

A Soluble Multimeric Recombinant CD2 Protein Identifies CD48 as a Low Affinity Ligand for Human CD2: Divergence of CD2 Ligands during the Evolution of Humans and Mice

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Summary

To search for possible ligands of CD2 distinct from CD58 (lymphocyte function-associated antigen 3), we have produced a soluble pentameric CD2-immunoglobulin (Ig) fusion protein (spCD2) linking the 182-amino acid human CD2 extracellular segment with CH₂-CH₃-CH₄ domains of human IgM heavy chain, thus enhancing the micromolar affinity of the CD2 monomer through multimeric interaction. Using quantitative immunofluorescence and standard stringency wash conditions, we observed that the binding of spCD2 to human B lymphoblastoid JY cells and red blood cells is virtually inhibited by anti-CD58 TS2/9 monoclonal antibody, even though these cells express levels of CD48 and CD59 comparable to CD58. Consistent with these results, spCD2 did not show any binding to Chinese hamster ovary (CHO) cells transfected with human CD48 or CD59. However, binding studies on CD48-, CD58-, or CD59-transfected CHO cells with spCD2 under low stringency wash conditions revealed that human CD48 is a low affinity ligand of human CD2 compared with CD58 ($K_d \sim 10^{-4}$ vs. $\sim 10^{-6}$ M, respectively). The findings are noteworthy given that in the murine system CD48 is the major ligand for CD2. No detectable binding was observed to CD59-transfected CHO cells despite a report suggesting that CD59 may bind to the human CD2 adhesion domain. Importantly, in cell-cell adhesion assays between CD2⁺ Jurkat T cells and CD48- or CD59-transfected CHO cells, there was no conjugate formation, whereas binding of Jurkat T cells to CD58-transfected CHO cells was readily detected. Collectively, our findings provide evidence for a conservation of the CD2-CD48 interaction in the human species that may be of limited, if any, functional significance. Given the importance of the CD2-CD48 interaction in the murine system and CD2-CD58 interaction in humans, it would appear that there has been a divergence of functional CD2 ligands during the evolution of humans and mice.

The CD2 molecule on T lymphocytes is a transmembrane surface glycoprotein that facilitates cell-cell contact (1-3). The amino-terminal domain of CD2 (domain 1) mediates its adhesion function by binding to LFA-3 (CD58) (3), another cell surface glycoprotein widely expressed on various cell types, including hematopoietic and epithelial cells (4). Both CD2 and CD58 are members of the Ig gene superfamily (5). The importance of CD2 function in the normal human response has been well documented: (a) for the process of cognate recognition involving Th cells and APC (6, 7); (b) for the cytolytic effector function of NK cells and CTL (8,

9); and (c) as a basis of the thymocyte-thymic epithelial cell interaction (10, 11). Moreover, in T lymphocytes perturbation of the extracellular segment of CD2 with specific mAbs leads to activation via a TCR-dependent mechanism (12, 13). Also, engagement of CD2 by its ligand CD58 together with mAbs specific for the membrane proximal second domain of CD2 (domain 2) can activate T cells (14), indicating that coordinate binding of ligands to both domains of CD2 generates an intracellular activation signal.

Whether there are other ligands of CD2 domain 1 or any natural ligands of CD2 domain 2 is presently uncertain. How-

ever, in view of known alternative ligands of the integrin receptor LFA-1 (15–17) as well as the Ig supergene family receptors intercellular adhesion molecule type 1 (ICAM-1) (15, 18) and B7 (19, 20), this is a distinct possibility. To search for CD2 ligands distinct from CD58, we have produced a pentameric form of soluble CD2 (spCD2)¹ as a probe. This protein is a fusion product comprising the entire 182-amino acid (aa) extracellular segment of human CD2 and the CH₂, CH₃, and CH₄ domains of the human μ heavy chain. The avidity of this multimeric CD2 construct overcomes the low (micromolar) affinity of the monomeric CD2 extracellular segment for its ligand(s) and makes it possible to utilize the extracellular CD2 segment as a probe to define alternative ligands.

Analysis of the binding of biotinylated spCD2 to JY cells and other B lymphoblastoid, monocytic, and erythroleukemic cell lines showed a striking correlation between CD58 expression and spCD2 binding: all spCD2 binding cells expressed CD58 and were inhibited from binding spCD2 by anti-CD58 mAb. Since human CD48 is structurally related to CD58, sharing 31% aa identity (21), and it has been clearly demonstrated that the major ligand of CD2 is CD48 in the murine system (22), we examined the ability of human CD48 to bind to human CD2. Not surprisingly, we were able to detect binding of spCD2 to human CD48. However, we observe that in contrast to the CD2–CD58 interaction, which promotes cell–cell adhesion, the CD2–CD48 interaction fails to support functional conjugates between CD2⁺ T cells and CD48-expressing cells and is characterized by an \sim 100-fold weaker affinity. A third possible CD2 ligand, CD59 (23, 24), was not confirmed in either cell-based or spCD2-based binding assays.

Materials and Methods

mAbs

The following previously described murine mAbs to human antigens were used. TS2/9 (anti-CD58, IgG1) (25) was generously provided by Dr. T. Springer (Center for Blood Research, Boston, MA). 3T4-8B5 (anti-T11₁, IgG2a) (12), 1 mono2A6 (anti-T11₃, IgG3) (12), 7T4-7A9 (anti-T11₁, IgM), 2Ad2A2 (anti-CD3, IgM), and an anticonotypic mAb 11C5 (IgG1) were derived in our laboratory. H19 (anti-CD59, IgG2a) (26) was provided by Dr. A. Bernard (Centre Hospitalier, Universitaire de Nice, France). YTH.53.1 (anti-CD59, rat IgG_b) (27) was a gift from Dr. H. Waldmann (Oxford University, Oxford, England), and 6.28 (anti-CD48, IgG3) (28) was provided by Dr. D. Thorley Lawson (Tufts University, Medford, MA). mAb 133-7 (anti-human B7, IgM) (29) was kindly provided by Dr. L. Nadler (Dana-Farber Cancer Institute, Boston, MA) and used as a control IgM for SDS-PAGE.

