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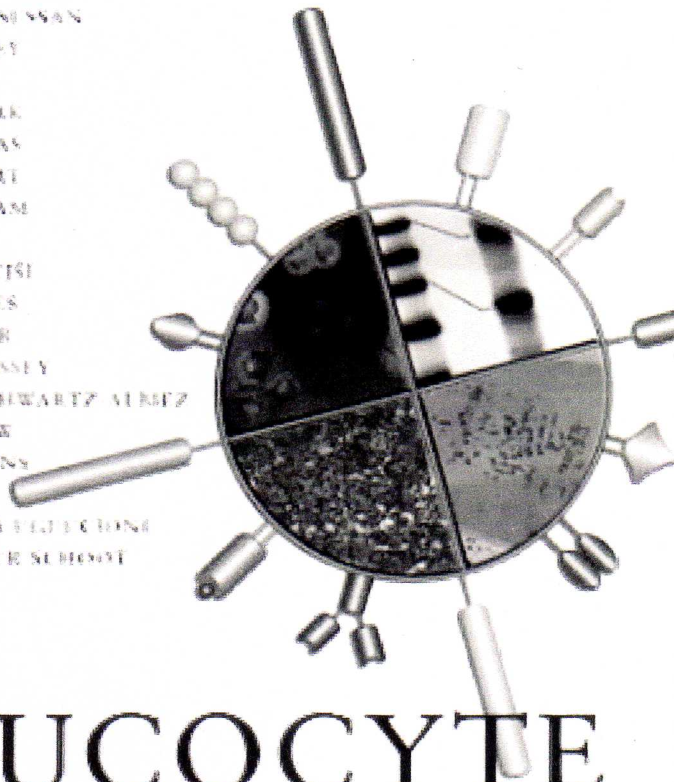

LEUCOCYTE TYPING VII

were well correlated with titres of DNP-specific antibody. The result suggests that sCD100 might play a role in humoral immune responses.

In order to explore the possible pathological involvement of CD100/Sema4D in autoimmune diseases, we then examined serum levels of sCD100 in MRL/lpr mice. Serum concentrations of sCD100 were significantly elevated in MRL/lpr mice (127 ± 89 ng/ml), while C57BL/6, BALB/c and MRL/n mice did not have detectable levels of serum sCD100 (< 4 ng/ml). We compared sCD100 and anti-ssDNA antibody levels in MRL/lpr mice at various weeks of age. Although neither sCD100 nor anti-ssDNA autoantibodies were detectable before eight weeks of age in MRL/lpr mice, they gradually increased with age (data not shown). Moreover, serum levels of sCD100 seemed to be well correlated with ssDNA levels in each MRL/lpr mouse at 14 and 16 weeks of age ($r = 0.773$, $p < 0.01$ for 14-week-age mice; $r = 0.697$, $p < 0.01$ for 16-week-age mice).

Our findings demonstrated that sCD100 proteins released from activated lymphocytes have activities which stimulate B cells. Large amounts of sCD100 were detected in sera of immunized mice as well as autoantibody-producing MRL/lpr mice. Functional sCD100 might contribute for abnormal B-cell proliferation and autoantibody production in MRL/lpr mice. The present study provides clear evidence for the involvement of this semaphorin in disease progression, and also suggests that it might be feasible to treat autoimmune diseases by blocking co-stimulatory pathways.

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SF5 The identification of critical adhesiotopes on the N-domain of human CEACAM1 required for homophilic interactions

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The human carcinoembryonic antigen (CEA; CD66) family is composed of 29 different genes, and classified into the CEACAM, pregnancy specific glycoprotein (PSG) and CEACAM-pseudogene (ps) subgroups [1]. The CEACAM proteins encompass CEA, and CEACAM1, 3, 4, and 6–8 [1]. CEACAM1 molecules on leukocytic, endothelial and epithelial cells function as homophilic adhesion molecules, tumour suppressors, regulators of adhesion and immunosurveillance, and as heterophilic adhesion receptors for E-selectin and for *Neisseria meningiditis, Neisseria gonorrhoeae* and murine coronaviruses [1–12]. The eight transmembrane isoforms of human CEACAM1 all possess an extracellular N-terminal IgV-set domain, followed by no (CEACAM1-1L and-1S), two (A1, B for CEACAM-3L and-3S) or three (A1, B, A2 for CEACAM-4L and-4S) IgC2-set domains, or have the A2 domain replaced by a serine-threonine rich non-Ig sequence (Y, Z for CEACAM1-3AL and-3AS). Alternative splicing of the cytoplasmic exons generates long (L) or short (S) CEACAM1 variants [1].

