CD6 as a cell surface receptor and as a target for regulating immune responses.

Running title: CD6 as a target.

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Abstract: CD6 has been exploited as a drug target as its expression is restricted, primarily to T cells, it has a well characterised cell surface ligand, CD166 and regulates T cell activation through a long cytoplasmic tail. CD6 can affect both the initiation and maintenance of T cell function in a negative and positive manner respectively so that it is important to understand these dual effects of a potential drug target. The effective mode of action of clinical monoclonal antibodies (mAbs) that recognise cell surface receptors including CD6 is commonly cytotoxic depletion of cells. It is not clear how current therapeutic strategies to target CD6 perturb function. With the benefit of new structural data, this review provides a critical analysis and interpretation of experiments in which various reagents have been tested and offers some suggestions as how more effective drugs may be developed.

Keywords: CD6, CD166, SLP-76, T cells, activation, inhibition, immunotherapy, signal transduction

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1. CD6 AS A CELL SURFACE RECEPTOR

CD6 is expressed on lymphocytes of the adaptive immune system. CD6 is expressed on all types of T cells and thymocytes indicating a conserved fundamental role in regulating T cell responses. The significance of expression on other cells including B cells and some cells in the brain will

become clear once we understand its specific niche (1, 2). CD6 is expressed on both CD4 and CD8 T cells and does not seem essential for the specialised function of cytotoxicity (3). A recent characterisation shows low CD6 expression on natural regulatory T (nTeg) cells (4). An inverse correlation between CD6 and FOXP3 expression suggests CD6 is not relevant for the specialisation necessary for suppressor cells (4). Levels of CD6 on the cell surface did not appear to be as dramatically regulated as its close relative, CD5 and a lack until more recently of reagents specific for mouse CD6 hindered progress (1, 5). CD6 and CD5 show structural similarity and both contain three scavenger receptor cysteine rich (SRCR) domains in their extracellular regions (Fig. 1) (6). SRCR domain containing proteins are generally associated with the innate immune system with the exceptions of forms of CD163 expressed on γδ T cells and CD5 and CD6 on αβ T cells (7). There appears to have been strong evolutionary pressure to conserve CD5 and CD6 in the adaptive immune system in mammals and birds but it is not yet clear why the particular topology of the extracellular region has been maintained. Another distinguishing feature of CD6 is its extraordinarily long cytoplasmic region, the longest of a lymphocyte receptor without catalytic activity (8). A prerequisite to exploiting CD6 as a therapeutic drug target is to understand engagement at the cell surface and the mechanism of signal transduction and under what circumstances perturbing CD6 enhances or suppresses immune responses.

2. INTERACTIONS OF CD6 AT THE CELL SURFACE

2.1 CD6 binds CD166

The extracellular region of CD6 binds a cell surface ligand, CD166 for which there are many other names, the most commonly used being “Activated Leukocyte Cell Adhesion Molecule” (ALCAM) (Fig. 1). CD166 contains five immunoglobulin superfamily domains which have been classified from membrane distal to proximal as VVCCC (Fig. 1). CD166 is expressed on innate and adaptive immune cells and also nonhaematopoetic cells including thymic epithelial cells which regulate development of immunity. The distribution and levels of expression of receptor and ligand are of key importance when considering therapeutic intervention. Low expression of CD6 on natural T regulatory cells indicates their suppressor function will not be affected which is an important consideration in developing an immunosuppressive drug (4). CD6 negative cells which showed low alloreactivity may be natural T regulatory cells (9).

The interaction between CD6 and CD166 has been reviewed by the group who discovered and characterised the interaction including dissection of contributions of individual domains to the interaction and mutagenesis of single amino acid residues (10-12). To briefly reiterate the findings, the *trans* interaction between CD6 and CD166 is between the N-terminal domain of CD166 and the membrane proximal domain of CD6 (10-12) (Fig. 1). The N-terminal domain of CD166 and the membrane proximal domain of CD6 are sufficient to sustain the interaction. A subsidiary role for other domains in the interaction between CD6 and CD166 was hinted at, particularly domain 2 of CD166 in enzyme-linked immunosorbent assay (ELISA) and cell binding studies with domain deletion mutants (13). Soluble CD6 is unstable and this is exacerbated in mutants lacking domains or the stalk region and may explain some of the differences in binding to CD166 (13, 14). In measurements by surface plasmon resonance no detectable contribution of CD6 SRCR domains other than domain 3 to the interaction with CD166 was detected (15).