Construction and Expression of Recombinant spCD2

An 8-kb BamHI/HindIII genomic fragment containing the C region of IgM heavy chain was excised from pN. ψ .u. trinitrophenyl

(TNP) (30), which was a gift from Dr. M. Schulman (University of Toronto, Canada), and cloned into BamHI/HindIII-digested Bluescript II KSM13⁺ (Stratagene, La Jolla, CA). Most of the 5' sequences upstream of exon 1 were eliminated by linearizing the plasmid with HindIII, digesting with Bal31 nuclease, and gel purification of a 2-kb fragment containing all exons of the C region of IgM. This fragment was digested with XbaI to remove the exon encoding the transmembrane region and was subcloned into the XbaI site of pBluescript II KS(+), which lies adjacent to a BamHI and HindIII site. A 600-bp BamHI fragment of pAC373/T11_{ex2} (31) was cloned into the BamHI site of this plasmid. The HindIII/XbaI fragment of the resulting chimeric plasmid was then subcloned into M13mp19 digested with HindIII/XbaI. Oligonucleotide-mediated mutagenesis was then conducted with the synthetic nucleotide 5'-CACTTTGGGAGGCAGCTCAGCAATCAC-TTTCTCTGGACAGCTGACAGGCTCGAC-3' to loop out sequences (including CH₁ exon 1 of μ heavy chain), between the last T11_{ex2} codon coding for lysine 182 and the first codon of CH₂ encoding for valine. The sequence of the junction after mutagenesis was confirmed by dideoxy sequencing. The BamHI fragment containing the chimeric CD2-IgM gene was excised, blunt ended with klenow, and inserted into the XhoI site of the mammalian expression vector pM2 (M. Concino, unpublished results) in the appropriate orientation resulting in the plasmid pM2CD2IgM.

pM2CD2IgM and pSV2 dhfr (32) were linearized with FspI and then cotransfected into CHO-dhfr⁻ cells provided by L. Chasin (Columbia University, New York, NY) using the calcium phosphate method of DNA precipitation at a ratio of 25:1 μ g, respectively. Transfectants were selected in ribo- and deoxyribonucleoside-deficient α -MEM (Media Tech, Inc., Herndon, VA) supplemented with 10% dialyzed FCS. CHO cells expressing spCD2 were passaged two to three times in selective media containing 20 mM methotrexate (MTX; Sigma Chemical Co., St. Louis, MO). Survivors were assayed for spCD2 expression by testing supernatants with an alkaline phosphatase-conjugated goat anti-human IgM antibody (Southern Biotechnology Associates, Birmingham, AL) by sandwich ELISA on plates coated with anti-T11₁ mAb, 3T4-8B5. Positives were passaged at least three times in 50 mM MTX and tested each time for spCD2 secretion, and one stable secretor clone was expanded for the purification of spCD2.

Purification and Biotinylation of spCD2 and sCD2

spCD2 was purified by immunoaffinity chromatography on an anti-T11₁ (3T4-8B5) mAb-coupled Sepharose 4B column. CHO-derived culture supernatant containing spCD2 was passed through a column equilibrated with PBS, pH 7.4, and the column was washed extensively with PBS. The bound material was eluted with 0.2 M glycine-HCl, pH 2.7, and 2-ml fractions collected into tubes containing 0.5 ml 1 M Tris base, pH 9.0, were subsequently pooled and concentrated up to 1 mg/ml on a centrprep-10 (Amicon Corp., Danvers, MA). Biotinylation of spCD2 was conducted by adding 3.4 μ g of *N*-hydroxysuccinamide-long chain (NHS-LC) biotin (Pierce Chemical Co., Rockford, IL) to 100 μ g of spCD2 in PBS, pH 7.4, in a reaction volume of 100 μ l, and incubating this mixture for 1 h at room temperature. The reaction was quenched by adding 5 μ l of 1 M Tris/HCl, pH 7.4. A plasmid encoding monomeric sCD2 was expressed in CHO cells and purified on an anti-T11₁ (3T4-8B5) column as previously described (33). sCD2 was biotinylated by adding 143 μ g of NHS-LC biotin to 500 μ g of sCD2 (10:1 molar ratio) and incubating the mixture for 1 h at room temperature and subsequently quenching the reaction with 10 μ l of 1 M Tris/HCl, pH 7.4.

¹ Abbreviations used in this paper: aa, amino acid; spCD2, soluble pentameric CD2.

Cells and Media

The following human cell lines were used in this study: J77, JY, SLA, Arent, Daudi, Raji, K562, U937, and HL60, which were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% FCS (Sigma Chemical Co.), 1% glutamine, 1% penicillin-streptomycin (Gibco Laboratories), and 1% sodium pyruvate (Whittaker Bioproducts, Walkersville, MD). Human RBC were isolated from normal healthy blood donors (Blood Bank, Dana-Farber Cancer Institute). CHO cells expressing either CD59 or CD58 and CD59 were obtained from Dr. A. Bernard and cultured in Ham's F12 media as described previously (34). Untransfected CHO cells were cultured in media containing equal amounts of F12 nutrient mixture (Gibco Laboratories) and DME (Whittaker Bioproducts), 10% FCS (Sigma Chemical Co.), 1% penicillin-streptomycin, 1% glutamine, and 1% Hepes buffer (Gibco Laboratories). Trypsin-EDTA (Gibco Laboratories) was used to remove adherent CHO cells from culture flasks.

Flow Cytometric Analysis

spCD2 Binding. 2×10^5 cells were routinely incubated with 50 μ l of 10 μ g/ml biotinylated spCD2 for 30 min at 4°C. For blocking studies with inhibitors, cells were pretreated with 40 μ l of a 1:100 dilution of mAb ascites and in some cases, 1–2 mg/ml purified mAbs or 2 mg/ml concentration of sCD2 or sCD4 (35) for 1 h at 4°C before the addition of biotinylated spCD2. Subsequently, cells were washed once with wash media (HBSS containing 2% FCS), and 50 μ l of a 1:25 dilution of streptavidin-FITC (Caltag Labs, So. San Francisco, CA) was added and incubated further for 20 min at 4°C. Cells were washed once, fixed in a PBS solution containing 3.7% formaldehyde, and fluorescence was estimated using a flow cytometer.

spCD2 Binding under a Low Stringency Wash Condition. 4×10^5 cells were incubated with 50 μ l of 10 μ g/ml biotinylated spCD2 for 1 h at 4°C. For titration of spCD2, various concentrations of biotinylated spCD2 ranging from 0.01 to 100 μ g/ml were used. For blocking experiments either biotinylated spCD2 was preincubated with 10 μ g/ml anti-T11, F(ab')₂ or anti-T11₃ F(ab')₂, or in some cases cells were preincubated with a 1:50 dilution of mAb ascites or 20 μ g/ml purified mAb directed against cell surface molecules for 1 h at 4°C before the addition of spCD2. At the end of the 1-h incubation, cells were spun down and flicked once to remove most of the unbound spCD2, and 50 μ l of 1:20 dilution of FITC-conjugated Streptavidin (Caltag Labs) was added directly without washing the cells. After a further 1-h incubation at 4°C, cells were spun down after mixing with 150 μ l of media and analyzed immediately on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

mAb Binding. 2×10^5 cells were incubated with 50 μ l of either 1:100 dilution of mAb ascites or 10 μ g/ml of purified mAbs for 30 min at 4°C, washed once as described above, and analyzed by indirect immunostaining using fluoresceinated second-step reagents. 50 μ l of a 1:50 dilution of FITC-conjugated goat anti-mouse Ig (Whittaker Bioproducts) or goat anti-rat Ig (Caltag Labs) was added and incubated for 20 min at 4°C. Subsequently, cells were washed once in wash media and fixed in formaldehyde as described above, and cell-bound fluorescence was analyzed using a flow cytometer.