Key amino acid residues on the GFCC′C″ face of the CEACAM1 N-terminal domain involved in homophilic adhesion

The N-terminal domain of CEACAM1 has been implicated in mediating homophilic adhesion [1,8,12]. Both CEACAM1-4L and CEACAM1-4S, and to a lesser extent CEA, CEACAM3 or CEACAM6, transfectants are bound to soluble CEACAM1-4-Fc (Fig. 1). Reduced binding of CEACAM1-4L cells occurred if CEACAM1-4-Fc were replaced by CEACAM1-1-Fc and CEACAM1-3-Fc, suggesting that the IgC2 and long cytoplasmic domains stabilize or increase the avidity of binding [8]. The basic structure of

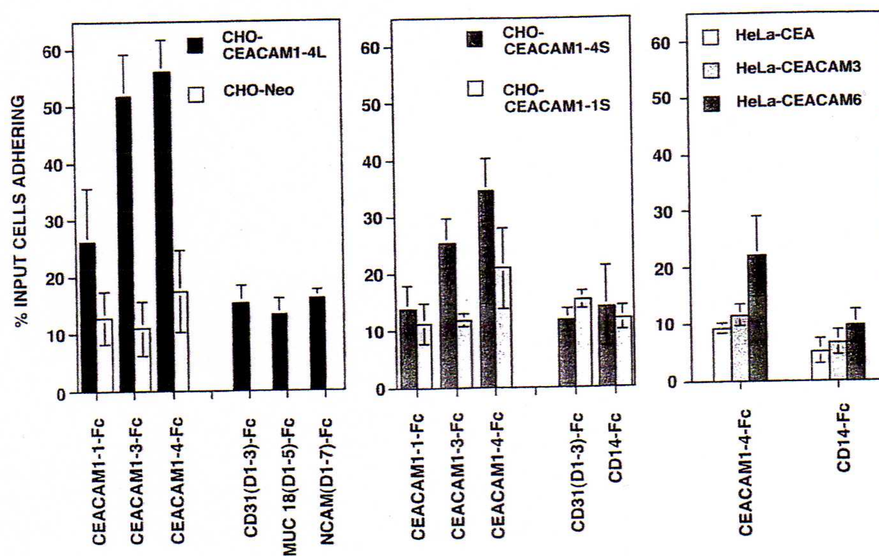


Fig. 1 CHO-CEACAM1-4L and -4S transfectants adhere preferentially to soluble recombinant human CEACAM1-3-Fc and CEACAM1-4-Fc domain deletion variants. Note weak binding of CEA, CEACAM3 and CEACAM6 transfectants to CEACAM1-4-Fc and of CEACAM1-4L to CEACAM1-1-Fc.

the N-terminal domain of CEACAM1 is a predicted tertiary fold of a stacked pair of β-pleated sheets, with strands, A, B, E and D lying anti-parallel in one sheet and strands C, C′, C″, F and G being anti-parallel in the other (Fig. 2). Amino acid sequences within the N-terminal domain of CEACAM1 were aligned with those of other human CEACAM family members and with human and rat CD2 and human CD58 on the basis of their X-ray crystallographic co-ordinates (Fig. 3) [13,14]. To identify key amino acids contributing to CEACAM1 homophilic adhesion, surface exposed and solvent accessible amino acids in the N-terminal domain of CEACAM1-3-Fc were mutated to alanine residues, nine on the GFCC′C″ face and four on the ABED face (Fig. 2 and 3).

