Frontline:

Optimal T cell activation requires the engagement of CD6 and CD166

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The T cell surface glycoprotein, CD6 binds CD166 in the first example of an interaction between a scavenger receptor cysteine-rich domain and an immunoglobulin-like domain. We report that in human these proteins interact with a K_D =0.4–1.0 μM and $K_{off} \geq$0.4–0.63 s^{-1}, typical of many leukocyte membrane protein interactions. CD166 also interacts in a homophilic manner but with around 100-fold lower affinity (K_D =29–48 μM and $K_{off} \geq 5.3$ s^{-1}). At concentrations, that will block the CD6/CD166 interaction, soluble monomeric CD6 and CD166 inhibit antigen-specific human T cell responses. This is consistent with extracellular engagement between CD6 and CD166 being required for an optimal immune response.

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1 Introduction

The control of immune responses and lymphocyte differentiation involves complex intracellular signals delivered through engagement of the antigen-specific receptor and interactions of other surface proteins with proteins at the surface of other cells and soluble factors. The majority of these leukocyte membrane molecules contain immunoglobulin superfamily (IgSF) domains. This is the most common domain type present on leukocyte cell surfaces [1]. In contrast the CD5 and CD6 lymphocyte membrane proteins contain a different type of domain, the scavenger receptor cysteine-rich (SRCR) domain. Although SRCR domains have been well-conserved throughout evolution, a unifying function or paradigm for this family has yet to be proposed. The group B SRCR superfamily are characterized by the presence of SRCR domains encoded by a single exon and normally containing eight cysteine residues [2]. Within this group are proteins, which contain exclusively SRCR domains in their extracellular regions, CD5, CD6, T19/WC1, SPα/AIM, M130/CD163, M160 and S4D-SRCRB/18-B [2, 3]. Proteins in this group can bind cell surface or soluble ligands. There are good molecular data showing that CD6 binds the cell surface protein, activated leukocyte cell adhesion molecule (ALCAM/CD166) [4] and preliminary data supporting the existence of cell surface ligands for WC1 [5] and CD5 [6–8]. In contrast, M130/CD163 binds the soluble complex, haptoglobin-hemoglobin [9]. CD5 and CD6 are the closest relatives within this group and the genes for these proteins map to contiguous regions of human chromosome 11q12.2 compatible with duplication of a common ancestral gene [10]. CD5 and CD6 are type 1 transmembrane glycoproteins with similar domain organization (Fig. 1A), each containing three extracellular SRCR domains, a transmembrane region and cytoplasmic tails well-suited for signal transduction. Expression of CD5 and CD6 appears to be coordinately regulated [11–13]. Both are primarily expressed by thymocytes and mature T cells [1, 2]. CD6 expression has not been well-characterized on $CD5^+$ B cells. Biochemical studies also support a physical and functional connection between CD5 and CD6 [14, 15].

Functionally, experiments with CD5 and CD6 mAb have suggested a costimulatory role for both these accessory proteins [12, 16–18]. However, CD5-deficient mice show that CD5 negatively regulates antigen receptor-mediated signals [19, 20]. Crossbreeding of these mice with other genetically deficient mice has provided further support for CD5 being a negative regulator of antigen receptor signaling during T cell development [21, 22]. These more recent data can be reconciled with early experiments in which T cells cross-linked with CD5 mAb, showed enhanced proliferation in response to TCR stimulation

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Abbreviations: CD4d3+4: Domains 3 and 4 of rat CD4 **CD6CD4d3+4:** Fusion protein containing full-length extracellular region of human CD6 and CD4d3+4 **CD6d1–2CD4d3+4:** Fusion protein containing domains 1 and 2 of human CD6 and CD4d3+4 **CD6d3CD4d3+4:** Fusion protein containing domain 3 and the stalk region of human CD6 and CD4d3+4 **CD166CD4d3+4:** Fusion protein containing full length extracellular region of human CD166 and CD4d3+4 **SRCR:** Scavenger receptor cysteine-rich (domain)

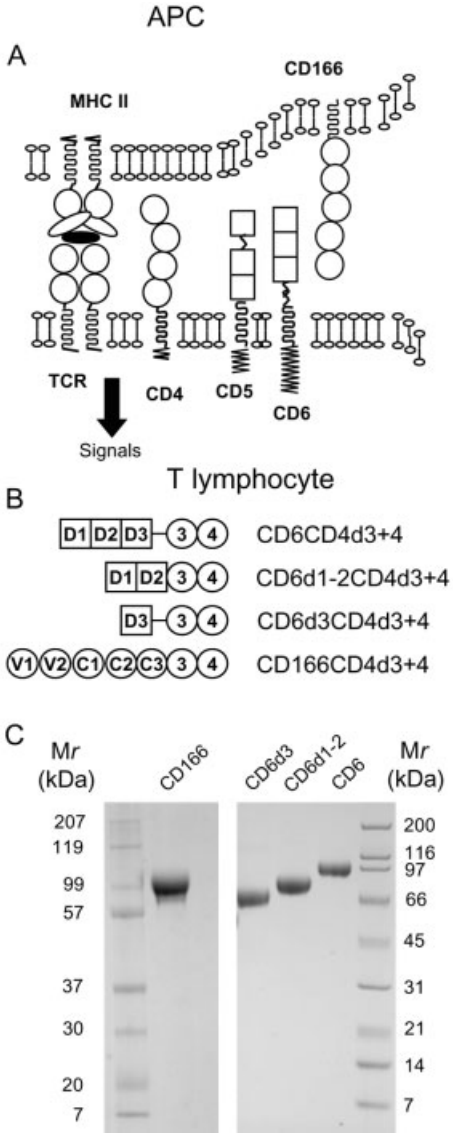

Fig. 1. Expression of soluble monomeric CD6 and CD166 fusion proteins. (A) Scale diagrammatic representation of some of the possible interactions between APC and T cells. The dimensions of the proteins are based on known structures as described for T cell interactions [1]. The distal V-like domain of CD166 contains the binding site for CD6 with the predicted topologies shown. (B) Schematic representation of the CD6 and CD166 extracellular regions expressed as chimeric fusion proteins with rat CD4d3+4. The CD6CD4d3+4 fusion protein is composed of three SRCR domains (d1–3) and the short membrane-proximal stalk region fused to rat CD4d3+4. Truncated fusion proteins contain CD6d1–2 or CD6d3. CD166CD4d3+4 fusion protein is composed of two V-like domains and three C-domains fused to rat CD4d3+4. (C) Monomeric fusion proteins were purified by affinity chromatography using an OX68 column and gel filtration and analyzed by SDS-polyacrylamide gel electrophoresis.

