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CD45-mediated signaling pathway is involved in *Rhizoctonia bataticola* lectin (RBL)-induced proliferation and Th1/Th2 cytokine secretion in human PBMC

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ABSTRACT

We earlier reported the mitogenic and immunostimulatory activities of *Rhizoctonia bataticola* lectin (RBL), purified from phytopathogenic fungus *R. bataticola* in human PBMC. The lectin demonstrates specificity towards glycoproteins containing complex N-glycans. Since CD45-protein tyrosine phosphatase that abundantly expresses N-glycans is important in T-cell signaling, the study aimed to investigate the involvement of CD45 in the immunomodulatory activities of RBL. Flowcytometry and confocal microscopy studies revealed that RBL exhibited binding to PBMC and colocalized with CD45. The binding was comparable in cells expressing different CD45 isoforms-RA, -RB and -RO. CD45 blocking antibody reduced the binding and proliferation of PBMC induced by RBL. CD45-PTPase inhibitor dephostatin inhibited RBL–induced proliferation, expression of CD25 and pZAP-70. RBL-induced secretion of Th1/Th2 cytokines were significantly inhibited in presence of dephostatin. Also, dephostatin blocked phosphorylation of p38MAPK and STAT-5 that was crucial for the biological functions of RBL. The study demonstrates the involvement of CD45-mediated signaling in RBL-induced PBMC proliferation and Th1/Th2 cytokine secretion through activation of p38MAPK and STAT-5.

1. Introduction

Leukocyte membranes are known to contain more than 200 different proteins, most of them being glycosylated. Despite having same glycosylation machinery each glycoprotein carries characteristic glycosylation patterns resulting in heterogeneous populations of N- and O-linked glycans at each glycosylation site referred to as the glycocalyx [1]. These cell surface glycosylation patterns are essential for the regulation of critical immunological responses such as T-cell activation, migration, and apoptosis [2].

The receptor-like protein tyrosine phosphatase-CD45, is the most abundant leukocyte transmembrane protein which constitute up to 10% of the total surface area on T lymphocytes [3]. It is expressed on the surface of all human nucleated hematopoietic cells and their precursors, with the exception of mature erythrocytes and platelets. Due to alternative splicing, CD45 can exist in various isoforms- CD45RA, CD45RB, CD45RC, CD45RAB, CD45RAC, CD45RBC, CD45RO, CD45R (ABC) ranging in molecular weight from 180 to 220 kDa [4]. The extended extra-cellular domains- A, B and C of CD45 contain numerous O-glycosylation sites and therefore the high molecular weight expressing cells have more O-glycans compared to low molecular weight isoform (CD45RO) [5]. CD45 also bears abundant N-glycans, most of which are found on fibronectin like domains situated at the membrane proximal region of the molecule, and are common to all CD45 isoforms [6]. Expression of different CD45 isoforms on T cell depends on the state of activation and differentiation [7]. Mature T cells express low molecular weight isoforms of CD45- CD45RO, CD45RA and CD45RB and thus have relatively fewer O-glycans compared with B cells, which express full length CD45RABC [8]. CD45 plays a key role in antigen receptor-mediated signaling in T and B cells and signaling through cytokine receptors [9–11]. Other reports have implicated CD45 as potential genetic modifier in autoimmune, infectious, and malignant diseases [4].

We have recently reported isolation and characterization of a complex sugar specific lectin- RBL from a phytopathogenic fungus *Rhizoctonia bataticola*. The carbohydrate-recognition profile of RBL as revealed from glycan array analysis demonstrated exclusive specificity for complex high mannose type N-linked glycans

Abbreviations: RBL, *Rhizoctonia bataticola* lectin; PBMC, peripheral blood mononuclear cells; MAPK, mitogen activated protein kinase; STAT, signal transducers and activators of transcription; IL-2Rα, interleukin-2 receptor alpha; ZAP-70, zeta chain associated protein; DS, dephostatin.

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including tri- and tetra- antennary high mannose oligosaccharides. [12]. Studies also revealed that RBL is highly mitogenic towards normal human peripheral blood mononuclear cells (PBMC) and induces secretion of Th1/Th2 cytokines such as IL-2, IFN-γ, IL-4 and IL-10 [13]. In the present study we explored whether CD45 is involved in RBL-mediated immunomodulatory activities in human PBMC.

2. Materials and methods

2.1. Isolation and purification of RBL

Purification of the lectin and preparation of the fluorescein isothiocyanate (FITC)-labeled lectin was according to previously described methods [12].