CEACAM1 N-domain reactive and conformationally dependent CD66/CEACAM Mabs

Using CHO or HeLa transfectants expressing CEACAM1-4L, CEACAM1-4S, CEACAM1-1S, CEA, CEACAM3L, CEACAM6 and CEACAM8 or soluble recombinant CEACAM1 isoforms, two mAbs, 26H7 and 5F4, appeared to be specific for CEACAM1 (11). The 26H7, 5F4, 12-140-4, 4/3/17, COL-4, YG-C28F2, D14HD11, 34B1, B18.7.7, D11-AD11, HEA 81, CLB-gran-10, F34-187, T84.1, B6.2 and B1.1 mAbs all recognize the N-terminal domain of CEACAM1. The F36-54, YG-C94G7, 12-140-5 and TET-2 mAbs, although not CEACAM1 specific, react with both the CEACAM1-3-Fc and CEACAM1-4-Fc constructs, but not the CEACAM1-1-Fc protein. Only one mAb, F34-187, reacted equally well with the native and denatured forms of the CEACAM1-4-Fc protein. Eight mAbs, CLB-gran 10, T84.1, B18.7.7,

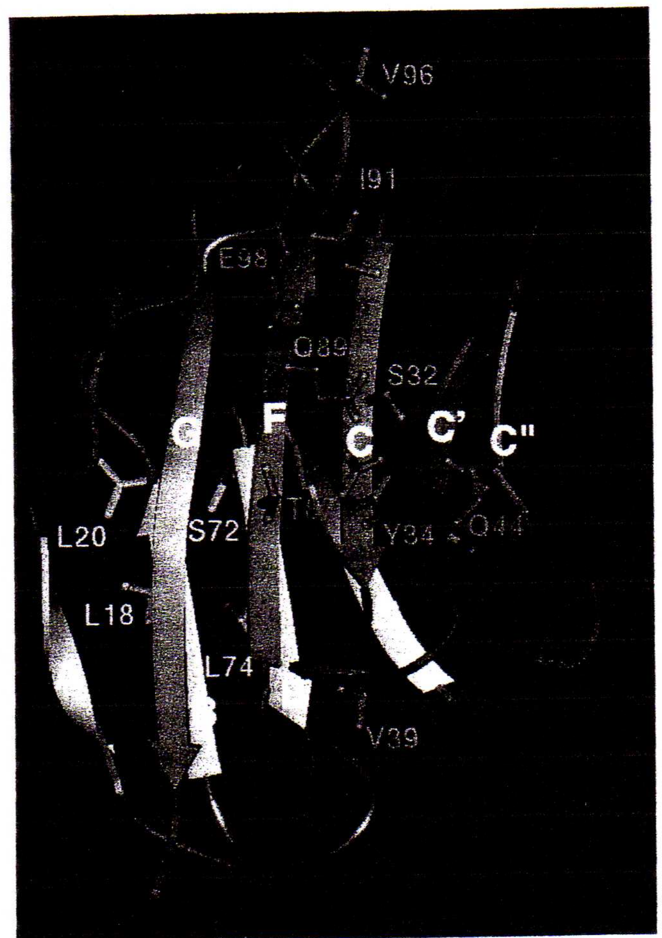
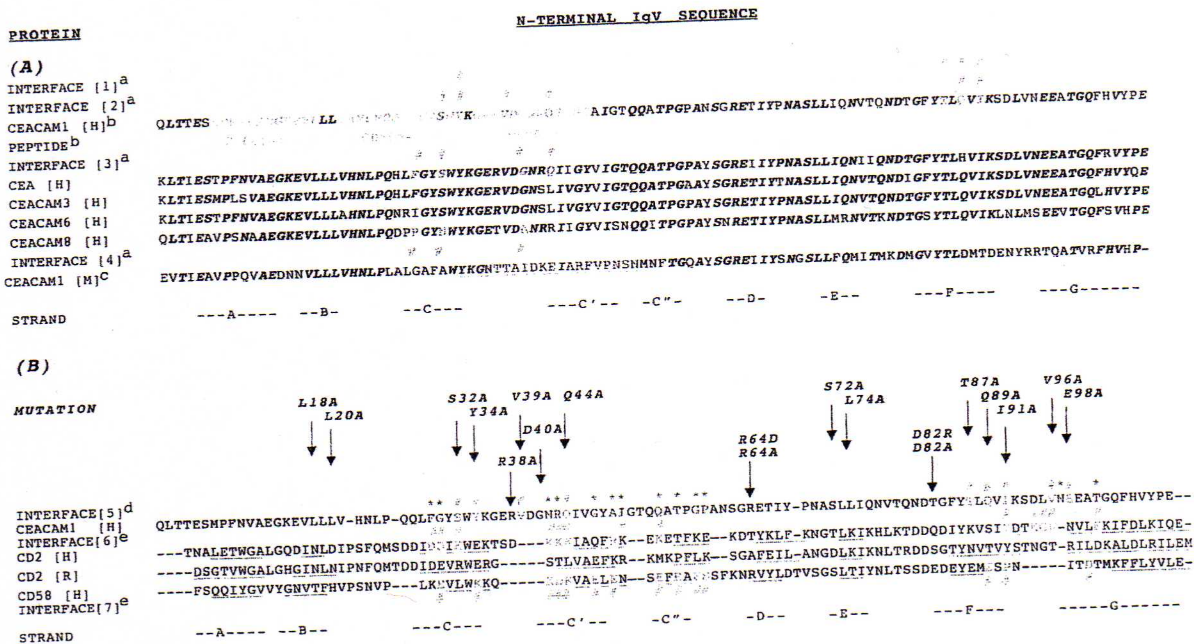