which may be explained by sequestering of CD5 [23]. Negative regulatory effects of CD5 are mediated by the cytoplasmic region and can be observed in the absence of the extracellular region of CD5 [24]. Correlation of CD6 expression on thymocytes with resistance to apoptosis [13] is consistent with a negative regulatory role for the CD6 cytoplasmic tail.

The extracellular region of CD6 has been shown to interact with CD166, a widely expressed protein with five IgSF domains and thus is the first example of an interaction between a SRCR domain and an IgSF domain (Fig. 1A) [4]. Biochemical analysis of this interaction using Ig fusion proteins suggests that the third (membrane proximal) SRCR domain of CD6 (CD6d3) specifically bound the N-terminal IgSF domain of CD166 (CD166d1) and that the other SRCR domains were not required for the interaction [25, 26]. Currently there are no data to propose a functional role for the engagement of these molecules. The use of whole antibodies have not been sufficient to address this as both nonblocking and blocking [12] CD6 mAb enhance the mixed lymphocyte response consistent with the mAb exerting their effects by cross-linking CD6 and influencing signaling. In addition to its ability to bind CD6, CD166 mediates homotypic CD166/CD166 interactions [27, 28].

We measured the affinity and kinetics of the heterophilic CD6/CD166 and homophilic CD166/CD166 interactions. We used soluble monomeric CD6 and CD166 proteins as highly selective tools in a T cell assay to show inhibition of a T cell response. These observations show that CD6/CD166 engagement provides a positive contribution during specific immune responses.

2 Results

2.1 Kinetic analysis of the CD6/CD166 interaction

Previous studies have shown that the CD6/CD166 interaction is mediated through CD6d3 and the N-terminal domain of CD166 (reviewed in [29]). The membrane proximal stalk region of CD6 enhanced the interaction but no contribution of the N-terminal domains of CD6 was detected [25, 26]. We analyzed the CD6/CD166 interaction and the contribution of the various CD6 domains quantitatively by surface plasmon resonance (SPR) using monomeric proteins and a BIAcore™. Recombinant fusion proteins were expressed containing rat CD4 domains 3 and 4 downstream of the complete CD6 extracellular region (CD6CD4d3+4), the first two SRCR domains (CD6d1–2CD4d3+4), the third SRCR domain and stalk region (CD6d3CD4d3+4) and the complete extracellular region of CD166 (CD166CD4d3+4) (Fig. 1B and C).

The affinity of the CD6/CD166 interaction was measured at 37°C by injecting a series of different concentrations of purified soluble monomeric CD6CD4d3+4 over CD166CD4d3+4-biotin and a negative control (CD4d3+4-biotin) immobilized on a streptavidin-coated chip in a BIAcore. Specific binding is represented by the difference in response units (RU) observed in the CD166 and control flow cell (Fig. 2A) once equilibrium has been reached. This is then plotted as a binding curve (Fig. 2C). Non-linear curve fitting of the binding data using the Langmuir model yielded a K_D =1.0 μM. Scatchard analysis gave the same result and shows the interaction follows a one-to-one binding model. Kinetic analysis of the interaction at 37°C yielded a K_{off} =0.4 s^{-1} for both high and low levels of CD166 immobilization, indicating that kinetic measurements were not grossly affected by rebinding or mass transport effects (Fig. 2E). The on-rate for this interaction was calculated as 4×10^5 $M^{-1}s^{-1}$ and the half-life as 1.7 s.

We measured the affinity of soluble monomeric CD6d3CD4d3+4 protein for CD166 at 37°C in the same way as with CD6CD4d3+4. A series of different concentrations of purified soluble monomeric CD6d3CD4d3+4 was injected over CD166CD4d3+4-biotin and the negative control (Fig. 2B). A K_D =2.9 μM was calculated for this interaction (Fig. 2D). Kinetic analysis yielded a K_{off} =0.7 s^{-1} for both high and low levels of CD166 immobilization (Fig. 2F). The measurement of K_{off} is independent of the concentration of protein passed over the chip. Therefore a higher K_{off} for CD6d3CD4d3+4 supports the K_D data suggesting a slightly lower affinity of this protein relative to CD6CD4d3+4 when binding CD166. The on rate and half-life for this interaction were calculated as 2.4×10^5 $M^{-1}s^{-1}$ and 0.99 s, respectively. In the complementary experiment of testing CD6d1–2 in the absence of CD6d3, no binding was observed when concentrations of up to 40 μM soluble monomeric CD6d1–2CD4d3+4 were passed over CD166CD4d3+4-biotin (1,000 RU) at 37°C compared with a negative control (data not shown). This level of immobilized CD166 was capable of specifically binding 235 RU CD6CD4d3+4 (6.5 μM). These results show that CD6d1–2 has no measurable affinity for CD166 but may play a minor role in stabilizing the interaction with CD166. We have not detected binding of fluorescent beads coated with CD6d1–2 to cells to suggest an alternative ligand for CD6d1–2 (unpublished data). There have been suggestions that CD6 binds to an alternative ligand [30–32].

