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Modulation of CD6 function through interaction with Galectin-1 and -3 ☆

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ABSTRACT

CD6 is a lymphocyte glycoprotein receptor that physically associates with the antigen-specific receptor complex at the center of the immunological synapse, where it interacts with its ligand CD166/ALCAM. The present work reports the carbohydrate-dependent interaction of CD6 and CD166/ALCAM with Galectin-1 and -3, two well-known soluble mammalian lectins. Both galectins interfered with superantigen-induced T cell proliferation and cell adhesion phenomena mediated by the CD6-CD166/ALCAM pair, while CD6 expression protected cells from galectin-induced apoptosis. The results suggest that interaction of Galectin-1 and -3 with CD6 and CD166/ALCAM might modulate some relevant aspects of T cell physiology.

Structured summary of protein interactions:
Gal-3 binds to **CD6** by pull down (View interaction)
Gal-1 binds to **CD6** by pull down (View interaction)
Gal3 binds to **ALCAM** by pull down (View interaction)
Gal1 binds to **ALCAM** by pull down (View interaction)
Gal-3 binds to **CD6** by enzyme linked immunosorbent assay (View interaction)
Gal-1 binds to **CD6** by enzyme linked immunosorbent assay (View interaction)
ALCAM binds to **Gal3** by enzyme linked immunosorbent assay (1, 2)
ALCAM binds to **Gal1** by enzyme linked immunosorbent assay (View interaction)
ALCAM physically interacts with **Gal1** and **CD6** by competition binding (View interaction)
CD6 binds to **ALCAM** by enzyme linked immunosorbent assay (View interaction)

1. Introduction

Galectins are a family of evolutionarily conserved animal lectins serving as pattern-recognition receptors (PRRs) [41], which have attracted recent interest as regulators of immune responses [19,30]. The carbohydrate-recognition domains (CRDs) of galectins specifically bind to β-galactoside-containing cell surface receptors and subsequently trigger signaling events, modulating cellular processes such as cell growth, survival, adhesion, cytokine secretion or migration [19].

Galectin (Gal)-1 and -3 are the most ubiquitously expressed and most widely studied members of the galectin family. Within the immune system, they are co-expressed in many cells from primary and secondary lymphoid organs, with their expression levels depending on the cell activation and differentiation status [36]. These include thymic epithelial cells (TEC) [45,4], activated T cells

Abbreviations: ALCAM, Activated Leukocyte Cell Adhesion Molecule; Gal-1/-3, Galectin-1/-3; PRRs, pattern-recognition receptor; CRD, carbohydrate-recognition domains; DC, dendritic cell; TEC, thymic epithelial cells; SRCR-SF, scavenger receptor cysteine-rich superfamily; rshCD5/6, recombinant soluble human CD5/6; o/n, overnight; TMB, 3,3′,5,5′-tetramethylbenzidine; RT, room temperature; LTA, lipoteichoic acid; PGN, peptidoglycan; PFA, paraformaldehyde; PE, phycoerithrin; SEB, staphylococcal enterotoxin B; K_d, dissociation constant

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[5,15,31], activated B cells [48,1], inflammatory macrophages [40,29,36], dendritic cells (DCs), endothelial cells and fibroblasts [32]. Both Gal-1 and -3 bind to T cells through recognition of still not fully characterized and not fully overlapping surface glycoproteins, including CD29, CD43 and CD45 [44], among others. Through these interactions, Gal-1 and -3 have been shown to modulate multiple T cell functions, both in vitro and in vivo [30,20].

CD6 is a surface receptor belonging to the scavenger receptor cysteine-rich superfamily (SRCR-SF), another ancient and highly conserved group of innate-like receptors, characterized by the presence of one or several repeats of a cysteine-rich extracellular domain named SRCR [21,33]. CD6 is mainly expressed on lymphocytes (thymocytes, mature T cells, NK cells and B1a cells) [3,16,9], but also on hematopoietic cell precursors [10] and certain brain regions [17]. There it plays a double role as a PRR and also as an accessory molecule involved in cell signaling and cell–cell adhesion contacts taking place during lymphocyte activation and differentiation [21,8,28]. The latter is essentially achieved through its physical association to the TCR/CD3 complex [11], as well as its interaction with CD166/ALCAM (Activated Leukocyte Cell Adhesion Molecule), an adhesion molecule of the Ig superfamily [27]. Moreover, CD6 co-localizes with the TCR/CD3 complex at the centre of the immunological synapse [11], where its interaction with CD166/ALCAM results critical for maturation and stabilization of this signaling structure as well as for subsequent T cell proliferative responses [11,13,47]. This, together with multiple functional studies with anti-CD6 mAbs, has resulted in CD6 being considered as an important T cell co-stimulatory molecule [34]. However, this view was recently challenged by data suggesting that CD6 could also act as a negative regulator of T cell activation [25].

Given that CD6 is a glycoprotein, and that some members of the SRCR-SF interact with galectins [14,23,39], potential interactions between CD6 and Gal-1 and -3 and their consequences have been explored. The results indicate that both galectins interact not only with CD6 but also with its ligand CD166/ALCAM in a carbohydrate-dependent manner. In this way, Gal-1 and -3 are shown to modulate several lymphocyte functions dependent on CD6- and CD166/ALCAM-mediated interactions.