Binding of Radioiodinated sCD2 to JY Cells

100 μ g of sCD2 was radioiodinated by the solid phase lactoperoxidase/glucose oxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, CA) method as described previously (31). For the cold com-

petition assay, 5×10^6 cpm of ¹²⁵I-sCD2 at 0.125 μ M in 50 μ l (3.1×10^8 cpm/nmol) was added to 2×10^6 JY cells preincubated with either increasing concentrations of cold sCD2 (0.025–12.5 μ M) or a 1:50 dilution of TS2/9 anti-CD58 mAb ascites in a final volume of 200 μ l, which had been previously overlaid onto 0.2 ml of a 1.5:1 mixture of dibutyl phthalate/dioctyl phthalate (Aldrich Chemical Co., Milwaukee, WI) in 0.5-ml plastic microcentrifuge tubes (Sarstedt, Numbrecht, Germany). After a 1-h incubation at 4°C, the tubes were centrifuged in a microfuge (152; Beckman Instrs., Inc., Fullerton, CA) for 2 min, and tips of tubes containing cell pellets were cut and cell bound radioactivity was determined in a gamma counter.

Transfection of CD58 and CD48 into CHO Cells

CD58 cDNA in expression vector CDM8 was a gift from Dr. B. Seed (Massachusetts General Hospital, Boston, MA) (36). CD48 cDNA in expression vector CDM8 was provided by Dr. D. Thorley-Lawson (Tufts University, Boston, MA) (37). Plasmid DNAs were purified by the Qiagen column method according to the manufacturer's recommendations (Qiagen, Studio City, CA). 50 μ g of KpnI-linearized CD58-CDM8 or CD48-CDM8 was cotransfected with 5 μ g of PvuI-linearized pSV2-neo-Sp65 (38) into CHO cells by electroporation using an electroporator (Bethesda Research Laboratories, Bethesda, MD) at settings of 250 V and 1,600 μ F (39). Transfectants were selected in growth media containing 400 μ g/ml G418 and screened for expression of CD58 or CD48 by indirect immunofluorescence using anti-CD58 or anti-CD48 mAbs. Stable CHO cell lines expressing comparable levels of CD58 or CD48, now termed CHO-CD58 and CHO-CD48, were established by cell sorting on an Epics V flow cytometer.

Cell Binding Assays

CHO cells were plated in triplicates of 10⁵ cells/well in 24-well plates (Nunc, Roskilde, Denmark) and used for binding assays 3 d later when the wells were fully confluent. Confluent CHO cells were washed twice with 1 ml of wash media (RPMI 1640 containing 2% FCS) and were pretreated with blocking mAbs at a 1:100 or 1:50 dilution of ascites or 5–10 μ g/ml of purified mAb for 15 min at 37°C. 30–45 $\times 10^6$ Jurkat cells were labeled with 0.8–1 mCi of ⁵¹Cr (New England Nuclear, Boston, MA) for 1 h at 37°C, washed twice in wash media, and 10⁶ cells were added into each well and incubated for 90 min at 37°C. Subsequently, cells were washed three to four times by gently dropping 1 ml of wash media each time to remove unbound cells, and cell-bound radioactivity was determined by lysing cells with 200 μ l of 1% NP-40 solution and counting 180 μ l of each sample on a gamma counter.

Results

Construction and Expression of a Pentameric Form of the Human CD2 Extracellular Segment. In an initial effort to search for alternative ligands of CD2, we produced a recombinant monomeric form of the 182-aa extracellular segment of CD2 in baculovirus (31) and subsequently in CHO cells (sCD2) (33). However, because the affinity (*K_a*) of monomeric sCD2 for the known ligand CD58 was only 0.4 μ M, it was not possible to develop a sensitive direct binding assay with either CD2 protein. Consequently, we herein produced a multimeric CD2 protein to improve the avidity of the CD2 interaction with its ligand(s). Since it is known that μ heavy chains can associate into pentamers in the absence of Ig light chains

(40), we produced a fusion protein between the human CD2 extracellular segment and the human CH₂-CH₃-CH₄ domains of μ heavy chain in CHO cells using the plasmid termed pM2CD2IgM depicted in Fig. 1 A and as described in Materials and Methods. Recombinant protein was purified on an anti-T11₁ mAb affinity column from culture supernatants containing ~ 4 – 5 $\mu\text{g/ml}$ spCD2. As shown by Coomassie staining of the reducing SDS-polyacrylamide gel in Fig. 1 B, the multimeric construct (spCD2) runs at ~ 90 kD, slightly larger than the μ heavy chain that migrates at ~ 80 kD. In contrast, the monomeric 182-aa recombinant CD2 extracellular segment protein, sCD2, migrates at ~ 36 kD. On nonreducing gels, spCD2 runs at ~ 900 kD as compared with purified IgM, which migrates at $\sim 1,000$ kD (Fig. 1 B, inset). This mobility is consistent with the pentameric nature of spCD2 and the IgM molecule.

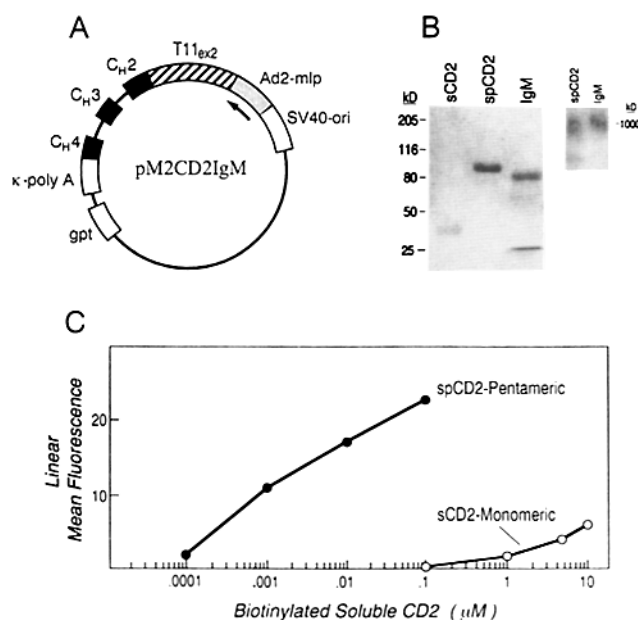


Figure 1. (A) pM2CD2IgM expression plasmid construct for production of spCD2 in CHO cells. T11_{ex2} encodes aa 1–182 of human CD2. CH₂, CH₃, and CH₄ encode the C region of domains 2, 3, and 4 of human μ heavy chain. (Ad2-mlp) Adenovirus 2 major late promoter and tripartite leader; (SV40-ori) DNA segment containing the SV40 origin of replication; (κ polyA) polyadenylation signal derived from the murine κ light chain gene; (gpt) *Escherichia coli* xanthine-guanine phosphoribosyl transferase gene. (B) SDS-PAGE analysis of spCD2. The above indicated samples (1–3 μg) were analyzed by 7% polyacrylamide gels under reducing conditions and subsequently stained with Coomassie blue. (Inset) Indicated samples run under nonreducing conditions in a 3% polyacrylamide gel. (C) Binding of biotinylated spCD2 and sCD2 to JY cells. The specific binding of the indicated micromolar concentrations of biotinylated spCD2 and sCD2 binding to JY cells was analyzed by flow cytometry using Streptavidin-FITC and measured as mean linear fluorescence channel after subtracting background fluorescence from cells that were incubated with only Streptavidin-FITC. The binding of biotinylated spCD2 (at 1 and 0.1 μM) and sCD2 (at 10 μM) was specific for CD58 as shown by the complete blockade of detectable fluorescence by the anti-CD58 mAb (data not shown).

