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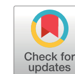
Changes in the function and phenotype of resident peritoneal macrophages after housing in an enriched environment



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ABSTRACT

Exposure to an enriched environment (EE) affects not only brain functions but also immune responses upon viral or bacterial infections. In this study, we examined changes in the phagocytic response and chemokine production of resident peritoneal macrophages after mice had been housed under EE conditions for 6 or 8 weeks, and then explored the possibility that EE could cause a change in the macrophage phenotype by means of flow cytometry as well as quantitative RT-PCR. The percentages of EE macrophages phagocytosing *S. aureus* and apoptotic neutrophils were significantly larger than those of standard environment (SE) macrophages. After coculturing with *S. aureus*, EE macrophages tended to produce greater amounts of chemokines such as MIP-2, KC and MCP-1 than SE ones, although the increases for MIP-2 and KC were not statistically significant. As compared with SE macrophages, EE macrophages included more CD40-positive cells (M1 marker), and expressed more mRNAs of IL-6 (M1 marker) and IRF4 (M2 marker), and less mRNA of CD38 (M1 marker), suggesting either the possibility that EE macrophages are a mixed population of M1 and M2 macrophages or the possibility that they are a unique population with a mixed M1 and M2 macrophage phenotype.

1. Introduction

Enriched Environment (EE) is the concept of the modification of the environment of captive animals to enhance their physical and psychological well-being by providing stimuli meeting the species-specific needs of experimental animals [1].

Exposure to EE affects brain functions [1]. In one study, following exposure to EE, mice showed enhanced production of brain-derived neurotrophic factor (BDNF) in the hypothalamus, thereby suppressing leptin production by adipocytes through sympathoneural β-adrenergic signaling and thus reducing tumor growth [2].

Exposure to EE also modulates immune functions [1]. After mice had been kept under EE conditions for three months, less virus-induced encephalitis was observed in association with less viral antigens, more T cell infiltration and less microglial activation [3]. In another study, following exposure to EE, mice showed a reduction of the viral load in the lungs, possibly due to augmentation of NK cell activity [4]. There has been another report of an increase in the frequency of NK cells infiltrating a tumor upon exposure to EE, by which EE reduced glioma growth in the brain [5]. Following exposure to EE, mice also exhibited an improved capacity to resolve systemic microbial infection through increased recruitment of neutrophils and monocytes to the site of inflammation due to an increase in the ratio of circulating neutrophils to lymphocytes [6].

When infected with bacteria, tissue resident macrophages initiate innate immunity by producing chemokines to attract neutrophils to the site for phagocytosis and killing. Neutrophils then undergo apoptosis, and they are phagocytosed by macrophages without second inflammation being provoked through a mechanism involving either TGF-β or NO [7,8]. We therefore examined changes in the phagocytic response and subsequent chemokine production of resident peritoneal macrophages after mice had been housed under EE conditions. Because M1/M2 dichotomy has been used to explain the changes in the functions of macrophages under physiological and pathological conditions [9], we then explored the possibility that exposure to EE could cause a change in the macrophage phenotype in terms of M1 and M2

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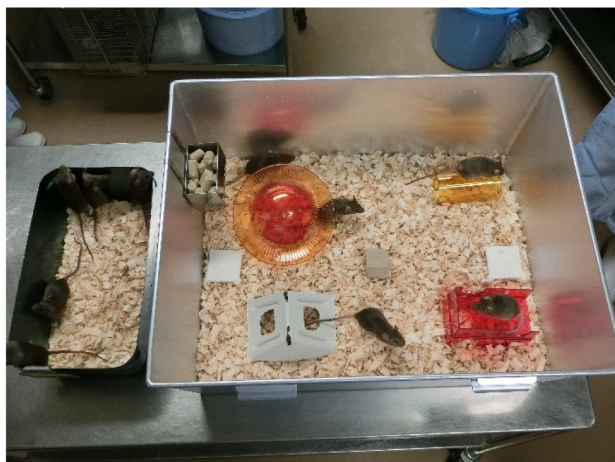

Fig. 1. Comparison of an enriched environment (EE, right) with a standard environment (SE, left).

macrophages.

2. Materials and methods

2.1. Mice

Male B6C3F1/Cr1 mice (3 weeks) were purchased from Charles River Japan and five mice per one cage were housed for either 6 or 8 weeks in a cage with either EE or a standard environment (SE) in a temperature-, humidity- and light-controlled facility (23 ± 1 °C, 45 ± 5%, 12 h-light/12 h-dark) with food and water available ad libitum. An EE cage was equipped with a running wheel, tunnel, bio-hut, wood gnawing block, shelter and nesting sheet (Fig. 1). Animal care and experimental schedules were approved by the National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST) of Japan, and were in strict accordance with the guidelines of the Institute.

In a separate experiment, male C57BL/6 mice (6 weeks) were purchased from SLC Japan and used for isolation of a neutrophil-rich population (thioglycolate broth-induced peritoneal cells). Animal care and experimental schedules were approved by the animal committee of Toho University.

