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Short Communication

Adjuvant and antigenic properties of *Mycobacterium avium* subsp. *paratuberculosis* on experimental autoimmune encephalomyelitis

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease in ruminants, has been linked as a possible risk factor for human multiple sclerosis. In the current study we investigated the adjuvant effect of MAP on experimental autoimmune encephalomyelitis (EAE). Groups of C57BL/6 mice were actively immunized with myelin oligodendrocyte glycoprotein (MOG) $_{35–55}$ peptide emulsified in incomplete Freund's adjuvant modified containing heat-killed MAP (MIFA). MOG-MIFA immunized mice showed an early disease onset and more severe clinical scores in comparison with MOG-CFA immunized mice, demonstrating for the first time the adjuvant effect of MAP on EAE development.

1. Introduction

Several studies have suggested a possible causal relationship between *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS) mainly mediated by T-cells autoreactive to myelin (Cossu, Yokoyama, & Hattori, 2018). Experimental autoimmune encephalomyelitis (EAE) is a T-cell mediated autoimmune disease commonly employed as a model for studying MS. EAE can be actively induced in mice by sensitization with the immunodominant epitope of myelin oligodendrocyte glycoprotein (MOG) $_{35–55}$ in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis*. MOG $_{35–55}$ peptide or incomplete Freund's adjuvant alone are not sufficient to induce disease, the mycobacterial component is necessary because activates mononuclear phagocytes inducing the phagocytosis of these molecules and the secretion of cytokines, resulting in the prolongation of the presence of antigens and a more efficient transport of these to the lymphatic system (Bittner, Azali, Wiendi, & Meuth, 2014). The present study sought to elucidate the adjuvant property of MAP antigens on EAE.

2. Materials and methods

2.1. Experimental animals and MAP antigens

C57BL/6J female mice (The Jackson laboratory, Charles River Laboratories Japan, INC.) 9–10 weeks old were maintained under specific pathogen free conditions in accordance with the guidelines of NIH and the Juntendo University Graduate School of Medicine.

MAP strain ATCC 19698 was grown in Middle brook 7H9 liquid medium (Difco Laboratories, MD, USA) enriched with BBL Middle brook OADC (Becton Dickinson, Tokyo, Japan) and 2 mg/L of mycobactin J (Allied Laboratory, MO, USA) for two weeks. After heat-inactivation of the bacterium for 1 h at 80 °C, MAP cultures were centrifuged and the weighed pellets were dissolved in PBS, triturated and centrifuged again. MAP was then lyophilized by Freeze Dryer (Eyela, Tokyo, Japan) 4 h at −50 °C under vacuum, and accurately weighed quantities of dry MAP were suspended in incomplete Freund's adjuvant (Difco Laboratories) at concentration of 10 mg/mL.

2.2. Induction and clinical assessment of EAE

Mice were immunized under anesthesia via sub-cutaneous injection at two sites (hind footpads) with 200 μg/mouse of $MOG_{35–55}$ peptide

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(BEX CO., LTD, JAPAN) emulsified in 0.2 mL of MIFA containing 4 mg/ml of MAP or CFA containing 4 mg/mL of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, MD, USA), with co-administration of 200 ng/mouse of pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection on day 0 and 2 post immunization (d.p.i.). CFA/MIFA injected control mice were used as negative controls. Mice were monitored daily for clinical signs using the following scoring system: 0 = no clinical signs; 1 = flaccid tail; 2 = hindlimb paresis; 3 = complete bilateral hindlimb paralysis, and 4 = moribund state or death of an animal.

2.3. Histological analysis

For histological evidence of EAE, three mice from each group were sacrificed on acute phase (15–20 d.p.i). Brains and spinal cords were removed, fixed in 10% neutral buffered formalin and embedded for sectioning. Serial transverse sections (5 mm in thickness) were stained with hematoxylin and eosin (H&E) or Luxol Fast Blue (LFB) to evaluate inflammation or demyelination, respectively.

