Cellular Immunology 251 (2008) 86–92

Contents lists available at ScienceDirect

Cellular Immunology

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

Expression of OX40 ligand in microglia activated by IFN-γ sustains a protective CD4$^+$ T-cell response *in vitro*

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ARTICLE INFO

Article history:
Received 2 Feburary 2008
Accepted 4 April 2008
Available online 15 May 2008

Keywords:
OX40L
Microglia
T cell
Central nervous system
Co-stimulation
Antigen-presenting cell

ABSTRACT

T-cell-dependent immunity in the central nervous system (CNS) is beneficial for neuroprotection, neurogenesis and even behavior. As a highly specialized site, the CNS is speculated to possess the means to maintain T-cell immune responses through its own resident cells. Therefore, we investigated whether microglia, the most potent antigen-presenting cells residing in the CNS, could sustain T-cell responses *in vitro*. We showed that interferon-γ (IFN-γ)-activated microglia ($MG_{IFN-\gamma}$) inducibly expressed an important immune co-stimulatory molecule, OX40 ligand (OX40L). Co-culture of activated CD4$^+$ T cells with $MG_{IFN-\gamma}$ significantly increased T-cell proliferation and decreased apoptosis, and these effects were markedly inhibited by addition of a neutralizing anti-OX40L monoclonal antibody. In addition, ligation of OX40L in $MG_{IFN-\gamma}$ enhanced their production of insulin-like growth factor I (IGF-I). These results suggest that the expression of OX40L in microglia provides a molecular basis for the maintenance of T-cell survival, expansion of T cells and increased secretion of remedial growth factor from $MG_{IFN-\gamma}$, which may contribute to the protective effect in the CNS.

1. Introduction

T-cell-dependent immunity in the central nervous system (CNS) is known to be critical in various pathological conditions, including autoimmune inflammation, microbial infection, and neurodegenerative processes [1–3]. Recently, accumulating evidence has indicated that it is required for post-injury neuronal survival and functional recovery [4–6]. Impairment of neurogenesis and spatial learning and memory abilities were observed in mice devoid of T cells [7]. This implies that a beneficial immune-associated mechanism underlies different aspects of brain plasticity and cell renewal, therefore, the longevity and activity of T cells in the CNS are more important than previously expected. On account of the tight blood–brain barrier (BBB), the CNS is a relatively independent system; once peripherally activated CD4$^+$ T cells cross the BBB endothelium and invade the CNS, their degree of activation and polarization is influenced by the CNS microenvironment [8]. Thus, the resident cells in the CNS must be the major participants in the modulation of T-cell immunity. However, the cellular and molecular events underlying the proliferation and survival of infiltrating T cells are still poorly defined.

Microglia is a resident population of cells found throughout the CNS, which play a pivotal role during CNS immune responses [9,10]. In response to certain stimuli, such as injury or infection, microglia may become activated, and perform phagocytosis and antigen presentation [11,12]. For an effective T-cell response, at least two signals are needed: one that is delivered by T-cell receptor (TCR) interaction with major histocompatibility complex (MHC) and peptides to initiate immune activation; and a co-stimulatory signal provided by antigen-presenting cells (APCs), which is crucial for growth, survival, differentiation and frequency of persisting memory cells, and their cytokine commitment [13]. Thus, various co-stimulatory molecules regulate T-cell responses at different temporal and spatial stages. Activated microglia have been characterized by increased expression of MHC class I and class II antigens, as well as co-stimulatory molecules B7-1, B7-2 and CD40 [14,15], which are essential for T-cell activation and initiation of adaptive immunity at an early stage. However, whether and how activated microglia have important roles in directing later stages of the immune response to promote T-cell survival and proliferation, and induce increased T-cell effector function remains unclear.

OX40 ligand (OX40L), originally termed glycoprotein 34 kDa (GP34), and its cognate receptor OX40 belong to the tumor necrosis factor (TNF) and TNF receptor superfamilies, respectively. OX40 is preferentially expressed by activated CD4$^+$ T cells, whereas OX40L is mainly expressed by APCs, including activated dendritic cells

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doi:10.1016/j.cellimm.2008.04.002

(DCs), B cells, macrophages, and Langerhans cells, as well as by T cells and endothelial cells [16,17]. Unlike another co-stimulatory molecule CD28, OX40/OX40L interactions maintain later rather than initial $CD4^+$ T-cell priming, and they have been shown to be crucial for T-cell expansion and survival, and for the generation of memory T cells [13]. OX40 is a promising therapeutic target for allergic, inflammatory and autoimmune diseases and tumors [17]. Interestingly, the OX40/OX40L pair of co-stimulatory molecules is a bi-directional signaling system; engagement of OX40L has been shown to promote cytokine production, proliferation of DCs, B cells and vascular endothelial cells [18–20]. Therefore, we imagine that modulation through the expression of OX40L in microglia is important in controlling the fate and function of $CD4^+$ T cell in the CNS.

In an attempt to better understand the mechanism of maintaining protective T-cell responses in the CNS, we first investigated whether interferon-γ (IFN-γ), a proinflammatory cytokine, mainly produced by activated T cells, could induce OX40L expression in cultured microglia, and then evaluated its effects on both activated $CD4^+$ T cells and microglia. The results offer an explanation for the maintenance of infiltrating T cells in the CNS to exert beneficial effects, and shed light on the relationship between the microglia and T cells.

2. Materials and methods

2.1. Mice

C57/BL6 mice were obtained from the Experimental Animal Center of Third Military Medical University (Chongqing, PR China). All mice were housed in pathogen-free conditions. They received standard diet and water *ad libitum*, and the protocol was fully accredited by the Chinese Ministry of Science and Technique for Accreditation of Laboratory Animal Care.

