatment, Department of Clinical and Experimental Medicine (Neurology), University Hospital of Sassari, Italy.

HCs were recruited at the Blood Transfusion Centre of Sassari, Italy. Ethical approval for all subjects was achieved from the Ethics Committees of the Local Health Authority, AOU Sassari, in 2017 (Prot. No. 6, 2015). All methods were carried out in accordance with the approved guidelines and written consent was obtained from all participants.

2.6. ELISA assays and statistical analysis

ELISA was performed according to a previously described protocol (Caggiu et al., 2016). The obtained absorbance values (at 405 nm) were normalized to a highly positive control serum common to all assays with absorbance reactivity set at 1.0 arbitrary units (AU/ml). Optimal cut-off values to discriminate between positive and negative samples were identified based on the receiver operating characteristic (ROC) curves with specificity established at 90%. Statistical significance of the data was determined through the Mann-Whitney *U* test (95% CI) using Graphpad Prism Version 6.02 software (GraphPad Software Inc., La Jolla, CA 92037, USA). Comparison of positivity to the assessed patients between MS and NMOSD patients compared to HCs was performed through Fisher's exact test.

3. Results

3.1. Map proliferation and immunofluorescence analysis

Map was unable to proliferate in astrocytes as no proliferation was recorded for a period of 72 h post-infection (data not shown). Moreover, secretion of PtpA or PknG was not observed during the same period of time even though some Map cells were internalized, suggesting that Map is not able to mount a stable infection within astrocytes (data not shown).

3.2. Immunoresponse against PtpA and PknG in MS and NMOSD patients

The humoral response and antibody seropositivity against two virulence factors of Map, PtpA and PknG, were evaluated in all MS patients at onset, NMOSD subjects and in HCs. Total IgG was analyzed in all patients. Concerning PknG analysis of the antibody response showed a statistically significant difference between MS patients and HCs, with a p-value < .0001 analyzed by Mann-Whitney test (Fig. 1A). Observed seropositivity was 61% in MS patients and 11% in HCs, determined by ROC analysis with a cut-off value of 0.568, AUC = 0,8024. In the same way, upon evaluation of antibodies reactivity against PtpA, we observed a 61% and 24% of seropositivity in MS patients and HCs, respectively (Fig. 1B), calculated by ROC analysis with a cut-off value of 0.28, evaluated by the Mann-Whitney with a p -value of 0.0005, AUC = 0,6621.

Antibodies against PknG were found in 9 out of 34 (26.47%) NMOSD patients compared to 3 out of 38 (7.9%) positive HCs cases (AUC = 0.6997, p = .0032; Fig. 1C). Much higher positivity among NMOSD patients, with a strong statistical significance, was registered regarding antibody positivity against PtpA found in 15 out of 34 (44.11%) subjects and only 3 out of 38 (7.9%) HCs (AUC = 0.8367,