2.2 Kinetic analysis of the CD166/CD166 interaction

In addition to its heterophilic engagement, CD166 interacts in a homophilic manner in *cis* or in *trans* [27, 28]. There is evidence that CD166 clusters on the cell surface enhancing avidity for CD166 on an opposing cell and that this is reduced using a CD166 blocking mAb [27]. The membrane proximal IgSF domains are involved in the *cis* clustering effect [27] while the *trans* homophilic interaction is mediated through the N-terminal domain [28]. The *cis* effect is dependent on cell surface expression thus it is the affinity of the *trans* homophilic interaction between the N-terminal domains we attempted to measure in solution to compare the kinetics of the interaction of CD166 with itself and with CD6. We purified CD166CD4d3+4, which behaved as monomer on gel filtration (data not shown). The purified monomeric CD166CD4d3+4 (Fig. 1) was passed over approximately equivalent molar amounts of immobilized CD166CD4d3+4 and CD6CD4d3+4 and a control flow cell. The sensogram traces in Fig. 3A show that CD166 binds to both CD166 and CD6. Binding is higher to CD6 until saturation is approached in both the CD166 and CD6 flow cells at the highest concentration of soluble CD166. In measuring homophilic interactions by SPR we have previously developed equations which take into account potential interaction between soluble molecules (K_{D1}), soluble and immobilized molecules (K_{D3}, assumed to be equal to K_{D1}) and between immobilized molecules (K_{D2}) [33]. Our previous analyses showed that for the CD150/CD150 homophilic interaction the best fit was obtained with K_{D1} =K_{D2} [33]. Initial analysis of specific equilibrium binding by CD166 to itself for four separate determinations including the data shown in Fig. 3B by standard non-linear curve fitting using a Langmuir model suggested that the homophilic interaction had a K_D in the range 24–35 μM. This assisted in choosing a value for initial substitution in the equilibrium homophilic binding equation 7 [33]. For the data shown in Fig. 3B, substitution of K_{D1} =42 μM resulted in K_{D1} =42±0 μM ~ K_{D2} =42.5±18 μM with A_f =121±18 μM (A_f =available concentration of immobilized CD166CD4d3+4). Fitting the data in the same way from the four sets of injections of CD166 over the same flow cell gave a range of K_D values from 32–48 μM and 114–122 μM for A_f with errors similar to those for Fig. 3B for K_{D2} and A_f (Table 1). To allow the fit, the concentration of immobilized CD166 was increased threefold above the calculated concentration (see Sect. 4). This can be explained by uneven coating of the chip at the low concentrations of immobilized CD166 used. Consistent with this explanation, the discrepancy was more marked at lower concentrations of immobilized material (Table 1).