2.4. T-cell proliferation assay

T-cell proliferative responses were assessed in splenocyte isolated from mice during acute phase. Cells were cultured in flat-bottomed 96-well plates at a density of 4×10^5 per well for 72 h in presence of varying concentration of antigens. During the final 18 h of culture, 1 μCi/well of [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added, and incorporation was measured by liquid scintillation.

2.5. Flow cytometry

Cells from spleen were isolated according to standard procedures. For surface staining, stimulated cells were incubated with Fc Block (2.4G2; BD Biosciences) for 15 min prior to staining with each fluorochrome labeled monoclonal antibody (mAb) (Table 1), or appropriate isotype-matched controls for 20 min on ice. For the detection of immune cells, the following antibodies were used: CD3 (17A2; Biolegend, San Diego, CA, USA), CD4 (GK1.5; BD Pharmingen), CD8a (53–6.7; Biolegend), TCR γ/δ (GL3; Biolegend), NK1.1 (PK136; Biolegend), CD11b (M1/70; Biolegend), CD115 (AFS98; Biolegend), CD11c (N418; Biolegend), and I-A/I-E (M5/114.15.2; Biolegend). Flow cytometry was performed on a BD LSRFortessa™ and analyzed using FlowJo software (FlowJo LLC, OR, USA).

2.6. Statistics

The unpaired two-sided Student's *t*-test was used for determining differences in clinical EAE characteristics and invasion of CD3$^+$ T cells into the CNS in the different groups of mice by using Prism 8.0 software (GraphPad, LA Jolla, CA, USA). *P* values of < 0.05 were considered significant.

3. Results

3.1. Clinical signs of EAE in mice immunized with MIFA or CFA

Active immunization with $MOG_{35–55}$ in CFA or MIFA, together with injection of pertussis toxin, resulted in induction of EAE (Fig. 1a). C57BL/6 mice showed a typical EAE clinical pattern with non-significant difference in clinical signs (Table 1). However, immunization with MOG-MIFA accelerated the onset of EAE, that began with moderate signs of disease from 7 to 8 d.p.i. (i.e. limp tail), whereas in the CFA-immunized mice it began from 9 to 11 d.p.i. ($p = 0.001$). Further, mice immunized with MOG-MIFA showed a significantly ($p = 0.03$) more severe EAE than MOG-CFA-immunized mice. The peaks of disease were seen in the acute phase exhibiting hind limb paresis or complete hind limb paralysis about 6–8 days after EAE onset for both groups, and it lasted 1–3 days. A remission occurred before 30 d.p.i. when clinical symptom was significantly improved and no difference were observed between groups. Control mice did not demonstrate neurological deficits at any point following CFA or MIFA injection without antigen.

Histopathological analysis revealed typical lesions, characterized by an intense perivascular inflammatory infiltrate in the brain and also in both, cervical and lumbar sections of the spinal cord (Fig. 1d). We did not observe any difference in the number of inflammatory lesions or demyelination in the spinal cord of MIFA-immunized compared with CFA-immunized mice; however, the density of immune cell per mm^2 quantified by ImageJ software (US NIH) was higher in the brain lesions of MIFA-immunized mice (345 ± 168) than in the CFA-immunized group (198 ± 41).

3.2. MOG-MIFA immunization increased CD4$^+$, monocytes and dendritic cells numbers

At acute phase, MOG-MIFA-immunized mice with EAE had a higher proportion of CD8$^+$ and CD4$^+$cells in the spleen than MOG-CFA immunized mice (Fig. 1c). Furthermore, MOG-MIFA immunization significantly increase the percentage of dendritic cells (CD11c $^+$ I-A/I-E$^+$) and monocytes (CD11b $^+$ CD115$^+$). No significant difference in CD8$^+$, γ/δ (CD3$^+$ TCR γ/δ$^+$), and Natural Killer T cells (CD3$^+$ NK1.1$^+$) was measured between the two groups.