Enhanced Binding Efficiency of Biotinylated spCD2 to B Lymphoblastoid Cells and Survey of Hematopoietic Cell Lines for Reactivity. A comparison of the binding of biotinylated spCD2 and sCD2 to the JY B lymphoblastoid cell line was made by flow cytometric analysis after developing CD2-reactive cells with Streptavidin-FITC and quantitating staining by linear mean fluorescence. As shown in Fig. 1 C, 10 μM monomeric sCD2 is required to achieve a clearly detectable binding pattern over the negative control stained with Streptavidin-FITC alone (linear mean fluorescence values). In contrast, <1 nM spCD2 is required to achieve the same level of binding on JY cells. The binding of spCD2 to cells was specific as judged by subsequent inhibition studies with selected mAbs and proteins as described in Fig. 2. Similarly, the binding of sCD2 to cells was specific as described in Table 1 (also see reference 31). These results unequivocally show that the detectable binding efficiency of biotinylated spCD2 is substantially greater (by four orders of magnitude) than that of biotinylated sCD2 for the CD2 ligand(s) on JY cells. It is thus possible to use biotinylated spCD2 in sensitive direct binding assays to search for additional CD2 ligands.

As shown in Table 2, spCD2 reactivity on a panel of hematopoietic cell lines correlated with the level of CD58 expression as judged by reactivity with an anti-CD58 mAb; the greater the anti-CD58 mAb reactivity, the greater the spCD2 reactivity. B lymphoblastoid JY, SLA, and Arent cells, which express ~ 7 – 21 -fold more CD58 compared with Daudi and Raji cells, show a comparable ~ 9 – 27 -fold increase in spCD2 reactivity. Similarly, erythroleukemic K562 cells, which express ~ 2.5 – 4.5 -fold more CD58 compared with monocytic U937 and HL60 cells, show a comparable two- to six-fold increase in spCD2 reactivity.

The Binding of spCD2 and sCD2 to CD48⁺CD58⁺CD59⁺ Cells Is Completely Blocked by Anti-CD58 mAb We next conducted experiments on the B lymphoblastoid cell line JY using biotinylated spCD2 in the presence of various putative inhibitors of CD2 binding, including selected mAbs and recombinant T cell surface proteins. As shown in Fig. 2, A–C, the JY cell line is reactive with anti-CD58, anti-CD48, and anti-CD59 mAbs. However, a 1:100 dilution of ascites containing the TS2/9 anti-CD58 mAb is sufficient by itself to completely block spCD2 binding (F vs. G). Complete blockade was also obtained with purified anti-CD58 mAb (data not shown). This inhibition is specific to TS2/9 because neither an equivalent amount of mAb directed against CD59 (H19 mAb) shown to block rosettes between human T cells and RBC (26) nor CD48 (6.28 mAb) expressed on JY cells block spCD2 binding (H and I). Likewise, sCD4, a recombinant protein comprising domains 1 and 2 of the human CD4 extracellular segment (35), does not inhibit spCD2 binding to JY cells, whereas monomeric sCD2 (2 mg/ml) inhibits binding substantially (up to 83%; Fig. 2, J and K). The results of spCD2 binding suggest that CD2 does not interact directly with CD48 or CD59 expressed on JY cells. Because the source of purified CD59 used by others to demonstrate CD2 binding in an in vitro plate assay was derived from human RBC (23), we tested the possibility that differential glycosylation of CD59 on JY cells vs. human RBC might contribute to the lack of CD2

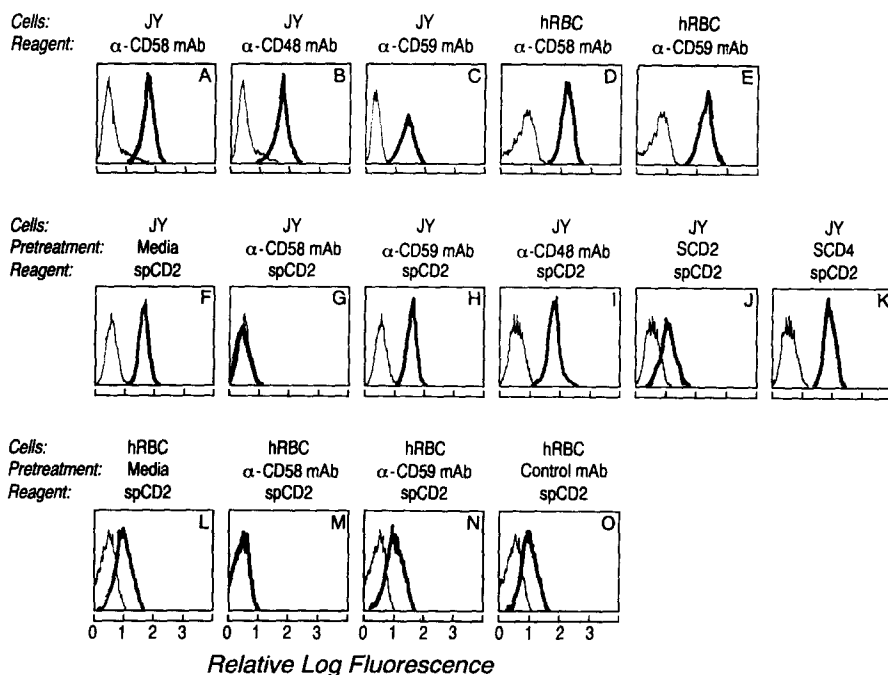


Figure 2. Flow cytometric analysis of spCD2 binding to JY and human RBC in the presence of various inhibitors. JY and human RBC were incubated with the following mAbs: 1:200 dilution of TS2/9 anti-CD58 ascites (A and D), 10 $\mu\text{g/ml}$ 6.28 anti-CD48 (B), and 10 $\mu\text{g/ml}$ H19 anti-CD59 (C and E). mAb binding to cells was analyzed using FITC-conjugated goat anti-mouse IgG. Background fluorescence was obtained by staining cells with the control 11C5 mAb and the same second-step Ab. JY cells were preincubated with the following: media (F), 1:100 dilution of TS2/9 anti-CD58 ascites (G), 1.2 mg/ml H19 anti-CD59 (H), 1 mg/ml 6.28 anti-CD48 (I), 2 mg/ml sCD2 (J), and 2 mg/ml sCD4 (K). Human RBC preincubations were as follows: media (L), or 1:100 dilution of TS2/9 anti-CD58 ascites (M), 1.2 mg/ml H19 anti-CD59 (N), and 1:100 dilution of control mAb 11C5 ascites (O). After preincubation with the various inhibitors the binding of biotinylated spCD2 to JY and human RBC was assessed on a FACScan[®] using Streptavidin-FITC as described in Materials and Methods.

binding to the former. Therefore, we next investigated the binding of biotinylated spCD2 on CD58⁺CD59⁺ human RBC (Fig. 2, D and E) in the presence of anti-CD58 mAb TS2/9. As shown in Fig. 2, L vs. M and O, the binding of spCD2 is completely and selectively inhibited by TS2/9. The H19 anti-CD59 mAb does not block the binding of spCD2 to human RBC (N). However, we could not, at this stage, exclude the possibility that H19 is directed at an epitope distinct from the CD2 binding site. Notwithstanding, these results collectively suggest that CD48 and CD59 molecules expressed on human JY B lymphoblastoid cells and human RBC may not bind CD2, whereas the interaction between CD2 and CD58 molecules is readily detected.