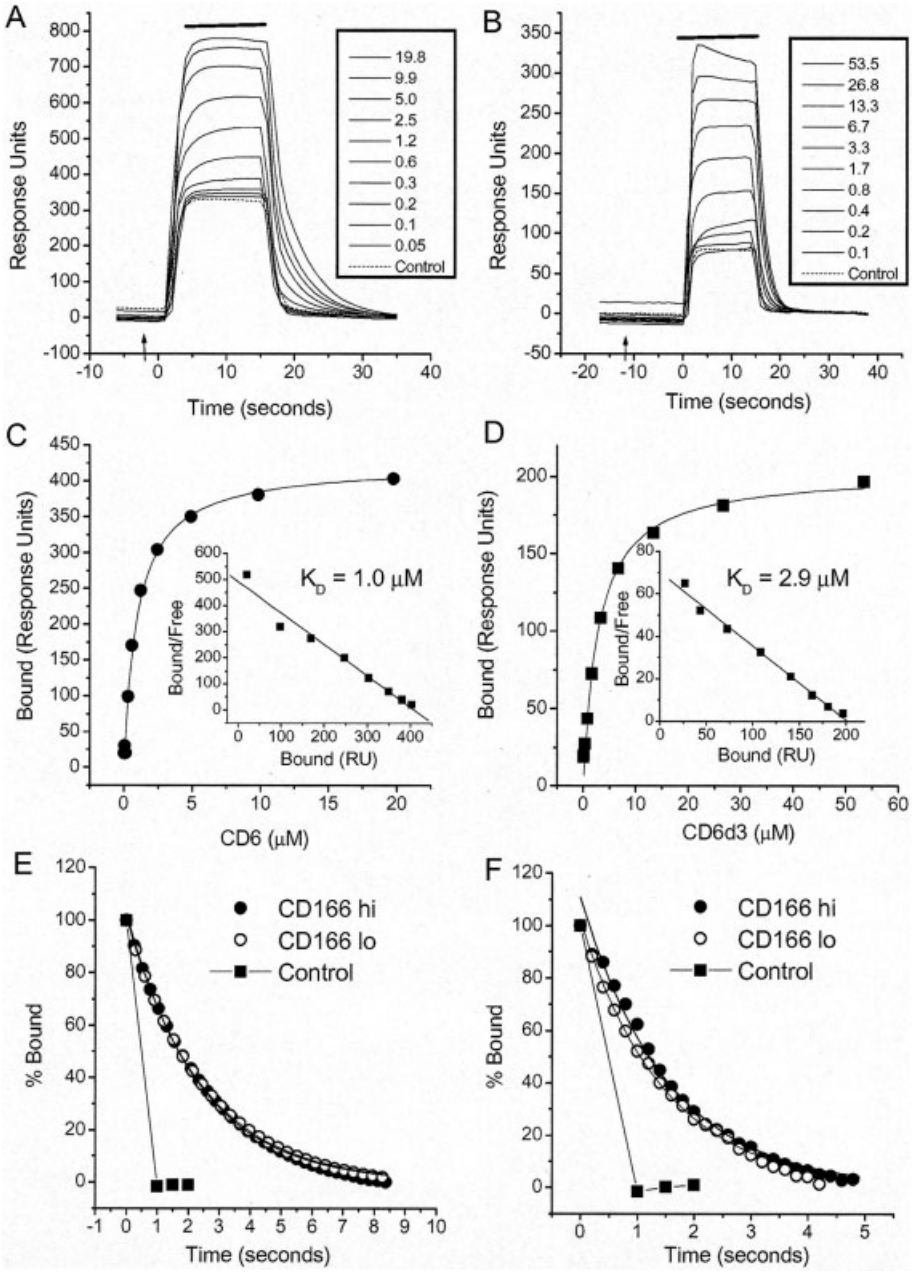

Fig. 2. The affinity and kinetics of the human CD6/CD6d3-CD166 interaction. (A) CD6 binds CD166: The indicated concentrations (μM) of CD6CD4d3+4 (CD6) was injected at 20 μl/min over immobilized CD166CD4d3+4-biotin (1250 RU) or CD4d3+4-biotin-negative control (2600 RU). The results from a series of experiments are superimposed. (B) CD6d3 binds CD166: Similarly, concentrations (μM) of CD6d3CD4d3+4 (CD6d3) were injected over immobilized CD166 (430 RU) or CD4d3+4-biotin-negative control (980 RU). (C and D) Specific equilibrium binding of CD6 or CD6d3 to CD166 is plotted. The data fit to a Langmuir model of binding and corresponds to a K_D of 1.0 μM for CD6/CD166 (C) and 2.9 μM for CD6d3-CD166 (D). Scatchard transformations of the binding data are shown inset in (C) and (D), and the linear fits show similar affinities to the curve fittings. (E) Dissociation constant of the CD6/CD166 interaction was measured by injecting 5 μl of soluble CD6CD4d3+4 (3 μM) at 100 μl/min over immobilized CD166CD4d3+4-biotin at high (1,250 RU) and low (600 RU) levels and also a negative control of CD4d3+4-biotin (2600 RU). Data were collected at 10 Hz. The data were then normalized (100% at the start of the dissociation phase), and first order exponential decay curves were fitted (lines) to the CD6 data that yield K_{off} values of 0.4 s^{-1}. (F) The dissociation constant of the CD6d3/CD166 interaction was measured under the same conditions as described for (E). CD6d3CD4d3+4 (6.38 μM) was injected over immobilized CD166CD4d3+4-biotin at high (400 RU) and low (200 RU) levels and also the negative control CD4d3+4-biotin (980 RU), yielding a K_{off} value of 0.7s^{-1}. Only every second dissociation data point is shown.

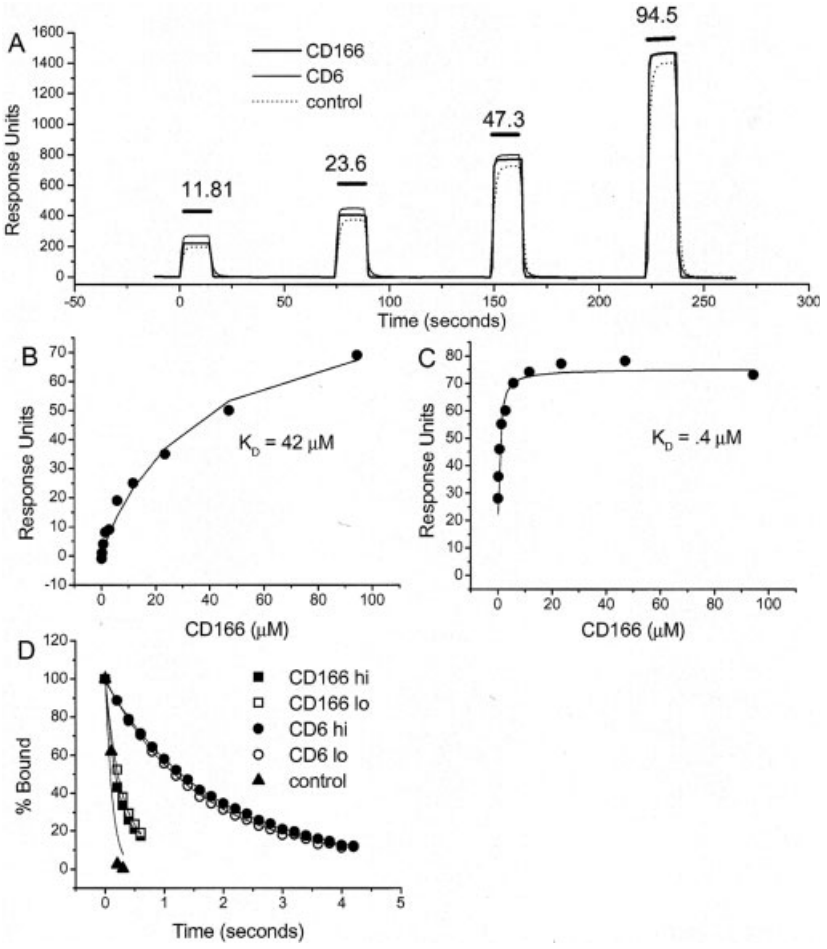

Fig. 3. CD166/CD166 homophilic interaction has a lower affinity and faster dissociation rate compared with the CD166/CD6 interaction. (A) Soluble CD166CD4d3+4 was injected at 20 μl/min at the indicated concentrations (μM) over immobilized CD166CD4d3+4-biotin (536 RU), CD6CD4d3+4-biotin (450 RU) and a negative control, CD4d3+4-biotin (595 RU). A selected range of soluble CD166 concentrations is shown to demonstrate saturation of immobilized CD166CD4d3+4-biotin and CD6CD4d3+4-biotin. (B) Fitting CD166/CD166-specific equilibrium binding with the homophilic binding equation 7 [33], substitution of K_{D1} =42 μM gave K_{D2} ~ 42.5±17 μM gave a good fit with a concentration of immobilized CD166CD4d3+4, A_f =120±18 μM. (C) CD166/CD6-specific binding was fitted using the Langmuir model giving a K_D =0.4 μM. (D) Specific dissociation of CD166 (6.3 μM) from $CD166^{hi}$ (536 RU) K_{off} =5.3 s^{-1}, $CD166^{lo}$ (211 RU) K_{off} =3.8 s^{-1}, $CD6^{hi}$ (1,133 RU) K_{off} =0.55 s^{-1}, $CD6^{lo}$ (450 RU) K_{off} =0.63 s^{-1} and from control CD4d3+4 (595 RU) K_{off} =8.6 s^{-1} at 100 μl/min was calculated by exponential decay curve fitting (lines). Only every second data point for CD6 is shown.

