Molecular Immunology 70 (2016) 1–7

Contents lists available at ScienceDirect

Molecular Immunology

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

Functional analysis of membrane-bound complement regulatory protein on T-cell immune response in ginbuna crucian carp

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ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form
25 November 2015
Accepted 29 November 2015
Available online 11 December 2015

Keywords:
Membrane-bound complement regulatory protein
CD46-like protein
T-cell
Teleost
Ginbuna crucian carp
PHA

ABSTRACT

Complements have long been considered to be a pivotal component in innate immunity. Recent researches, however, highlight novel roles of complements in T-cell-mediated adaptive immunity. Membrane-bound complement regulatory protein CD46, a costimulatory protein for T cells, is a key molecule for T-cell immunomodulation. Teleost CD46-like molecule, termed Tecrem, has been newly identified in common carp and shown to function as a complement regulator. However, it remains unclear whether Tecrem is involved in T-cell immune response. We investigated Tecrem function related to T-cell responses in ginbuna crucian carp. Ginbuna Tecrem (*gTecrem*) proteins were detected by immunoprecipitation using anti-common carp Tecrem monoclonal antibody (mAb) and were ubiquitously expressed on blood cells including CD8α$^+$ and CD4$^+$ lymphocytes. *gTecrem* expression on leucocyte surface was enhanced after stimulation with the T-cell mitogen, phytohaemagglutinin (PHA). Coculture with the anti-Tecrem mAb significantly inhibited the proliferative activity of PHA-stimulated peripheral blood lymphocytes, suggesting that cross-linking of Tecrems on T-cells interferes with a signal transduction pathway for T-cell activation. These findings indicate that Tecrem may act as a T-cell moderator and imply that the complement system in teleost, as well as mammals, plays an important role for linking adaptive and innate immunity.

1. Introduction

Complements have long been considered vital only to innate immunity, without any role in the adaptive immune responses, until approximately 40 years ago. From the early 1970s, growing evidence in mammals has suggested that complements may be involved in the instruction and regulation of adaptive immunity, especially in B-cell-mediated antibody responses (Carroll, 2004; Carroll and Isenman, 2012). In addition, several researches have shown the involvement of the complement in the regulation of T-cell responses (Astier et al., 2000; Carroll, 2004; Longhi et al., 2006; Morgan et al., 2005; Kemper and Atkinson, 2007; Heeger and Kemper, 2012; Clarke and Tenner, 2014).

In recent years, multiple functions of CD46, a membrane-bound regulator of complement activation (RCA) protein in mammals, on T-cell immunity have been documented (Russell, 2004; Liszewski et al., 2005; Heeger and Kemper, 2012; Kemper and Kohl, 2013; Yamamoto et al., 2013). Binding of CD46 ligands triggers alterations in cytokine, chemokine (Vaknin-Dembinsky et al., 2008; Cardone et al., 2010) and nitric oxide production in T-cells, proliferation of T-cells when cross-linked with CD3 (Zaffran et al., 2001; Astier et al., 2006), isotype switching in cooperation with interleukin (IL)-4 and alterations in T-cell morphology (Marie et al., 2002). Furthermore, some recent studies have shown that CD46 is a potent costimulator for the induction of interferon-γ (IFN-γ)-secreting effector T helper type 1 (Th1) cells and their subsequent switch into IL-10-producing regulatory T cells (Cope et al., 2011; Le Friec et al., 2012; Ghannam et al., 2014).

In teleost, a CD46-like molecule, termed Tecrem, has been recently identified in common carp and was shown to function as a complement regulator (Tsujikura et al., 2015). However, it is still unclear whether Tecrem is involved in T-cell activation or regulation. The ginbuna crucian carp has been shown to be an ideal model fish for comparative immunology research, especially for studying

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http://dx.doi.org/10.1016/j.molimm.2015.11.010
0161-5890/

T-cell-mediated immunity, because of the availability of clonal fish strains and antibodies specific for T-cell subset markers (Nakanishi et al., 2011; Somamoto et al., 2013, 2014a,b, 2015). In a previous study, we found three isoforms of membrane-bound RCA protein termed ginbuna Tecrem (*gTecrem*-1, *gTecrem*-2 and *gTecrem*-3) and characterised at the mRNA level in ginbuna crucian carp (Nur et al., 2013). *gTecrem*-1, in particular, has a tyrosine phosphorylation site in its cytoplasmic tail and is expressed in T-cell subsets. These evidences suggest that *gTecrem*-1 possesses functions modulating T-cell activation or/and regulation.

The monoclonal antibody (mAb) specific for common carp Tecrem (cTecrem) has recently been established using *cTecrem*-expressing CHO cells as an immunogen (Tsujikura et al., 2015). Because common carp and ginbuna crucian carp are close species, the mAb-recognising cTecrem is expected to be cross-reactive to *gTecrem*. In this study, we first confirmed the cross-reactivity of the mAb against *gTecrem*. Furthermore, to learn whether gTecrem is involved in T-cell immunomodulation, the function of *gTecrem* proteins after stimulation with a T-cell mitogen, phytohaemagglutinin (PHA), has been analysed using the mAb.

2. Materials and methods

2.1. Animals

Clonal triploid ginbuna crucian carp (S3n strain), weighing approximately 40–120 g, was obtained from the National Research Institute of Aquaculture and maintained in a circulating water tank at 24 °C, fed with commercial diets at Kyushu University.