2.2. Reagents and antibodies

Following anti-human antibodies -pan CD45, CD45RA, CD45RB, CD45RO, phospho p38 and FITC labeled anti-CD25 and anti-CD3 conjugated to PE were purchased from BD Biosciences (USA). Phospho ZAP-70 (tyr^{319}), phospho STAT-5 (tyr^{694}) and STAT-5 antibodies were purchased from Cell Signaling Technologies (USA). p38 antibody was obtained from Santa Cruz Biotechnologies (CA, USA). β-actin antibody was purchased from MP Biomedicals (USA). Species specific HRP-labeled secondary antibodies were procured from Biorad (USA) and fluorescent labeled secondary antibodies were procured from Molecular probes (USA). Th1/Th2 cytokine ELISA kit was obtained from eBiosciences (USA). Pharmacological inhibitor of CD45 dephostatin was procured from Sigma (USA).

2.3. Isolation of PBMC

Human PBMC were isolated by density centrifugation of heparinized blood of healthy donors using Histopaque 1077 (Sigma Chemicals). Cells collected from interface were suspended in RPMI 1640 medium supplemented with heat inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and maintained at 37 °C under 5% CO_2 in humidified incubator. The study was approved by the ethics committee of NCCS.

2.4. Flow cytometry

PBMC were treated with 3% BSA for blocking and stained with FITC-labeled RBL and CD45 antibodies followed by Cy3-conjugated secondary antibody. Data was acquired for 10,000 events on BD FACS Calibur cytometer (Becton Dickinson, San Jose, CA) and analyzed using cell quest-pro software. Unstained cells processed similarly were used as negative control. For blocking studies, PBMC were treated with CD45 mAb for 1 h followed by RBL-FITC staining and acquisition by flow cytometry. To study the expression of CD25 in presence or absence of CD45-inhibitor dephostatin, PBMC were exposed to dephostatin followed by RBL (1.25 μg/ml) stimulation and harvested at 24 h. The cells were stained with FITC-labeled anti-CD25 antibody and PE-labeled CD3 antibody and flow cytometry analysis was performed.

2.5. Expression of phospho-ZAP-70 (tyr^{319})

To determine the expression of phosphorylated proteins by flow cytometry BD phosflow system was used. PBMC treated with RBL for the specific time periods were fixed in pre-warmed fixation buffer to maintain their phosphorylation state. The cells were then permeablized by adding Perm Buffer followed by washing and incubation with appropriate concentration of primary and fluorochrome-conjugated secondary antibody. Cells were then washed and data acquired on flow cytometer (FACS Calibur).

2.6. Confocal laser scanning microscopy

For double immunofluorescence, PBMC were stained with FITC-conjugated RBL and anti-human CD45 mAb followed by cy3-conjugated anti-mouse secondary antibody and visualized using confocal laser scanning microscope Ziess (USA). Nuclei were stained using DAPI.

2.7. Proliferation assay

The dose and time kinetics of RBL stimulation of PBMC were determined in our previous studies [13]. PBMC in the presence or absence of CD45 blocking antibody or dephostatin (12.5 and 25 μM) were stimulated with 1.25 μg/ml of RBL for 72 h. During the last 18 h, tritiated thymidine 1 μCi/well (Board of Radiation and Isotope Technology, India) was added and the stimulation was measured as counts per minute (CPM).

2.8. Cytokine ELISA assay

Culture supernatants were collected from PBMC stimulated with RBL (1.25 μg/ml) in presence or absence of CD45 (25 μM) and analyzed for IL-2, IFN-γ, IL-4 and IL-10 secretion using cytokine sandwich ELISA kit (eBiosciences), following the manufacturer's protocol. Conditioned medium from unstimulated PBMC was used as negative control.

2.9. Western blotting

PBMC were stimulated with RBL in presence or absence of dephostatin for 12 h. The cells were lysed using RIPA lysis buffer (120 mM NaCl, 1.0% Triton X-100, 20 mM Tris–HCl, pH 7.5, 100% glycerol, 2 mM EDTA, protease inhibitor cocktail, Roche, Germany) and total protein was electrophoresed on 10% SDS-polyacrylamide gels and blotted onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% BSA, the blots were probed with antibodies to phospho-p38 and -STAT-5 (tyr^{694}). The bands were visualized by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, USA). The blots were stripped and reprobed for the total forms of the proteins and actin was used as loading control.

2.10. Statistical analysis

Statistical analysis was performed using Student's t test and Mann–Whitney rank sum test. A p-value <0.05 was considered to be statistically significant.

3. Results

3.1. Binding of RBL to CD45 and its isoforms on human PBMC

The binding of RBL to CD45 positive PBMC was determined by dual color flow cytometry analysis. Cells were stained with FITC-labeled RBL and pan-CD45 mAb followed by appropriate secondary antibody conjugated to PE, the data was acquired on the FL-1 and FL-2 channels of a flow cytometer. The dot plots shown in Fig. 1A revealed that 99% of the PBMC were positive for both CD45 and RBL binding. Characterization of localization of RBL-binding sites and CD45 on PBMC was done by confocal laser scanning microscopy, with cells stained with FITC-RBL and anti-pan-