Table 1. Affinity of CD166/CD166 interaction[a)]

K_{D1} μM	K_{D2} μM	A_f (eq.7) μM	Chi^2	A_f (eq.14) μM
42	42.5 ± 17	121 ± 18	9	36
48	48 ± 17	122 ± 17	6	36
35	35 ± 17	115 ± 21	15	36
32	32 ± 12	114 ± 16	10	36
29	29 ± 13	74 ± 10	8	14
31	31 ± 13	74 ± 9	7	14

a) The value of K_{D1} was chosen for K_{D1} = K_{D2}. The apparent concentration of CD166CD4d3+4 on the chip was calculated by using equation 7, A_f (eq. 7) and equation 14, A_f (eq. 14) [33].

Table 2. Affinity and kinetics of CD6/CD166 and CD166/CD166 interactions

soluble	immobilized	K_D μM	k_{off} s^{-1}	$t_{1/2}$ s
CD6	CD166	1 ± 0.1	0.4 ± 0.02	1.7
CD166	CD6	0.4 ± 0.06	≥ 0.6 ± 0.02	1.2
CD6d3	CD166	3 ± 0.23	0.7 ± 0.01	1.0
CD166	CD166	29-48	≥ 5.3 ± 0.01	0.1

Comparison of the CD166/CD166 interaction with the CD166/CD6 interaction revealed a 100-fold difference in affinity (Table 2). CD166 bound to CD6 with a K_D=0.4 μM (Fig. 3C). This value is similar to results shown in Fig. 2C for the opposite orientation and ensures that the majority of the soluble CD166 is active and the K_D values are accurate. It can be seen in Fig. 3A that CD166 dissociates more slowly from CD6 than CD166. A more detailed kinetic analysis of dissociation of CD166 from CD6 is shown in Fig. 3D giving a K_{off} ≥0.63 s^{-1} that fits well with the data from the opposite orientation (Fig. 2E). CD166 dissociates tenfold faster from CD166, K_{off} ≥5.3 s^{-1} (Fig. 3D). Comparison with the value for washing through the flow cell shows these values are at the limits of detection of the BIAcore machine (Fig. 3D). This weak homophilic binding is more comparable with the CD150 homophilic interaction (K_D =200 μM) we have measured [33].

2.3 CD166 and CD6 compete for binding to CD166

It is the N-terminal domain of CD166, which mediates both its heterophilic and homophilic interactions with CD6 and itself, respectively. There are data to suggest the CD166 binding sites for CD6 and CD166 are different [27, 28]. We tested whether there was competition between CD166 and CD6 for binding to CD166. When soluble CD166 and CD6 were premixed and passed over immobilized CD166, specific binding was reproducibly reduced relative to the response observed with CD6 alone (Table 3). This result is consistent with soluble CD166 competing with immobilized CD166 for binding to CD6 and more compatible with a model in which the heterophilic and homophilic binding sites on CD166 are identical or at least overlapping.

2.4 Blocking CD6/CD166 extracellular engagement reduces antigen-specific T cell responses

To understand the contribution of CD6/CD166 extracellular engagement in regulation of immune responses, we investigated the effects of specifically blocking this interaction between human cells using soluble monomeric protein. This reagent provides a powerful tool to isolate the effects of ligand engagement from potential cross-linking events that would occur when using antibodies or Ig fusion proteins. We checked the expression of CD166 and CD6 on resting human PBL. CD166 was expressed on monocytes (Fig. 4A) with minimal expression on lymphocytes (Fig. 4C). Within the lymphocyte population, the majority of T cells were negative for CD166 (Fig. 4B). $CD166^+CD3^+$ cells only increased from 1% to 4% after 24 h polyclonal activation with Con A in agreement with previous data [4]. CD6 had a complementary distribution (Fig. 4) being highly expressed on T cells (Fig. 4B and C) and low on monocytes (Fig. 4A). Thus the distribution of CD6 and CD166 is compatible with a role of the CD6/CD166 interaction in controlling T cell activity.

We stimulated purified PBL with tetanus toxoid antigen in the presence or absence of monomeric CD6CD4d3+4, CD6d3CD4d3+4 or control CD4d3+4. Using concentrations of protein twofold greater than the measured affinities (Fig. 2), we observed a reduction in antigen-specific proliferation of PBL relative to a negative control at the same protein concentration (Fig. 5A). A 1.7-fold reduction in proliferation was observed when blocking with either CD6CD4d3+4 or CD6d3CD4d3+4 suggesting that CD6 domain 3 is primarily responsible for these effects. This is consistent with our kinetic study and previously reported data, which show that domain 3 is the main

Table 3. CD166 and CD6 compete for binding to CD166[a)]

Soluble (μM)	RU (CD166lo)	RU (CD166hi)
CD166(2)	28	36
CD6(1)	57	133
CD166(2) + CD6(1)	20	29

[a)] Soluble CD166CD4d3+4 and CD6CD4d3+4 were passed over immobilized CD166CD4d3+4-biotin^{lo} (207 RU), CD166CD4d3+4-biotin^{hi} (553 RU) and CD4d3+4-biotin (607 RU). Specific equilibrium binding is shown. Concentration of soluble CD166CD4d3+4 or CD6CD4d3+4 is shown in parentheses.