2.2. Blood cell separation

Erythrocytes and leucocytes were isolated using a Histopaque (d = 1.083, Sigma–Aldrich) gradient method as previously described (Nur et al., 2013). Cell suspensions were applied to Histopaque and centrifuged for 30 min at 380 × g to separate erythrocytes and leucocytes. Leucocytes on the Histopaque were collected, and erythrocytes below the Histopaque were harvested. Separated cells were washed twice with RPMI 1640 (Nissui Pharmaceutical) and adjusted to suitable concentration in each experiment.

2.3. Detection of Tecrem protein on blood cells by flow cytometry

PBLs and erythrocytes ($1–5 \times 10^6$ cells/mL) were incubated in the presence of 5% foetal bovine serum (FBS) on ice with 1/10 diluted hybridoma supernatant of anti-Tecrem mAb (1F12, isotype IgG_1) (Tsujikura et al., 2015) for 45 min, washed twice with RPMI 1640 and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (diluted 1/100, Sigma Japan) on ice for 1 h. The cells were then washed twice with RPMI 1640. Cells without anti-Tecrem mAb treatment were used as negative controls. Expressions of Tecrem were analysed with a Beckman Coulter Epics XL Flow Cytometer equipped with System II software (Beckman Coulter). The mean fluorescent intensity of Tecrem was normalised by that of the negative control and expressed as the mean fluorescence intensity rate (MFIR).

The expression of Tecrem protein on $CD4^+$ and $CD8\alpha^+$ T cells was analysed by two-colour immunofluorescence using anti-CD4 and anti-CD8α mAbs (mouse ascites: 6D1 and 2C3) (Toda et al., 2011). PBLs were first incubated with 1F12 mAb for 30 min, washed twice with RPMI 1640 and then incubated with Alexa Fluor 488-labelled goat anti-mouse immunoglobulin G (IgG; diluted 1/500, Life technology) on ice for 30 min. After washing twice, the cell suspension was equally divided, and the portions were incubated with 6D1 or 2C3 mAb for 30 min. Each labelled cell was washed twice with medium and incubated with PE-labelled goat anti-rat IgG (diluted 1/100, Abcam). The cells were then washed three times with medium. The expression of these cell surface proteins in the lymphocyte fraction was analysed by flow cytometry, as described earlier. It was confirmed that these secondary antibodies did not cross-react with rat or mouse IgG.

2.4. Immunoprecipitation of Tecrem protein on cell surface

Erythrocytes were purified using a Histopaque gradient, as described earlier. The purified erythrocytes were washed twice with phosphate-buffered saline (PBS) and then adjusted to a density of 10^8 cells/ml in PBS, to be used for biotinylation of cell surface proteins with 10 mM sulfo-NHSBiotin (Pierce), according to the manufacturer's instructions. After washing three times with PBS, the biotinylated cells were lysed and sonicated using 1 ml of NP40 lysis buffer (1% Nonidet P40, 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 10 mM NaF, 0.5 mM EDTA and 1 mM PMSF) to each millilitre of cell suspension. The cell lysates were centrifuged, and supernatants were used in the immune precipitation assay using 1F12 (5 μg/ml of the cell lysate) that was coupled to the swollen protein G Sepharose 4 Fast Flow beads (GE Life Sciences) according to the manufacturer's instructions. The beads were incubated with 1× SDS–PAGE sample buffer and boiled for 3 min; then, the supernatant obtained was directly loaded on to 12% SDS-polyacrylamide gel under reducing conditions. The proteins in the gel were electro-transferred onto nitrocellulose membranes, treated with avidin and peroxidase-conjugated biotin (Vectastain ABC kit; Vector Laboratories) and then visualised using 3,3′-diaminobenzidine (DAB).

2.5. Proliferation assay of PBLs after PHA-stimulation

PBLs (3×10^5 cells) in 100 μl of RPMI 1640 medium supplemented with 2% ginbuna serum were seeded in each well of 96-well flat-bottomed microtitre plates (Nunc, Denmark). These PBLs were then stimulated by the addition of PHA 10 μg/ml (Sigma–Aldrich Japan). Unstimulated PBLs served as the negative control. Then, cells were incubated at 25 °C in a 5% CO_2 humidified incubator for 72 h. Thereafter, the cells were subjected to a colorimetric MTT assay for cell proliferation assessment. Briefly, 10-μl MTT solution (5 mg/ml in PBS) was added directly to each well and incubated for 4 h in the dark at 37 °C. The cells were then solubilised with 100 μl of acidic isopropanol (0.1 N HCl in absolute isopropanol) and incubated overnight at room temperature. The absorbance was measured at 590 nm using a microplate plate reader (ImmunoMini NJ-2300; Nunc Japan, Tokyo, Japan).

2.6. Detection of Tecrem protein on PHA-stimulated PBLs

The PBL suspension in RPMI 1640 medium with 5% FBS was adjusted to 1.5×10^6 cells/ml and then stimulated by adding 10 μg/ml PHA. Non-stimulated PBLs served as the control. The suspension (0.5 ml) was seeded onto a 48-well plate and incubated for 5 h at 25 °C in a humidified air containing 5% CO_2. Cells were then collected, washed twice with RPMI 1640 and treated with anti-Tecrem mAbs and FITC-labelled second antibody, as described earlier. The mean fluorescent intensity of Tecrem was normalised by that of the negative control and expressed as the MFIR.

2.7. Effects of anti-Tecrem mAb on proliferation triggered by PHA

PBLs were cultured in RPMI 1640 medium in 96-well flat-bottomed microtitre plates (Nunc, Denmark) supplemented with 2% ginbuna serum and 10 μg/ml PHA, in the presence of 5, 10 or 15 μg/ml of purified anti-Tecrem IgG or 15 μg/ml of an isotype-matched irrelevant antibody (normal mouse IgG_1). PHA stimulated with PBLs without anti-Tecrem was used as the control. Then, cells