Fig. 2 Molecular model of the N-terminal IgV set domain of human CEACAM1. Ribbon diagram of the N-terminal domain of CEACAM1 showing the predicted homophilic interface.



a. Sites of Opa interactions with CEACAM1; b. Peptides regulating CD11/CD18 & L-selectin expression on neutrophils; c. Critical sequence for mouse ceacam1 binding to coronaviruses; d. Amino acid residues predicted to mediate homophilic interactions on CEACAM1; e. Amino acids at the human CD2 and CD58 interfaces [13, 14].

Fig. 3 Amino acid alignments of the N-terminal CEACAM1 IgV domain. Amino acids in CEACAM1 conserved in other family members are indicated in bold type. Strand positions are underlined and marked under the sequence and lettered A to G. Mutated residues are indicated by arrows.

D14HD11, HEA 81, B1.1, 34B1 and 4/3/17, reacted with the native form and, to a lesser extent, with the denatured protein. Nine mAbs, YG-C94G7, TET-2, 12-140-5, COL-4, 26H7, 5F4, B6.2, YG-C28F2 and 12-140-4, of which the latter six are N-terminal domain reactive, reacted preferentially with the native protein and were conformation dependent [8].

The GFCC′C″ face and CC′ loop of N terminal domain of CEACAM1 are crucial for mediating homophilic adhesion

Analyses of mutant proteins with conformation dependent mAbs indicated that the majority of mutations did not substantially affect the structural integrity of CEACAM1. Exceptions were: COL-4, which did not react with the I91A or T87AQ89AI91A mutants; 12-140-4, which failed to bind to the Y34A or S32AY34AV39A mutants; and 5F4, which did not bind the Y34A, S32AY34AV39A or T87AQ89AI91A mutants and showed reduced adhesion to T87A, Q89A and I91A.

The mutated soluble recombinant molecules were screened for their ability to mediate adhesion to CHO-CEACAM1-4L transfectants (Fig. 4). Most notably, the V39 and D40 residues on the CC′ loop play a critical role in homophilic adhesion, with a lesser contribution from the S32 residue on the Cβ strand. Adhesion was abrogated if residues T87, Q89 and I91 on the Fβ strand were mutated together. Since these residues are all located on the non-glycosylated GFCC′C″ face of CEACAM1, the N-domains of CEACAM1 may interact in a so-called 'hand-shaking' fashion [13,14]. Conservative mutations of exposed amino acids on the ABED face of CEACAM1 did not significantly affect homophilic adhesion (Figs. 2–4).