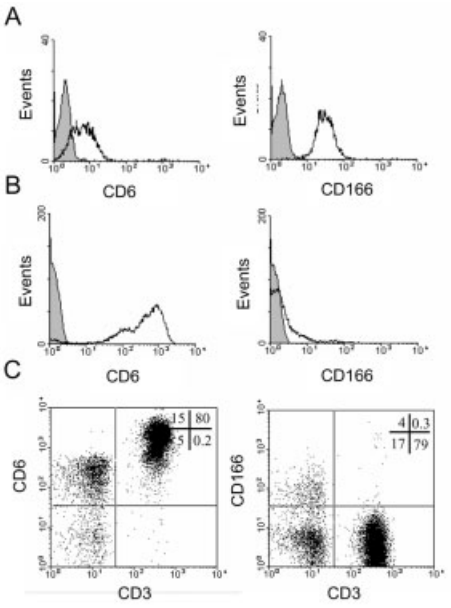

Fig. 4. Distribution of CD6 and CD166 on human PBL. Human PBL were gated for monocyte (A) or lymphocyte (B) populations and stained with CD6 mAb (clear histograms *left* A and B) or CD166 mAb (clear histograms on *right* A and B). Isotype-matched negative controls are represented by the shaded histograms. (C) The lymphocyte population was double-stained for CD6 (*left*) or CD166 (*right*) and CD3.

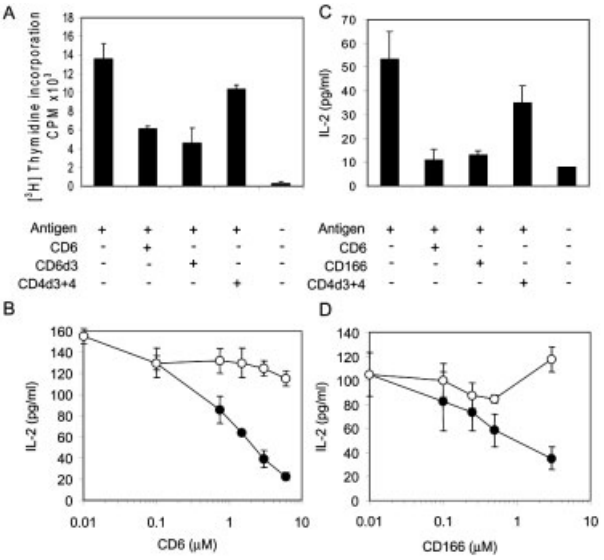

Fig. 5. Inhibition of antigen-specific T cell responses with soluble monomeric CD6 and CD166. (A) CD6 and CD6d3 reduced proliferation of tetanus toxoid stimulated PBL to similar levels. PBL were activated *in vitro* with 1 U/ml of tetanus toxoid antigen in the presence or absence of equal protein concentrations (0.4 mg/ml) of CD6CD4d3+4 (5 μM), CD6d3CD4d3+4 (6 μM) or CD4d3+4 (16 μM). (B) Inhibition by CD6 is concentration-dependent. IL-2 responses were measured 24 h after stimulation of PBL with 1 U/ml tetanus toxoid antigen in the presence of different concentrations of soluble monomeric CD6CD4d3+4 (solid circles), or the negative control CD4d3+4 (open circles) at 6 μM, 3 μM, 1.5 μM, 0.75 μM and 0.1 μM. (C) CD6 and CD166 reduced IL-2 production to similar levels. IL-2 responses were measured 24 h after stimulation of PBL with 0.25 U/ml tetanus toxoid antigen in the presence or absence of equal molar concentrations (2.5 μM) of CD6CD4d3+4, CD166CD4d3+4 or the negative control CD4d3+4. Two experiments produced similar results. (D) Inhibition by CD166 is concentration dependent. IL-2 responses were measured 24 h after stimulation of PBL with 0.25 U/ml tetanus toxoid antigen in the presence of different concentrations of soluble monomeric CD166CD4d3+4 (solid circles), or the negative control CD4d3+4 (open circles) at 3 μM, 0.5 μM, 0.25 μM and 0.1 μM.

region responsible for binding CD166 [25, 26]. The same trends were observed when using an alternative antigen (streptodornase kinase) for stimulation (data not shown). The concentration of tetanus toxoid antigen used in the experiment shown in Fig. 5 was 1 U/ml. When using a lower amount of specific antigen, 0.25 U/ml, the blocking effects of soluble CD6d3CD4d3+4 increased to 2.2-fold relative to the negative control (data not shown).

The proliferation assay described measures effects of incubating monomeric CD6 or CD6d3 with PBL, 6 days after exposing PBL to specific antigen. Since CD6 is highly expressed on resting T cells (Fig. 4B and C) we expected CD6/CD166 engagement to have greater influence during the early stages of the immune response. In addition, we sought to ensure that the functional effects observed were dependent on the amount of soluble monomeric protein present in a concentration-dependent manner. To test these hypotheses a similar assay was performed as described previously, but supernatants were harvested 24 h following incubation of purified PBL with 0.25 U/ml antigen in the presence or absence of increasing amounts of soluble monomeric CD6CD4d3+4 and the negative control protein (CD4d3+4 at equivalent molar concentrations). The supernatants were then assayed for interleukin-2 (IL-2) and the results shown in Fig. 5B. Clearly soluble monomeric CD6CD4d3+4 inhibited IL-2 production in a dose-dependent manner with about a sevenfold reduction observed at the maximum concentration of soluble CD6 giving a greater magnitude than in the proliferation assay (Fig. 5A).