Table 1. Competitive Inhibition of Radioiodinated sCD2 Binding to JY Cells

Inhibitor	Bound to JY cells	
	Exp. 1	Exp. 2
μM	<i>cpm</i>	
sCD2		
0.025	46,310 \pm 752	35,122 \pm 446
1.25	20,096 \pm 1,090	20,414 \pm 396
12.5	14,059 \pm 651	14,673 \pm 358
Anti-CD58 mAb	14,374 \pm 606	14,387 \pm 132

JY cells were preincubated with either increasing micromolar concentrations of sCD2 or a saturating 1:50 dilution of anti-CD58 mAb ascites and competed with 0.125 μM of ¹²⁵I-sCD2 (3.1 \times 10⁸ cpm/nmol) at 4°C for 1 h. Cells were pelleted by centrifugation and radioactivity bound to the pelleted cells was determined as described in Materials and Methods. Shown are mean and SD obtained from duplicate samples.

Although it was conceivable that the spCD2 fusion protein failed to adopt a native CD2 conformation required for CD48 and CD59 binding, this possibility seems remote since spCD2 reacts with anti-T11₁, anti-T11₂, and anti-T11₃ mAbs directed at conformationally sensitive epitopes (data not shown). Nevertheless, to examine the contribution of CD58 to CD2 binding on lymphoid cells, a radiolabeled binding assay was used with sCD2 and JY cells. The binding of ¹²⁵I-sCD2 to JY cells was tested in the presence of either

Table 2. Reactivity of Anti-CD58 mAb and spCD2 on Cell Lines

Cell line	Origin	Specific linear fluorescence intensity	
		Anti-CD58 mAb reactivity	spCD2 reactivity
JY	B lymphoblastoid	85.0	39.6
SLA	B lymphoblastoid	99.0	64.0
Arent	B lymphoblastoid	87.1	24.2
Daudi	B lymphoblastoid	4.8	2.4
Raji	B lymphoblastoid	13.7	6.7
K562	Erythroleukemic	16.8	11.2
U937	Monocytic	6.9	5.9
HL60	Myeloid	3.8	1.9

The anti-CD58 mAb and spCD2 reactivity with various cell lines was analyzed by flow cytometry as described in Fig. 2. Specific mean linear fluorescence values for anti-CD58 and spCD2 reactivity were obtained by subtracting from the total linear fluorescence values the mean fluorescence linear values obtained when cells were stained with control 11C5 mAb or with only streptavidin-FITC, respectively.

increasing micromolar amounts of unlabeled sCD2 or saturating amounts of anti-CD58 mAb. As shown in Table 1, the binding of ^{125}I -sCD2 is inhibited to the same level by both a 100-fold excess of unlabeled sCD2 and anti-CD58 mAb TS2/9, indicating that the binding of CD2 to JY cells is entirely through CD58. Therefore, by at least two independent assays using both pentameric and monomeric soluble CD2, we find that the only detectable surface CD2 ligand expressed on JY cells is CD58 as defined by TS2/9 mAb.

Anti-CD58 mAb Does Not Crossreact with CD48 or CD59. The possibility that the TS2/9 anti-CD58 mAb might also crossreact with human CD48 and CD59 was tested on a set of CHO cell transfectants expressing selectively CD58, CD48, CD59, or CD58 and CD59, termed CHO-CD58, CHO-CD48, CHO-CD59, or CHO-CD58+59 cells, and an untransfected control cell line, CHO. As shown in Fig. 3, the TS2/9 anti-CD58 mAb showed binding to CHO-CD58 (A) but not to CHO-CD48 cells (B), which express comparable levels of CD48 as judged by reactivity with the 6.28 anti-CD48 mAb (E). Incubation with a 100-fold higher concentration of anti-CD58 mAb still failed to detect reactivity with CHO-CD48 cells (data not shown). These results indicate that the TS2/9 anti-CD58 mAb does not crossreact with the closely related CD48 molecule and that soluble CD2 binding to JY cells is restricted to CD58. In addition, a direct analysis of CD2 binding to CD48 was conducted on CHO-CD48 or control CHO-CD58 cells using the biotinylated spCD2 binding assay. As shown by flow cytometric analysis (Fig. 3, G, H, and I), under the standard washing condition utilized for mAb binding biotinylated spCD2 binds CHO-CD58 but not CHO-CD48 or parental CHO cells.

As shown in Fig. 4, the TS2/9 anti-CD58 mAb binds only CHO-CD58+59 cells and showed no reactivity with CHO-CD59 cells, which bind two independent anti-CD59 mAbs,

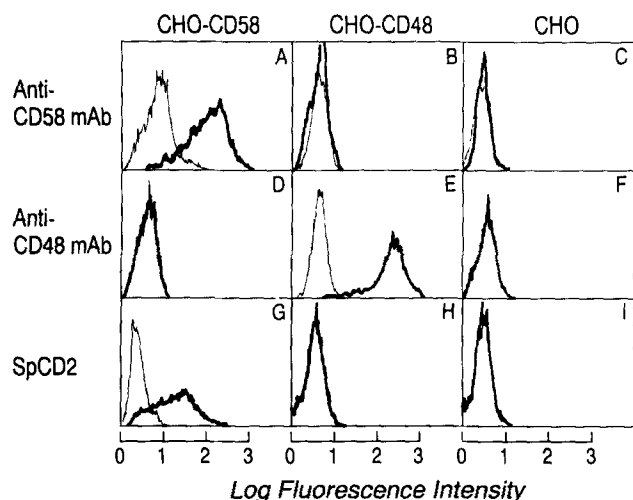


Figure 3. spCD2 binding to CHO-CD48 and CHO-CD58 transfectants. CHO cells or CHO cell transfectants were tested for their reactivity with TS2/9 anti-CD58 mAb (A-C), 6.28 anti-CD48 mAb (D-F), and biotinylated spCD2 (G-I), and binding was analyzed on a FACScan[®] as shown in Fig. 2.

H19 and YTH53.1 (Fig. 4, A, B, and E, and data not shown). This finding excludes the possibility that the TS2/9 anti-CD58 mAb crossreacts with CD59. Hence, the detectable binding of biotinylated spCD2 to CD58⁺CD59⁺ JY cells and human RBC, which is blocked by anti-CD58 mAb, must be restricted to the cell surface CD58 molecule. Consistent with this notion was the further finding that spCD2 bound to CHO-CD58+59 but not CHO-CD59 transfectants (Fig. 4, G and H, respectively). Neither a prolonged incubation period (overnight) nor alteration of incubation temperatures (4, 20, and 37°C) results in detectable spCD2 binding to CHO-CD59 cells (data not shown).