These studies support the view that the GFCC′C″ faces of the Ig family members may have evolved as a sticky patch to recognize a variety of protein–protein interactions. The interface of CEACAM1 is much less hydrophilic than that involved in CD2 interactions with either CD2 or CD58, where charged residues are engaged in a complex salt bridge network ensuring high specificity with interacting co-receptors. For CEACAM1, only one salt bridge occurs at the adhesive interface. Hydrophobic residues at the CEACAM1 adhesive interface are V39, I91, F29, I50, and V96. The amino acid residues involved in CEACAM1 interactions with the neisserial Opa proteins differ from, but also overlap to some degree, those required for homophilic

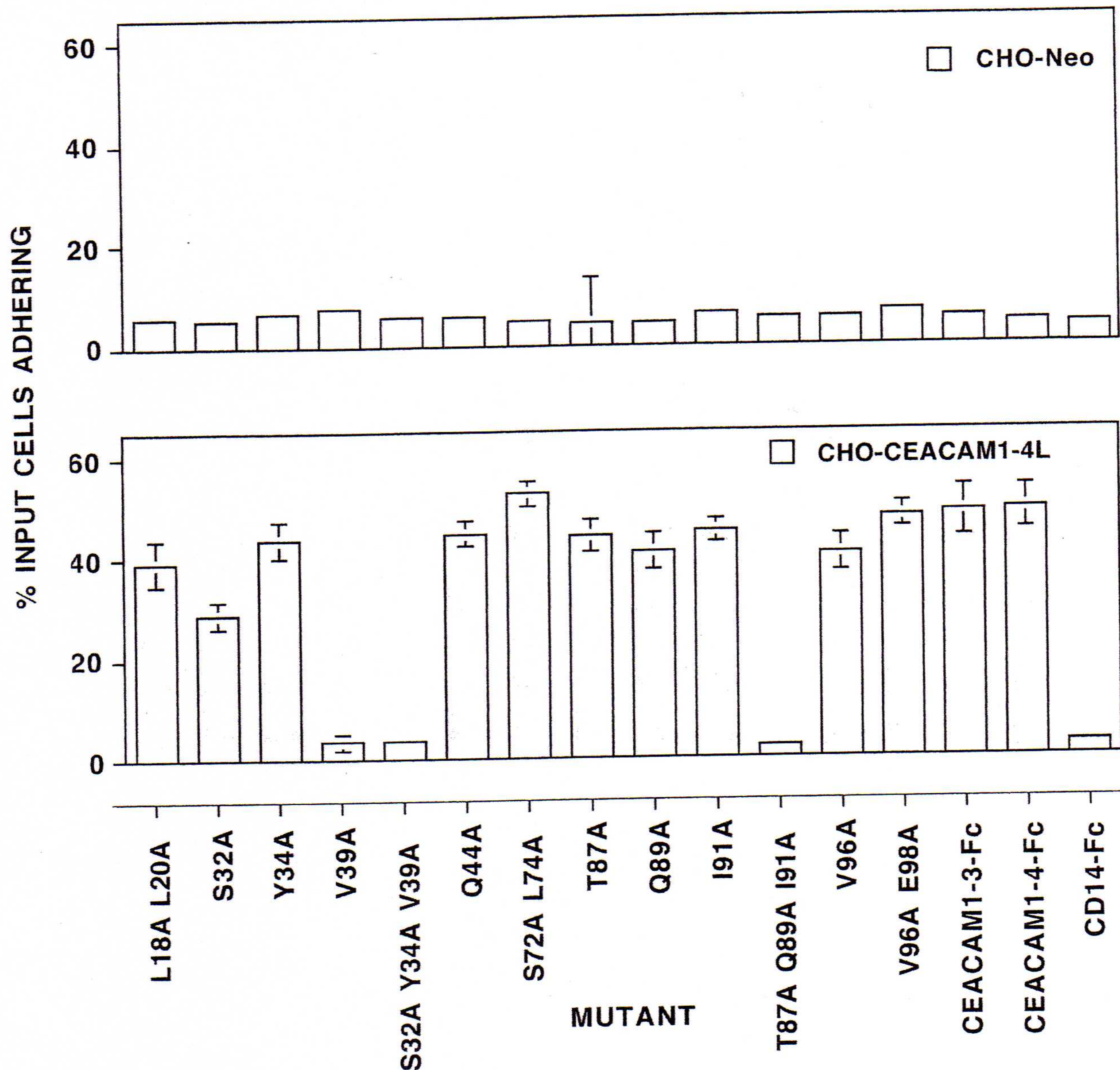


Fig. 4 The GFCC′C″ face of the CEACAM1 N terminal domain contributes to homophilic adhesion. Adhesion of mutated CEACAM1-3-Fc proteins or of the unmodified CEACAM1-3-Fc, CEACAM1-4-Fc or CD14-Fc to CHO-CEACAM1-4L transfectants (mean ± SD; $n=6$).

adhesion [2,7,8]. Both the homophilic and heterophilic CEACAM1 interfaces are more hydrophobic than hydrophilic in nature and may bind with greater affinity than interactions involving CD2 and CD58. A high degree of sequence similarity exists between the N-domains of CEACAM1 and CEA, with the GFCC′C″ faces showing few differences (Fig. 3). A model of CEA [15] has suggested that the Ig domains of CEA dimerize and subsequently align in parallel on the surface of the