2.2. Preparation and treatment of microglia and $CD4^+$ T cells

For preparation of primary microglia, cells were prepared from newborn (1–2 days old) C57/BL6 mouse brain by adherence and shaking methods essentially as we previously described [10]. In brief, mouse brains were dissected and the meninges were removed under a dissecting microscope. Cerebral cortical tissue was minced mechanically and digested with trypsin (0.25%) and DNase I (0.01%). Dissociated cells were cultured in a 5% CO_2 atmosphere at 37 °C, in basal Iscove's modified Dulbecco's medium (IMDM, Gibco Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was changed every 2–3 days. After seeding for 10–14 days, when the mixed culture cells were grown at 80–90% confluence, the flasks were shaken at 200 rpm in an orbital shaker for 12 h at 37 °C to free microglia and oligodendrocytes from the more adherent astrocytes. The floating cells were collected and plated in a new flask to allow adherence for 2 h, and then shaken gently to separate oligodendrocytes from the more adherent microglia. The purity of the microglia cultures was assessed by immunostaining with microglial cell surface markers CD11b and F4/80, and more than 95% of the cells were positively stained. For the induction of OX40L, primary microglia were incubated of recombinant murine IFN-γ (BD Pharmigen, San Diego, CA). For cross-linking of OX40L in microglia, microglia were seeded in six-well cell-culture plates at 2×10^5 cells/well, and cultured with or without IFN-γ (20 ng/ml) [21], recombinant fusion protein of mouse OX40 and the Fc portion of IgG (mOX40-Fc, 1 μg/ml, R&D Systems, Minneapolis, MN) or mouse IgG Fc fragment (mIg-Fc, Jackson ImmunoResearch Laboratories, West Grove, PA) [22], used alone or in combination for 48 h.

For preparation of $CD4^+$ Th cells, 6-week-old female C57/BL6 mice were sacrificed by cervical dislocation. Murine $CD4^+$ Th cells were negatively selected from the murine splenocytes by magnetic activated cell sorting (MACS) using a mixture of biotin-conjugated antibodies against CD8, CD11b, CD45R, CD49b, TER-119 according to the manufacturer's instructions (BD Pharmigen). Then, streptavidin-conjugated magnetic particles were added to the incubates to isolate the $CD4^+$ T cells. Purity of $CD4^+$ T cells was over 90%.

2.3. RT-PCR and real-time RT-PCR

Total RNA was collected from cultured cells using Trizol reagent (Sigma, St. Louis, MO) as described previously [23]. Quantification of RNA was performed by measuring absorbance at OD260. The quality of total RNA was controlled by running 1.5% agarose gels and assessed as acceptable if strong and intact 28S rRNA and 18S rRNA bands were visible under ultraviolet light after staining with ethidium bromide. No bands of genomic DNA were observed in agarose gels. The RT-PCR was performed using a commercially available kit (Takara Biotechnology Co. Ltd., Dalian, PR China). cDNA synthesis from total RNA (0.5 μg) was carried out in a reaction volume of 10 μl. Reverse transcription was performed at 42 °C for 30 min. The reaction was stopped by denaturing the enzyme at 99 °C for 5 min. cDNA was stored at −20 °C. For each RNA sample, a reaction tube was prepared as described above but without reverse transcriptase to serve as an RT-negative control. Cycle conditions were 94 °C for 2 min, then 30 cycles of: denaturation at 94 °C for 40 s, annealing at 65 °C for 30 s, and extension at 72 °C for 1 min each, at last 72 °C for 8 min. Semi-quantification of the PCR product was done after electrophoresis on 1.5% agarose gels. Real-time RT-PCR was carried out using SYBR Green PrimeScript RT-PCR Kit (TaKaRa) on a DNA Engine Opticon 2 system (MJ Research) according to manufacturer's instructions. Primer sequences were as follows:

OX40L (GenBank Accession No. NM 009452.1):
sense 5′-CCGCTCGAGATTGTGAAGATGG-3′,
anti-sense 5′-GCTCTAGAGCCCCTCAAAGGACAC-3′;
IGF-I (GenBank Accession No. NM 001111274.1):
sense 5′-CAGGCTCCTAGCATACCTGC-3′,
anti-sense 5′-GCTGGTAAAGGTGAGCAAGC-3′;
β-actin (GenBank Accession No. NM 007393.3):
sense 5′-TAAAGACCTCTATGCCAACACAGT-3′,
anti-sense 5′-CACGATGGAGGGGCCGGACTCAT-3′.

2.4. Western immunoblot analysis

Protein extracts were prepared from cells using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) and Complete Mini Protease Inhibitor Tablets (Roche, Indianapolis, IN), followed by centrifugation at 16,000*g* for 15 min at 4 °C. The supernatant was taken as the protein extract. Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein (20–100 μg depending on protein of interest) were separated on a 12–15% SDS–polyacrylamide gel and electrotransferred onto a PVDF membrane (Millipore, Bedford, MA). Nonspecific binding of proteins was blocked by saturating the PVDF membranes with 5% non-fat dry milk and 1% BSA in TBS (20 mM Tris–HCl, 137 mM NaCl, pH 7.6) −0.1% Tween-20 (TBST) for 2 h at room temperature. Primary antibody [rat anti-mouse OX40L (clone RM134L, eBioscience, San Diego, CA); mouse anti-mouse IGF-I (clone Sm1.2, Millipore); goat anti-mouse actin (Santa Cruz, CA)] incubation was carried out overnight at 4 °C. Membranes were washed and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:4000, Dako, Denmark) for 1 h at room temperature. Finally, membranes were washed and the blots were visualized with an enhanced chemiluminescence (ECL) kit (Millipore). Quantitative densitometric analyses were performed using a