Human CD48 Is a Low Affinity Ligand for Human CD2. A recent study using a mouse sCD2-Ig fusion protein revealed that the major ligand for mouse CD2 is CD48 (22). Since JY cells express human CD48 (Fig. 2) and the binding of spCD2 and ^{125}I -sCD2 to JY cells is entirely blocked by TS2/9 anti-CD58 mAb (which does not crossreact with CD48), this suggested to us that either CD48 is not a ligand or is a low affinity ligand for human CD2 that is not detectable under these assay conditions. We therefore used less stringent washing conditions as described in Materials and Methods to detect any low affinity binding. As shown in Fig. 5, top, under the latter condition, binding of spCD2 to CD58 as well as CD48 is detected (A and E) but no binding was detected to parental CHO cells (data not shown). The binding to CHO-CD58 and CHO-CD48 is specific and can be blocked by an anti-T11₁ F(ab')₂ reactive with the CD2 adhesion domain (domain 1) but not by an anti-T11₃ F(ab')₂ directed at the membrane proximal domain (domain 2) (Fig. 5, top, B, C, F and G). The inhibition of spCD2 binding to CHO-CD48 by anti-T11₁ F(ab')₂ suggests that the CD48 binding site on human CD2, like the CD58 binding site, is found on the adhesion domain. Furthermore, as expected, the binding of spCD2 to CHO-CD58 and CHO-CD48 cells was blocked by anti-CD58 and anti-CD48 mAbs, respectively (Fig. 5, top, D and H) but not by control mAbs (not shown).

To determine the relative affinity of the CD2-CD48 interaction compared with the CD2-CD58 interaction, we con-

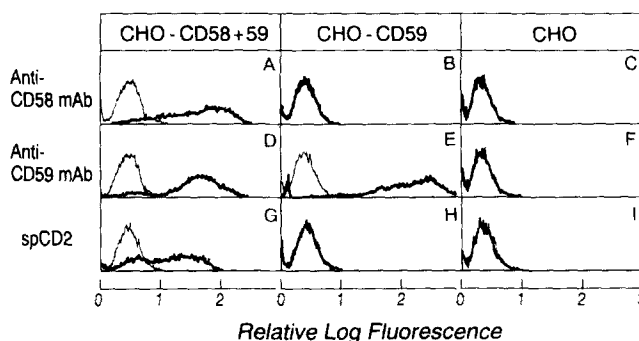


Figure 4. spCD2 binding to CHO-CD58+59 and CHO-CD59 transfectants. The reactivity of CHO cells or CHO cell transfectants was tested for binding to TS2/9 anti-CD58 mAb (A-C), H19 anti-CD59 mAb (D-F), and biotinylated spCD2 (G-I), and binding was analyzed as shown in Fig. 2.

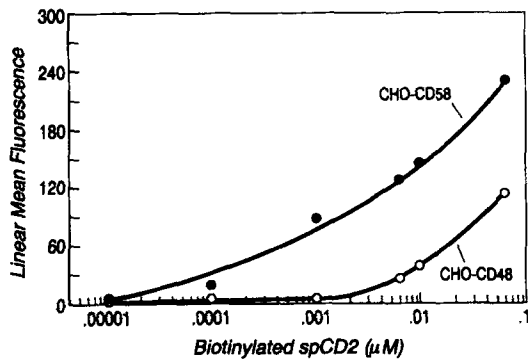
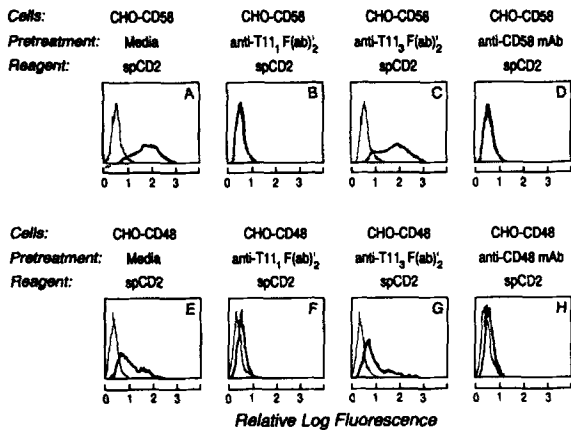


Figure 5. spCD2 binding to CHO-CD48 and CHO-CD58 transfectants under low stringency wash conditions. (Top) Binding of biotinylated spCD2 to CHO-CD48 and CHO-CD58 transfectants was performed under low stringency wash conditions as described in Materials and Methods. spCD2 was preincubated with either media (A and E), anti-T111 F(ab')₂ (B and F), or anti-T113 F(ab')₂ (C and G) before addition to CHO transfectants. In D and H, cells were preincubated with either anti-CD58 or anti-CD48 mAb and then incubated with biotinylated spCD2. (Bottom) Analysis of relative binding avidities of biotinylated spCD2 to CHO-CD48 and CHO-CD58 cells. The specific binding of the indicated micromolar concentrations of biotinylated spCD2 to CHO-CD48 and CHO-CD58 cells was conducted under low stringency wash conditions and analyzed by flow cytometry. Under these conditions, spCD2 showed no binding to the parental CHO cells. Binding was presented as the linear mean fluorescence channel as described in the legend for Fig. 2.

ducted binding studies with various concentrations of spCD2 on CHO-CD48 and CHO-CD58 cells expressing comparable levels of CD48 and CD58 molecules under the above low stringency conditions. As shown in Fig. 5, bottom, the relative binding avidity of spCD2 for CD48 was about two orders of magnitudes lower than that for CD58. Thus, to achieve a readily detectable linear mean fluorescence value of 40 on the linear segment of the curves, 10 nM spCD2 was required for CHO-CD48 and ~0.2 nM spCD2 was required for binding to CHO-CD58. Given the known micromolar affinity of the monomeric CD2-CD58 interaction and based on our assessment of the relative binding avidity of spCD2 for CD48 and CD58, we estimate that the affinity (K_d) of monomeric CD2 for CD48 to be in the 10^{-4} M range.

The Low Affinity CD2-CD48 Interaction Does Not Facilitate Adhesion between CD2⁺ T Cells and CD48⁺ Cells. As a means of examining the functional relevance of the human CD2-CD48 interaction, we conducted binding studies using CD2⁺ Jurkat cells on CHO-CD48 and CHO-CD58 cells. ⁵¹Cr-labeled CD2⁺ Jurkat cells failed to bind to CHO-CD48 cells and untransfected CHO cells but showed specific anti-CD2 (anti-T111) or anti-CD58 mAb inhibitable binding (>67%) to CHO-CD58 cells (Fig. 6). In contrast, anti-CD48 or anti-CD3 mAbs had no significant effect on the binding to CHO-CD58 cells. Given that anti-CD3 treatment is known to result in activation of Jurkat cells, the latter observation is consistent with our previous study demonstrating that CD2-mediated adhesion is unaffected by intracellular signals that upregulate the adhesion function of other molecules (41). Therefore our cell binding assay demonstrates that the low avidity human CD2-CD48 interaction is not sufficient to mediate adhesion between CD2⁺ T cells and CD48⁺ cells. Thus the physiologically relevant ligands for CD2 in the human and mouse appear to be distinct.