2.2. Preparation of cells

Apoptotic neutrophils and secondary necrotic ones were obtained according to the method previously described [10]. Briefly, peritoneal exudate cells (PECs) were obtained 6 h after injection of a thioglycolate broth solution into the peritoneal cavity of mice. The cells included > 95% of neutrophils, which were identified on flow cytometry analysis. The cells were washed with phosphate-buffered saline (PBS; saline containing 14 mM Na_2PO_4 and 6 mM KH_2PO_4, pH 7.4) twice, and then suspended in RPMI 1640 medium containing 7% FCS at a cell density of 2×10^6 cells/ml. The cells were then cultured for 2 and 16 h, respectively. The former included 15 ± 2% apoptotic cells and 20 ± 2% secondary necrotic cells ("apoptotic neutrophils"), and the latter 5 ± 1% apoptotic cells and 75 ± 2% secondary necrotic cells ("secondary necrotic neutrophils").

To prepare peritoneal resident macrophages, PECs were obtained from mice with cold PBS, and then a cell suspension with a cell density of 10^6 cells/ml (200 µl or 1 ml) was added to a 96-well black clear bottom plate.

(Corning) or a 24-well plate (Corning) to allow adherence for 1 h. The cells were then washed with PBS three times, the resultant cells including > 90% of macrophages, as determined on HE staining or flow cytometric analysis.

2.3. Phagocytosis assay

The phagocytosis of *S. aureus* by peritoneal macrophages was assayed by using pHrodo RED *S. aureus* (Invitrogen). According to the manufacturer's information, the fluorescence of the pHrodo Red dye dramatically increases as the pH decreases from neutral to acidic conditions and the fluorescence is negligible outside the cells, enabling the measurement of phagocytosis of *S. aureus*. Peritoneal exudate cells (PECs) were isolated from mice, followed by adherence to plastic wells of microplates (flexi PERM micro12) for 1 h at 37 °C (three wells per PECs from one mouse). In some experiments, plastic adherent cells were preincubated with 20 µg/ml of anti-MCP-1 monoclonal antibody (clone 2H5) or control hamster IgG (Thermo Fischer) for 30 min. The cells were then incubated with 0.25 mg/ml of pHrodo RED *S. aureus* for 1 h at 37 °C. The cells were then washed and fixed with 4% paraformaldehyde for 20 min at 4 °C. They were then mounted with MOWIOL and stored at 4 °C. They were observed under a confocal microscope using a Fluoview system (Olympus) (three areas in each well).

The assay for phagocytosis of apoptotic cells or secondary necrotic cells was described before [11]. Briefly, apoptotic cells or secondary necrotic cells were labeled with PKH Red (Sigma), followed by co-culturing with peritoneal macrophages labeled with PKH Green (Sigma) for 2 h at 37 °C in a 96-well plate. The cells were then washed, and non-engulfed cells were removed. The cells were then fixed with 4% paraformaldehyde for 20 min at 4 °C. They were then mounted with MOWIOL and stored at 4 °C. Cells were then examined in three different areas in each well under a confocal microscope.

2.4. Measurement of cytokines

After culturing, the supernatants were harvested, followed by centrifugation. Samples were stored at −80 °C until the assay. Then MIP-2, KC, MCP-1 and IL-6 levels were determined with ELISA Development Kits (PeproTech).

2.5. Flow cytometric analysis

Cells adhered to a plastic plate were washed with PBS containing 0.1% BSA and then collected in 2 ml of PBS containing 0.1% BSA. The cell density was then adjusted to 5×10^5 cells/ml in PBS containing 1% BSA. The cells were pretreated with Fc block and mouse IgG for 30 min on ice, followed by staining with PE-conjugated anti-CD40 mAb (BD Pharmingen), PE-conjugated anti-CD206 mAb (BioLegend), or biotin-conjugated anti-HLA mAb as a control, and subsequent staining with FITC-conjugated anti-F4/80 mAb or FITC-conjugated anti-HLA mAb as a control. The cells were then incubated for 15 min with PE-conjugated streptavidin to reveal the biotinylated reagents. The stained cells were analyzed by flow cytometry with a FACS Calibur (BD Biosciences).

2.6. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from plastic adherent cells with a FastGene™ RNA basic kit (Nippon Genetics). First-strand cDNA was synthesized from total RNA with ReverTra Ace (TOYOBO) and a random primer, and used for qRT-PCR. Using KOD SYBR qPCR mix (TOYOBO), the expression of mRNA for the 12S ribosomal RNA (r12S) gene [12], as an internal standard, and the expressions of mRNAs for the genes of interest were analyzed by real time PCR (AriaMx real-Time PCR System, Agilent Technologies, Japan), and the threshold cycles of the mRNAs were determined. The concentrations of the mRNAs were then determined by means of the standard curve method, and the relative expression of mRNAs for the genes as compared to that of the 12S ribosomal RNA gene was calculated. The PCR amplification program

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