2.2 Structure of CD6 consecutive SRCR domains

The X-ray crystal structure of the three SRCR domains shows that these domains are not arranged in a straightforward linear array but are kinked as shown schematically in the diagram (Fig. 1) (15). The joins between each domain are very similar indicating this is important. The consequence is that if the individual domains use the same binding face they would be in different orientations with respect to the cell surface. The membrane proximal unstructured stalk region is likely to provide flexibility and be important for facilitating the interaction between CD166 and CD6 at the cell surface. The isoform of CD6 found naturally that lacks the CD166 binding domain retains the stalk region (16). The orientation of CD6 domains 1 and 2 in the two and three domain forms of CD6 may differ. In addition to facilitating *trans* interactions

with CD166, orientation of CD6 domains may be important for lateral interactions in *cis* on the same cell surface. In the absence of domain 1, CD6 was not stable at the cell surface (MHB, unpublished data). A tendency of CD6 to dimerize may be important for stabilisation at the cell surface (15) as has been suggested for CD5 (17, 18).

2.3 Other Interactions of CD6 at the cell surface

Reports of host interactions of CD6 which are less well defined compared with the interaction with CD166 include calcium or carbohydrate dependent components (19-21). Calcium dependent and carbohydrate dependent interactions have been described for other SRCR domains (22, 23).

The evidence for one alternative ligand for CD6 on host cells was based on the lack of inhibition by a CD166 mAb (J4-81) on interactions between CD6 and CD166 expressing cells (24). In the same assay a CD6 mAb (T12) known to have the capacity to block CD6/CD166 interactions was ineffectual whereas a high affinity CD6 domain 1 mAb caused significant inhibition (12, 24). The lack of inhibition by the CD166 mAb in the same assay may also be due to inherent properties of the antibody such as affinity and the epitope recognised. These data as stated by the authors themselves are not conclusive in providing evidence for an alternative ligand for CD6 (24). Molecular characterization of the 3A11B antigen is needed to assess the significance of these findings (25, 26).

In the absence of clear data for additional ligands for CD6, at least with comparable affinity as that of the CD166 interaction, additional probably low affinity interactions may not be significant compared with the effects of perturbing CD6/CD166 interactions when considering CD6 as a therapeutic target.

2.4 Homophilic Interactions of CD166

CD166 mediates two types of *trans* interactions between cells, heterophilic with CD6 and homophilic with itself. Both heterophilic and homophilic interactions depend on the N-terminal V-like domain (13, 27-29). Images of interacting cells showed the CD166 homophilic *trans* interactions between cells (27, 29). In the second approach, atomic force microscopy with purified recombinant CD166 immobilized on a cantilever surface interacted with CD166 on the cell surface (28). These studies were carried out in cells which do not express CD6. The role of homophilic interactions of CD166 has been investigated in cancer and development (30, 31). Presence and absence of CD166 can promote tumour growth and metastases respectively, complicating any strategy to target its perturbation in cancer therapy (32).

A role for membrane proximal C domains in regulating avidity in *cis* on the same cell surface was demonstrated in two ways. Firstly, a CD166 mAb specific for the membrane proximal domains and secondly, expression of CD166 lacking the N-terminal domain in cells expressing wild type CD166 inhibited CD166 homophilic interactions (29, 32). Homophilic interactions of CD166 molecules between cells depend on the N-terminal domain and clustering through the membrane proximal domains (29). An interaction between the membrane proximal domains is not maintained in solution, there being no difference in the molecular species observed in gel filtration between the full length extracellular region and truncated versions of CD166 (15). Weak interactions below the level of detection in binding assays and surface plasmon resonance using soluble protein may have functional effects when the dimensions of interactions are restricted between cells (33, 34) . Homophilic interactions of cadherins have been modelled taking into account very weak *cis* interactions below the limits of biochemical detection but which influence interactions in *trans* (33, 34). Homophilic interactions of CD166 may be regulated in a similar manner. The predicted length of the complete CD166 molecule ~20 nm indicates there must be some flexibility in the molecule to accommodate interactions between cells which are compatible with signal transmission (8, 15).

2.5 Measuring interactions of CD6 and CD166

The heterophilic interaction is stronger than the homophilic. In surface plasmon resonance experiments, the affinity of CD166 for CD6 was 2 orders of magnitude higher than the homophilic interaction (14). Repetition of these measurements with His tagged proteins purified at neutral pH instead of fusion proteins purified at low pH gave K_D values with the same differential but a

magnitude lower, K_D=0.1 µM and K_D=10 µM for the heterophilic and homophilic interactions (and MHB, unpublished data). For another homophilic interaction between immunoglobulin superfamily domains of SLAM (CD150), calculation of affinities of homophilic interactions from surface plasmon resonance binding data using equations taking into account interactions between soluble and immobilized receptors correlated with data obtained by analytical ultracentrifugation (35). The dissociation rates for the CD166 homophilic interaction were in the order of K_{off}~ 5 s^{-1}, 5 -10 fold faster than the heterophilic interaction at K_{off}~ 0.5 s^{-1} (14). Repetition of these experiments with His-tagged proteins confirmed the values for the CD6/CD166 interaction, CD166 dissociated from immobilised CD6, K_{off}= 0.2 s^{-1} and vice versa, K_{off}= 0.4 s^{-1} ((15), Lee I Garner and MHB, unpublished data). The difference between the two types of interaction was shown independently by atomic force microscopy and dissociation rates measured by the two different techniques were similar (14, 28,).