Human CD2 Does Not Bind to CD59-transfected CHO Cells. To detect any low affinity binding of CD2 to CD59, we used the same low stringency wash conditions for spCD2 binding to CHO-CD59 cells as used to detect low affinity binding of CD2 to CD48 ($K_d \sim 10^{-4}$ M). Under these conditions, spCD2 showed no binding to CHO-CD59 cells (data not shown). In addition, we conducted cell-based binding assays in which we examined the ability of ⁵¹Cr-labeled CD2⁺ Jurkat cells to bind to CHO cell transfectants. As shown in Fig 7, CD2⁺ Jurkat cells showed no binding to CHO-CD59 cells as compared with untransfected CHO cells. In

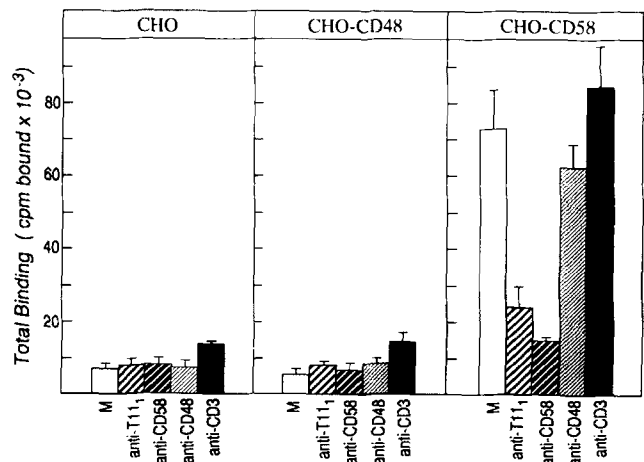


Figure 6. Binding of CD2⁺ Jurkat cells to CHO-CD58 and CHO-CD48 transfectants. 10⁶ Jurkat cells labeled with ⁵¹Cr were incubated for 1 h at 37°C with the indicated CHO cell populations in media or pretreated with the designated mAbs. At the end of this incubation, individual wells were washed extensively in wash media and binding of Jurkat cells was detected by lysing adherent cells in a 1% NP-40 solution and counting radioactivity on a gamma counter. Results shown are representative of three independent experiments, and the mean and SD were calculated from triplicate samples.

contrast, Jurkat cells bound to CHO-CD58+59 double transfectants. The binding to the CHO-CD58+59 cells was dependent on the CD2-CD58 adhesion pair as judged by the >87% blockade with anti-CD2 (anti-T11₁) or anti-CD58 (TS2/9) mAbs but no blockade by H19 anti-CD59 mAb (Fig. 7). This was further substantiated in rosetting assays involving human thymocytes and CHO-CD58+59 and CHO-CD59 cells in which rosetting was blocked by anti-CD2 (anti-T11₁) and anti-CD58 (TS2/9) mAbs but not by the H19 anti-CD59 mAb (data not shown). Thus, by two independent CD2 binding assays, it appears that CD59 is not a ligand for CD2.

Discussion

We have used a pentameric version of CD2 in which the entire extracellular segment of CD2 has been fused to CH₂-CH₃-CH₄ μ heavy chain as a probe to detect CD2 ligands in hematopoietic cells, including B lymphoblastoid cell lines. The binding of biotinylated spCD2 to various cell populations is directly correlated with their expression of CD58 (Table 2). In particular, the binding of both spCD2 and ¹²⁵I-sCD2 to JY lymphoblastoid cells was entirely blocked by TS2/9 anti-CD58 mAb, which suggests that the binding of CD2 to JY cells was restricted to CD58 (Fig. 2 and Table 1). Since JY cells also express comparable levels of CD48, the murine equivalent of which is a ligand of mouse CD2 (22), and CD59, which has recently been reported to be a second ligand for CD2 (23, 24), we wondered if the TS2/9 mAb crossreacted with CD48 and CD59 and blocked spCD2 binding to these molecules. Analysis of the binding of TS2/9 anti-CD58 mAb to CD48- or CD59-transfected CHO cells showed no such crossreactivity (Figs. 3 and 4). Moreover, standard binding experiments with both biotinylated spCD2 and CD2⁺ Jurkat cells show no detectable binding to CHO-CD48 or CHO-CD59 cells, while CD2 binding to control CHO-CD58 and CHO-CD58+59 cells is readily demon-

strated (Figs. 3, 4, 6, and 7). However, studies with spCD2 under low stringency wash conditions revealed binding to CHO-CD48 cells whereas no binding to CHO-CD59 cells was observed under any conditions tested (Fig. 5, and data not shown). The affinity of the CD2-CD48 interaction compared with the CD2-CD58 interaction was ~ 100 -fold weaker and was estimated to be $\sim 10^{-4}$ M, consistent with the lack of conjugate formation between Jurkat cells and CHO-CD48 cells. Collectively, our results indicate that the CD2-CD48 interaction, although conserved in the human, may be of limited physiological significance.

Using a murine CD2-IgG chimera the ligand for CD2 in the mouse has recently been identified as CD48 (BCM1) (22). Given the significant homology (31% aa identity) between human CD58 and CD48 (21), we investigated whether human CD2 could bind to human CD48. Binding studies with biotinylated spCD2 on CD48- or CD58-transfected CHO cells using less stringent washing conditions show that human CD48 is a low affinity ligand for human CD2. Based on the low affinity of CD2 for CD48, neither spCD2 under standard wash conditions nor the monomeric sCD2 show any detectable binding to CD48⁺ JY cells in the presence of anti-CD58 mAb (Fig. 2 and Table 1). Since this low affinity binding does not result in the adhesion of CD2⁺ Jurkat cells to CHO-CD48 cells, we consider the interaction of uncertain physiological significance, although we cannot rule out the possibility that at high copy number of CD2 and/or CD48 some functional cell-cell adhesion may occur. Of note, anti-CD3 mAb treatment, which is known to activate Jurkat cells and upregulate the adhesion function of other adhesion molecules such as LFA-1 (41, 42), did not have any effect on the CD2-CD48 interaction (Fig. 6). Hence, it is likely that the low affinity interaction between human CD2 and CD48 is a consequence of the evolutionary divergence of ligands for

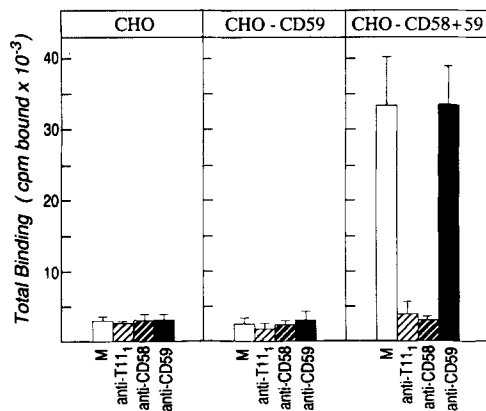


Figure 7. Binding of CD2⁺ Jurkat cells to CHO-CD58+59 and CHO-CD59 transfectants. Binding of Jurkat cells to individual CHO cell populations pretreated with media or the indicated mAbs was conducted as shown in Fig. 6. Results shown are representative of three independent experiments.