2. Experimental procedures

2.1. Production and purification of chimerical recombinant proteins

Recombinant soluble human CD5 (rshCD5), CD6 (rshCD6), and ALCAM-Fc proteins were produced and purified as described [11,24,38].

pGex constructs coding for WT (rhGal3) and mutant (R186S) human Gal-3, and for Glutathione S-transferase (GST) fusion proteins (GST-Gal1, GST-Gal3) were kindly provided by Fu-Tong Liu, and H. Leffler (Lund University, Sweden) and expressed in *Escherichia coli* BL21 strain. For protein production, bacteria were grown at 37 °C in 2YT broth supplemented with 0.1 mg/ml of ampicillin until absorbance at 600 nm reached 0.5–0.8. Then 0.2 mM Isopropyl β-D-1-thiogalactopyranoside was added and bacteria cultured at 25 °C overnight (o/n). Bacterial pellets were resuspended in 60 ml of lysis buffer (20 mM Tris–HCl pH 7.5, plus 5 mM EDTA, 20 mM β-mercaptoethanol (ME), 10 mM sucrose, and 2.5 ml Complete protease inhibitor (Roche), and sonicated three times. The supernatant was clarified by centrifugation and then incubated at 4 °C for 2 h with 1.5 ml of Glutathione Sepharose 4B (GE Healthcare) beads (50%, v/v). After extensive washings with lysis buffer (omitting EDTA) and 20 mM Tris–HCl, pH 7.5, GST proteins were eluted by addition of 50 mM Tris–HCl pH 8.0 plus 10 mM glutathione for 10 min at room temperature (RT). The same procedure was followed for production of WT and R186S rhGal3, except that bacterial solubilisates were loaded onto a Lactosyl-Sepharose column (Sigma–Aldrich) and protein eluted by addition of PBS plus 25 mM lactose and 10 mM β-ME.

Endo Trap® red columns (Hyglos GmbH) were used to remove endotoxin from all the recombinant and chimerical protein preparations used in this study, following manufacturer's instructions.

Chimerical or recombinant proteins were biotin-labeled using EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific), following manufacturer's instructions.

2.2. Pull-down assays

GST-Gal proteins adsorbed onto Glutathione Sepharose beads were blocked in TBS plus 5% BSA, and then incubated o/n at 4 °C with 0.1 μg biotin-labeled proteins (rshCD6, rshCD5) or ALCAM-Fc, in the presence or absence of increasing concentrations of lactose or sucrose. Beads were then washed three times with TBS and bound proteins were eluted with Laemmli's sample buffer and separated by 8% SDS–PAGE for further Western blot analysis with HRP-labeled streptavidin (Roche) or HRP-labeled $F(ab')_2$ anti-human Ig Fc antiserum (Jackson Immunoresearch), and development by chemiluminescence (Supersignal West Dura Extended Duration Solution, Pierce).

2.3. ELISA assays

96-well plates (Nunc, Roskilde, Denmark) coated o/n at 4 °C with purified GST-Gal1, GST-Gal3 or GST (1 μg/well) were blocked with PBS plus 3% BSA. Plates were then incubated with biotinylated rshCD5 or rshCD6 and bound protein was detected with HRP-labeled streptavidin (Roche). Color was developed by adding 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Sigma) and absorbance measured at 450 nm.

Interaction of galectins with ALCAM-Fc was analyzed as above, except that plates were coated with ALCAM-Fc (1 μg/well) and then assayed for binding to biotinylated GST-Gal1, GST-Gal3 or GST proteins.

Similarly, interaction of WT or mutant rhGal3 with CD6 or ALCAM-Fc was performed by coating plates with rhGal3 or R186S (1 μg/well) and incubating them with either biotin-labeled rshCD6 or unlabeled ALCAM-Fc. Bound proteins were detected with HRP-streptavidin or HRP-labeled $F(ab')_2$ anti-human Ig Fc antiserum (Jackson Immunoresearch), respectively.

Competition assays were performed by coating plates with lipopolysaccharide (LPS) from *E. coli* K12 O111:B4, lipotheichoic acid (LTA) or peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma) or ALCAM-Fc (all at 1 μg/well). Then, a fixed amount (500 ng) of biotin-labeled rshCD6 was pre-incubated with increasing concentrations of unlabeled purified GST-Gal1, GST-Gal3 or GST proteins and added to the wells for further detection with HRP-streptavidin and TMB as above.

2.4. Cell adhesion assays

96-well plates coated o/n at 4 °C with purified rshCD6 or ALCAM-Fc (1 μg/well) were blocked with RPMI 1640 plus 0.5% BSA. Then, 10^5 RAJI B-cells ($ALCAM^+$) or Jurkat T-cells ($CD6^+$) (American Tissue Culture Collection) were added to wells, respectively, and left to adhere for 30 min at 37 °C in the presence or absence of different amounts of purified GST, GST-Gal1, GST-Gal3, rshCD6 or ALCAM-Fc proteins. After washing, adhered cells were fixed with 4% paraformaldehyde (PFA) for 15 min and washed before adding crystal violet diluted in 2% ethanol. The plate was then extensively washed with water and cell adhesion was analyzed by adding 2% SDS and measuring absorbance at 620 nm.