Soluble CD6 will block both CD6/CD166 and CD166/CD166 interactions (Table 3). To ensure that we were observing effects of blocking CD6/CD166 and not CD166/CD166 in the functional assay we stimulated PBL with 0.25 U/ml tetanus toxoid in the presence or

absence of soluble monomeric CD6CD4d3+4, CD166CD4d3+4 or CD4d3+4 at a molar concentration (2.5 μM) capable of blocking the heterophilic and not the homophilic interactions of CD166, according to our kinetic data. CD6 and CD166 inhibited IL-2 production to similar levels (Fig. 5C), supporting our interpretation that we were specifically blocking CD6/CD166 in the assay. The blocking ability of CD166 was dependent on the concentration of protein used similarly to soluble CD6 (Fig. 5D).

3 Discussion

One of the challenges in immunology is to understand at a molecular level how the large numbers of proteins present in the human genome exert their function on the immune system. The cell surface of leukocytes is central to transmission of signals from the environment and the effect of pathogens to give signals to the cell. With so many different proteins their effects are likely to be subtle and be important in different situations. We describe the molecular analysis of the T cell protein CD6 and probing its function with highly selective recombinant proteins. Firstly the CD6/CD166 interaction between an SRCR domain and an IgSF domain was shown to be low affinity like most other leukocyte membrane protein interactions with a K_D of around 1 μM; secondly the membrane proximal CD6d3 bound almost as well as the three-domain form showing that this domain was responsible for most of the interaction; thirdly the CD166/CD166 homophilic interaction was around 100-fold weaker than the heterophilic interaction. Knowledge of the affinity and availability of large amounts of soluble monomeric CD6, CD6d3 or CD166 allowed functional assays to be probed. These proteins gave inhibition of T cell activation at concentrations around the K_D of the CD6/CD166 interaction. The high level of CD6 and CD166 on resting T cells and on antigen-presenting cells (APC), respectively show that the T cell/APC interaction is the primary site that the soluble CD6 is blocking in these assays. Soluble CD6 will also block CD166/CD166 interactions. However, we have shown in the functional assay that soluble monomeric CD166 used at concentrations that will only block CD6/CD166 inhibits T cell responses in a comparable manner to soluble CD6. Since we used soluble monomeric protein in this assay the effects observed can only be due to blocking the interaction and not cross-linking either molecule on the cell surface. Thus the functional effects observed are best interpreted as blocking CD6/CD166 and hence affecting the movement of CD6 on the T cell surface when in contact with CD166-positive cells such as APC. In contrast both nonblocking and blocking CD6 mAb [12] enhance the mixed lymphocyte response consistent with the mAb exerting their effects by cross-linking CD6 and having effects on signaling.

There have been very few functional studies using recombinant extracellular regions of proteins mediating such weak interactions because of the high protein concentrations needed. In studies on a soluble form of B7 it was not possible to rule out effects due to multimers of B7 and this protein tends to form dimers [34, 35]. The extracellular region of CD4 is efficient in blocking the high-affinity interaction with HIV gp120 protein but has no effect on T cell assays dependent on the weak interaction with MHC class II [36].

These findings suggest that extracellular CD6/CD166 ligand engagement serves to provide a positive contribution when T cells elicit an antigen-specific immune response. This appears contradictory to the negative regulatory role proposed for its close relative, CD5 [19, 20]. Two apparently opposing extracellular and intracellular functions can be reconciled if the dampening-down effects of a cytoplasmic region are necessary to regulate the level of T cell activation making it important to recruit CD6 during an immune response consistent with the CD6/CD166 interaction being relatively strong [37].

Recruitment of CD6 into the contact site in an antigen-specific response has been described [15]. The topology of the CD6/CD166 interaction suggests it spans a distance of approximately five tandem Ig-like domains that could potentially be recruited to the immunological synapse (Fig. 1A). The distance spanning the ten Ig-like domains formed as a consequence of CD166/CD166 *trans* interaction is too large to be present in the immunological synapse under the current theory [38]. Hence the localization of CD166/CD166 is probably different to CD6/CD166. The schematic drawing in Fig. 1A is supported by electron micrographs of a CD5CD4d3+4 in which IgSF and SRCR domains have similar dimensions and the three domains of CD5 formed a linear array [39]. Recruitment of CD6 to the immunological synapse is a prerequisite for optimal T cell activation and blocking the CD6/CD166 interaction prevents this recruitment from occurring.

4 Materials and methods

4.1 Monoclonal antibodies

The antibodies used were: anti-human CD3 (Leu-4) FITC (BD Immunocytometry Systems), phycoerythrin (PE)-labeled goat anti-mouse Ig (PharMingen). Anti-human CD166 domain C2 (HAL 47.1) and anti-human CD6 domain 3 (13C3–2A11) [12] were kindly provided by Michael Bowen.

OX68 (anti-rat CD4d3+4) and W6/32 (anti-human HLA) are referenced in the European Collection of Animal Cell Cultures (ECACC; Porton Down, Salisbury, GB).