3.3. T-cell proliferation from MOG-EAE immunized mice

All antigens were previously titrated to determine the optimal concentration, which we found to be 50 μg/mL for $MOG_{35–55}$, MAP lipophilic antigens (Otsubo et al., 2015), ovalbumin (OVA), and 5 μg/mL for phytohemagglutinin (PHA). Each MOG-immunized mouse tested during the acute phase of EAE responded well to $MOG_{35–55}$, regardless the adjuvant used, with a stimulation index higher in MOG-MIFA immunized mice (Fig. 1b). Moreover, a strong proliferative response against MAP lipophilic antigens was also observed in all groups, with a stimulation index comparable to that of the potent immunogen PHA.

4. Discussion

In the present study, we demonstrated for the first time that EAE can

Table 1
Characteristics of EAE following MOG-CFA or MOG-MIFA immunization.

	MOG + MIFA (*n* = 20)	MOG + CFA (n = 20)	MIFA/CFA (*n* = 5)
Incidence of EAE	80%	87%	0%
Mortality before disease onset	13%	6%	NA
Day of onset	8.4 ± 1.1[a]	9.6 ± 0.9	NA
Maximum mean disease score	2.7 ± 0.5[a]	2.4 ± 0.3	NA
Cumulative disease score to day 30	37 ± 10.6	33 ± 8.2	NA

[a] Statistically significant.