cell, making residues in the N-terminal domain of CEA accessible for homophilic adhesion in trans. Although biochemical studies [12] suggest that high affinity homophilic binding involves domains 1 and 6 of CEA, this does not preclude a first phase of lower affinity binding between just the N-terminal domains of CEA as the two cell surface membranes approach each other. The subtle sequence variations on the GFCC′C″ faces of CEA and CEACAM1 are a key feature for further study and X-ray crystallographic analyses.

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SF6 Selective anti-tetraspanin antibody activation of RBL cells transfected with human tetraspanins

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Monoclonal antibodies (mAbs) against some members of the tetraspanin superfamily of membrane proteins (CD63, CD9, CD81), have been shown to stimulate the degranulation of rat basophilic leukaemia cells (RBL2H3) transfected with human tetraspanins [1–4] The cellular response is stimulated by murine IgG1, but not by IgG2a or IgM mAb, and is not due to intercellular interaction of immobilized mAb with Fc receptors [4]. Degranulation requires cross-linking of the tetraspanin with the endogenous high affinity IgE receptor, FcεRI. One human tetraspanin, CD9, has been

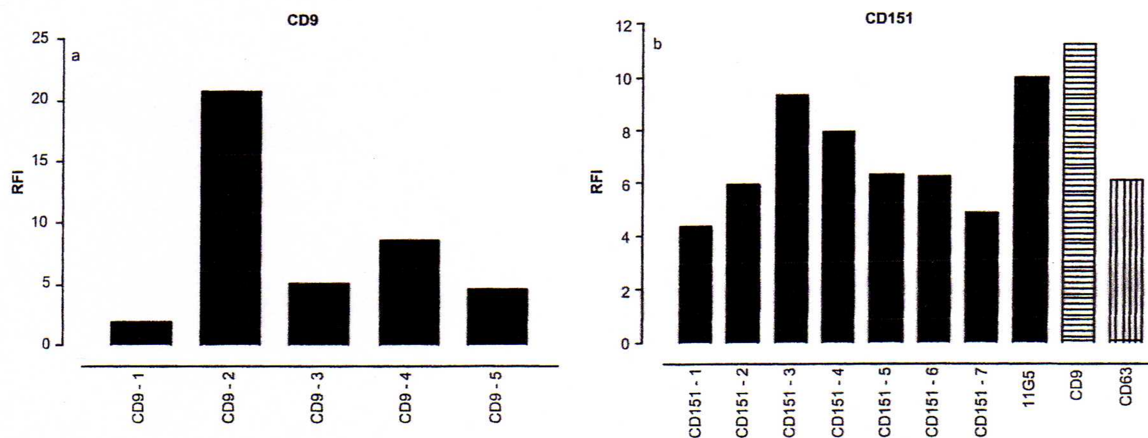

Fig. 1 Relative binding intensity of (a) CD9 and (b) CD151 panel antibodies to human tetraspanin-transfected RBL2H3 cells as determined by FACS analysis.