4.2 Cloning and expression of soluble CD6 and CD166 fusion proteins

Fragments encoding the complete or parts of the human CD6 extracellular region were amplified by PCR from a human CD6-pCDM8 template [40] using a final concentration of 10% DMSO in the PCR reaction mix. The full-length CD6 and domains 1 and 2 (CD6d1–2) fragments were amplified with primers (restriction sites are underlined) (sense) tagtagtctagaagccagagacagctccagac and (antisense) tagtaggtcgaccgagattccttgttc and (antisense) tagtaggtcgaccgcgccccctgtcaggcgcca, respectively to protein sequences Met^{1}-Arg^{397} and Met^{1}-Ala^{271} [40]. A fragment encoding CD6d3 and the membrane proximal stalk region (CD6d3) was amplified with (sense) tagtagtctagattgtcgacatcagagcaccagtcc and (antisense) tagtagctcgagcgagattccttgttctctatt to encode the protein sequence Ser^{260}-Arg^{397}. Products were digested with *Xba* I and *Sal* I or *Xba* I and *Xho* I for CD6d3 and ligated into a vector containing rat CD4 leader (CD4L) and domains 3 and 4 of rat CD4 (CD4d3+4) followed by a sequence which can be enzymatically biotinylated (CD4LCD4d3+4-biotin pEF-BOS-XB expression vector) [41] from which the CD4L had been removed with *Xba* I and *Sal* I. The CD4L was reinserted into the CD6d3CD4d3+4 vector cut with *Xba* I and *Sal* I. The resulting protein sequence at the junction with rat CD4d3 for CD6 and CD6d3 was KESR*STSIT* and that for CD6d1–2 was TGGA*STSIT.* The extracellular region of CD166 was amplified with (sense) tagtagtctagacaccaagaaggaggaggaat and (antisense) tagtaggtcgacgcctggtcattcacctttc from ALCAM-pCDM8 [4] to encode the protein sequence Met^{1}-Ala^{526} with the resulting sequence at the junction with rat CD4d3 of NDQA*STSIT*. *Xba* I-*BamH* I fragments encoding CD6CD4d3+4, CD6d1–2CD4d3+4, CD6d3CD4d3+4 and CD166CD4d3+4 were transferred to the PEE14 vector cut with *Xba* I and *Bcl* I. PEFBOS-XB constructs were transiently expressed and biotinylated [41]. Stable lines were established with PEE14 constructs [42] in Chinese hamster ovary cells and expressed protein purified by affinity chromatography with OX68 mAb [43]. Monomeric protein was eluted from a Superdex 200 (Pharmacia Biotech Ltd) column and used in BIAcore analysis without further concentration. Extinction coefficients for CD6CD4d3+4 (103,120 $M^{-1}cm^{-1}$), CD6d1–2CD4dD3+4 (81,250 $M^{-1}cm^{-1}$), CD6d3CD4d3+4 (53,120 $M^{-1}cm^{-1}$) and CD166CD4d3+4 (84,650 $M^{-1}cm^{-1}$) were calculated (www.basic.nwu.edu/biotools/Proteincalc.html).

4.3 BIAcore™ analysis of affinity and kinetics of the CD6/CD166 interaction

BIAcore analysis was carried out using a BIAcore™ 2000 biosensor instrument (BIAcore AB) as described [41, 44]. Approximately 2,000–3,000 response units (RU) streptavidin (0.2 mg/ml) was coupled to a research grade CM5 chip (BIAcore AB) in 10 mM sodium acetate pH 5 using an amine coupling kit and an activation time of 5 min. For equilibrium affinity measurements at 37°C, increasing and decreasing concentrations of monomeric purified proteins (5 μl injections at 20 μl/min) were passed over biotinylated protein immobilized on streptavidin [41, 44]. Kinetic measurements were made by injecting purified proteins for 3 s at 100 μl/min over high and low levels of immobilized protein [41, 44]. Homophilic binding was analyzed by substitution of a range of values for K_{D1} into equation 7 [33] and allowing K_{D2} and the concentration of immobilized material (A_f) to vary until K_{D1} = K_{D2}. K_{D1} and K_{D2} were initialized using values obtained from a rough estimate of K_{D1} from a Langmuir model. A_f was calculated using equation 14 and P =14, *i.e.* 14 RU =1 μM for CD166CD4d3+4 calculated experimentally [33]. Dissociation rates were measured [44] using Origin software, version 5.0 (Microcal Software, Inc., Northampton, MA).

4.4 Purification of PBL

Human PBL were isolated by centrifugation on Ficoll-Hypaque (density 1.077; Pharmacia, Uppsala, Sweden) for 30 min at 18°C. Mononuclear cells were collected from the interphase, centrifuged and residual red blood cells were lysed by 1 min incubation in water followed by addition of 2×PBS. After two washes in PBS, cells were resuspended in complete medium (RPMI-1640, 2 mM glutamine, 10% human AB serum, 100 U/ml Penicillin-Streptomycin) at 2×10^{6}/ml.

4.5 Proliferation and IL-2 assays

PBL (100 μl at 2×10^{6}/ml) were put into 96-well U-bottom plates (NUNC, Gibco). Tetanus toxoid antigen (anatoxine, GibcoBRL) was added, 100 μl of 1 U/ml or 0.25 U/ml in the presence or absence of soluble purified monomeric CD6CD4d3+4, CD6d3CD4d3+4, CD166CD4d3+4or CD4d3+4 proteins. For proliferation assays, cultures were incubated at 37°C for 144 h in 5% CO_2 and pulsed with 0.5 μCi [^{3}H]thymidine (Amersham International, GB) during the last 16 h of the culture. For IL-2 assays cells were incubated at 37°C for 24 h in 5% CO_2 and 100 μl supernatants were collected and used in IL-2 immunoassays as described (PharMingen).

4.6 Flow cytometry

PBL (100 μl at between 1×10^{6} and 1×10^{7} per ml) were centrifuged at 1,200 rpm (300×g in a 96-well U-bottom plate (NUNC, Gibco) for 2 min and resuspended in 25 μl of primary antibody (CD6 mAb, CD166 mAb or isotype controls at about 5 μg/ml of purified mAb or spent tissue culture supernatant) in PBS 1 % BSA, 0.02% NaN_3. The cells were incu-

bated at room temperature for 15 min, washed twice with 200 μl buffer and incubated with 25 μl (1/100) PE-labeled goat anti-mouse IgG for 15 min. The cells were washed with 2×200 μl buffer, 50 μl of 10% mouse serum added for 15 min to prevent nonspecific binding of antibodies and FITC-CD3 was added. After 15 min cells were resuspended in 200 μl and analyzed on a FACScan (Becton Dickinson) using WinMDI data analysis software (J. Totter, The Scripps Clinic, La Jolla, CA).

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