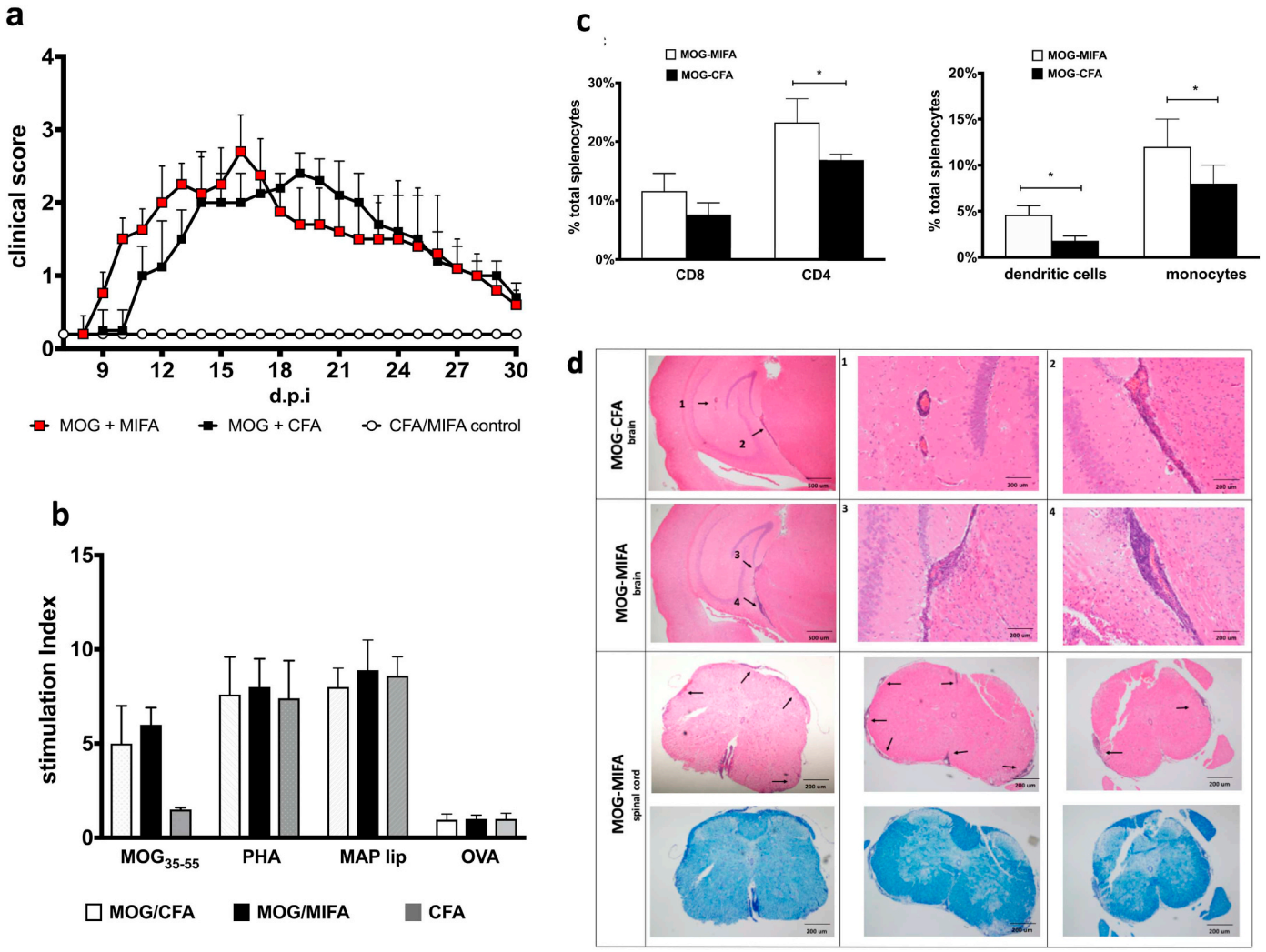

Fig. 1. Effect of MIFA on EAE. (a) Clinical signs. (b) T-cell proliferation. * statistically significant. (c) Flow cytometry of splenocytes from mice stimulated 48 h with $MOG_{35–55}$. (d) Comparison of representative H&E stain of brain, and H&E/LFB stain of spinal cord.
All data are representative results of three independent experiments.

be induced in C57BL/6J mice by $MOG_{35–55}$ with heat-killed MAP. Cell wall components present in the heat-killed mycobacteria are capable to induce a strong Th1 cell response (van Crevel, Ottenhoff, & van der Meer, 2002), which results in augmented delayed-type hypersensitivity against autoantigens (Kobayashi, Kaneda, & Kasama, 2001).

The fact that MOG-MIFA immunized mice developed early and more severe EAE when compared to MOG-CFA immunized mice, could be due to the presence of MAP specific pathogen-associated molecular patterns responsible for toll-like receptors activation (Ferwerda et al., 2007), which plays a critical role in promoting the innate and adaptive immune response necessary to develop EAE (Gooshe, Abdolghaffari, Gambuzza, & Rezaei, 2014).

Surprisingly, amongst the *Mycobacterium* species, only MAP has been related as causative factor of MS. MAP antigens were capable to elicit a strong T-cell mediated response in a cohort of Sardinian patients with MS (Cossu et al., 2015), and our data from EAE model also suggested an activation and proliferation of encephalitogenic T-cells following MAP immunization. These evidences from clinical and animal model studies support for the hypothesis that an increasing in newly diagnosed MS patients in Japan can be due to the exposure to MAP-contaminated dairy products (Cossu et al., 2016; Yokoyama et al., 2018). On the other hand, BCG appears to have a protective role in the progression of both MS and EAE (Cossu, Yokoyama, & Hattori, 2017). The mechanisms by which live BCG reduces the severity of EAE seems to be related to a traffic diversion of $MOG_{35–55}$ reactive T-cells to local BCG inflammatory sites, away from the CNS (Sewell et al., 2003), while we believe that the role of MAP in triggering autoimmunity is based on T-cell mediated molecular mimicry theory (Cossu et al., 2018).

To note, active suppression by regulatory T-cells (Tregs) plays an important role in the control of self-antigen-reactive T-cells in many disease models including EAE, and mycobacteria are strong adjuvants for Tregs induction (Rook et al., 2004). Considering that these immune cells influence both the innate and adaptive immune response, especially in BAFF-related peripheral tolerance (Mameli et al., 2016), further research across all aspect of mycobacteria-host interactions is necessary.

In conclusion, we have shown a strong adjuvant effect of MAP in in the development and progression of EAE, providing an alternative and reproducible method for understanding the mechanism responsible for the generation of autoimmune response of CNS.

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