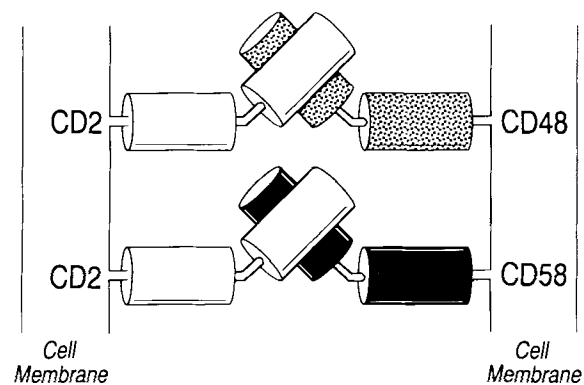


Figure 8. Illustration of the binding of CD2 to its known ligands. Based on the Ig-like structure of CD2, which consists of two extracellular domains, and the significant similarity among primary amino acid sequences of CD2, CD48, and CD58, it is likely that these three receptors have similar tertiary structures. The binding site on CD2 for CD58 is known to reside in the NH₂-terminal adhesion domain (domain 1). From mAb blocking studies, it appears that CD48 binds to this same CD2 domain. The major CD2-based ligand binding interactions are shown on the bottom and top for human and mouse, respectively.

CD2 in the human and mouse. Preliminary evidence from quantitative binding studies show that the affinity of mouse CD2 for mouse CD48 is substantially higher than that between the human homologues (data not shown). Hence it is likely that among the products of the CD2/CD48/CD58 receptor gene family (Fig. 8), the mouse CD2-CD48 interaction was largely replaced during evolution of the human species by the CD2-CD58 interaction. In conjunction with the data on murine system (22), these results provide among the first evidence that the major ligand-receptor pairs are distinct in humans and mice. As the CD58 gene locus of the mouse is undefined at present, it is not yet clear whether CD58 is nonfunctional in the mouse or, alternatively, whether both murine CD48 and murine CD58 are ligands for murine CD2. This latter possibility is of interest, particularly in view of the restricted hematopoietic expression of CD48 as contrasted with the more widespread distribution of CD58 (8, 21, 28).

Long-range restriction site analysis of a segment of human chromosome 1p and the distal end of mouse chromosome 3 encoding CD2 and ATP1A1 (*Atpa-1*) genes reveals that they form syntenic pairs (43). Likewise, human chromosome 1q and the distal end of mouse chromosome 1 encoding CD48 and ATP1A2 (*Atpa-3*) genes are syntenic (44). Hence, it is conceivable that a primordial gene segment duplicated to produce a CD2 adhesion receptor/CD48 ligand recognition pair (whose genes show significant sequence homology both in humans and mice [44, 45]) before the divergence of humans and mice. Subsequently, during evolution, the CD2 gene may have diverged in these two species and its product evolved to recognize distinct ligands, CD58 and CD48, respectively. Because human CD58 is more homologous to human CD48 than to human CD2 (21) and is also located on human chromosome 1 (46), it is likely that a further duplication of the CD48 gene gave rise to CD58 in the human whose product binds to CD2 with higher affinity. This duplication and divergence of the CD48 gene and its products may have also evolved to interact with a yet-to-be-identified ligand in the human. Based on the present study and that of Kato et al. (22), it would appear that the major ligands for CD2 in the humans and mice are distinct, even though human and mouse CD2 and human and mouse CD48 are ~50% and 59% homologous, respectively (44, 45). This is in contrast to other well-characterized lymphoid adhesion receptor/ligand interactions, including LFA-1/ICAM-1 (15, 47), CD4/class II MHC (48, 49), CD8/class I MHC (50, 51), and CD5/CD72 (52, 53), where the interacting receptor/ligand pairs are conserved across the mouse and human species. Together, our study on CD2 in the human and mouse systems shows that during evolution primary structural homology between species may not always lead to conservation of a functional receptor ligand interaction across species. This consideration needs to be borne in mind when interpreting the recent unexpected observation that thymocyte development is normal in CD2^{-/-} mice created by homologous recombination (54).

The discrepancies in the literature regarding the interaction between CD2 and CD59 are yet to be fully resolved.

An independent study of CD59 ligands using a chimeric CD59-IgG molecule revealed that the ligand for CD59 is primarily expressed on activated B lymphocytes and not found on either resting or activated T cells or T cell lines (55). Given the T cell-restricted tissue distribution of human CD2, these results are consistent with our finding that CD59 is not a CD2 ligand. In contrast to these results, two other recent studies suggest that CD59 is a second ligand for CD2. In fact, Hahn et al. (23) reported that the CD59 binding site is overlapping with, but distinct from, CD58. In that study, human CD2 transfectants of murine T cell hybridomas were shown to bind to plates coated with the 18–20-kD CD59 protein purified from human RBC. Because the binding of biotinylated spCD2 to both CD58⁺CD59⁺ JY cells as well as human RBC is completely blocked by anti-CD58 mAb, differential glycosylation of CD59 on RBC cannot account for the differences in results between our study and that of Hahn et al. (23). A second study by Deckert et al. (24) suggested that CD59 is a CD2 ligand based on the ability of anti-CD59 mAb, H19, to block rosetting between human thymocytes, CD2-transfected thymoma cells, or CHO cells and human RBC or CD59-transfected CHO cells. However, the percentage of rosetting cells was small and binding studies with ¹²⁵I-labeled soluble CD59 did not demonstrate saturable binding to CD2-expressing cells. The conflicting results reported in these studies are difficult to reconcile with our own. For example, even if CD58 contaminated the CD59 preparation used in the study by Hahn et al. (23), the difference in CD2 binding sites for CD58 and CD59 as revealed by site-directed mutagenesis cannot be explained. Furthermore, in both our study and that of Deckert et al. (24), H19 mAb was used with differing results. However, it is possible that binding of CD2 to CD59 is dependent on an as yet undefined T cell structure (X) that may be absent from Jurkat cells used here, and, hence, would not be detected in either our cell-based or soluble CD2 assay. If that were the case, then given the reports by Hahn et al. (23) and Deckert et al. (24), X must be present on CHO cells as well as murine T cell hybridomas, two disparate cell types from divergent species. We believe this explanation to be unlikely as we also observed no specific (i.e., blockable with an anti-CD59 mAb) rosettes between human thymocytes and CD59 CHO cell transfectants.

In summary, we have developed a pentameric version of CD2, spCD2, and used this in sensitive flow cytometric analysis to define alternative ligands for CD2. This analysis combined with more physiological, cell-based adhesion assays involving CD2⁺ T cells and CD59-expressing cells indicate that CD2 does not bind CD59. Furthermore, using spCD2 we identify that human CD48 is a low affinity ligand for human CD2 and has an estimated binding constant that is 50–100-fold less avid than that of CD58. Moreover, our cell-based adhesion assay using CD2⁺ Jurkat cells and CD48- or CD58-expressing CHO cells shows that there is no adhesion mediated by the CD2-CD48 interaction and provides evidence that the major ligands for CD2 in humans and mice are distinct. Collectively, our findings indicate that the major

human cell surface ligand mediating CD2-based cell-cell interaction is CD58. We also note that the sulfated polysaccharide dextran sulfate, which has been shown to bind human CD2, may be involved in the binding of CD2 to additional

ligands (56). Using the spCD2-based binding assay, it should now be possible to test if additional ligand(s) for human CD2 distinct from CD58 exist on other cell types either constitutively or upon activation.

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