2.6 CD6 and CD166 interactions are conserved across species

The CD6/CD166 interaction is conserved across species. Human CD6 binds human and mouse CD166 with the same affinity (5). The affinity of mouse CD6 for human CD166 has not been reported but all 14 residues identified in human CD6 as being involved in CD166 binding are conserved in mouse (36), (15). A mouse CD6-Ig fusion protein reduces adhesion between human cells (26). Human CD6 also bound to bovine CD166 (37). Binding of soluble recombinant human CD6 tetramers to cells expressing rat CD166 was not observed (38). These binding experiments with low affinity reagents are critically dependent on levels of expression and reduced expression of rat compared with human CD166 maybe the explanation for lack of cross species binding (38). Once there is a critical level of expression of CD166, engagement is observed and may not depend on species specific interactions (38). Cross species CD166 homophilic interactions have been not yet been quantified but as human and mouse CD166 are 93% conserved (30) it is likely that as for the heterophilic interaction, the cross species affinities will be similar.

As has been predicted from the high level of conservation among species, cross species homophilic interactions of CD166 have been demonstrated between human and rat CD166 (11, 30, 39). It has been shown that expression of CD166 influences engraftment of stem cells indicating cross species hCD166 homophilic interactions will be of importance in model systems (30, 40).

2.7 Heterophilic and homophilic interactions of CD166 domain 1 compete

The orientation of the interacting N-terminal domains in homophilic interactions is not yet resolved. Models of CD166 homophilic interactions acting in *trans* have depicted the two N-terminal domains interacting in an anti-parallel fashion (29, 41). A binding interface between two N-terminal domains was identified in the X-ray crystal structure of CD166 (15). This involved the β strands A'GFCC'C" as commonly found in other IgSF interactions and mutations which disrupted ligand binding to CD6 mapped to the interacting faces (15). However there is no evidence that the crystal contacts represent a physiological interface.

Whether it be by competition or steric hindrance, there is competition between heterophilic CD6/CD166 and CD166/CD166 homophilic interactions (15). Any reagent which targets CD166 is likely to affect heterophilic CD6/CD166 and homophilic CD166/CD166 interactions. Specific perturbation of CD6/CD166 interactions requires a reagent against CD6.

3. THERAPEUTIC POTENTIAL OF BLOCKING ENGAGEMENT OF CD6 AND CD166

The primary target of a strategy to perturb CD6 and CD166 in the immune system through blocking extracellular engagement is the heterophilic CD6/CD166 interaction which has the higher affinity (14, 28). Therapeutic strategies to target CD6 and CD166 in the immune system have focussed on antibodies to CD6 to distinguish between CD6/CD166 heterophilic and CD166 homophilic interactions (5, 42, 43). The relative importance of the two types of interactions of CD166 in the immune system has not been systematically studied. The rationale for current therapeutic regimes is immunosuppression (42, 44).

A reagent which blocks both interactions may have a more profound effect but it is not yet clear whether this would be beneficial or detrimental. The broad distribution of CD166 means the homophilic interactions occur in multiple organs. The effects of CD166 deficiency in genetically manipulated mice were first studied in the nervous system (45). CD166 is repeatedly selected as a neoplastic marker in cancers and *trans* interactions between CD166 molecules also affect tumour growth and dissemination (32). To reiterate, loss of CD166 reduces primary growth but promotes metastases complicating perturbation of CD166 homophilic interactions as a general approach to controlling cancer (31, 32).

3.1 Perturbing CD6 interactions with mAbs

The effects of heterophilic interactions between CD6 and CD166 have been distinguished from CD166 homophilic interactions using CD6 antibodies (Table 1). Perturbation with CD6 mAbs in experiments where the mode of action is interpreted to be blocking has revealed an overall inhibitory effect on immune responses indicating that engagement by CD166 is important for costimulatory effects of CD6 (5, 43, 46, 47).

It was only after the cloning of CD6 and the production of recombinant protein that CD6 domain 3 mAbs were produced (1, 5, 48). A CD6 mAb specific for the membrane proximal ligand binding domain blocked soluble CD166 binding to CD6 in biochemical analysis (5). The blocking mAb inhibited antigen specific responses in primary human T cells (5). In functional experiments, it is not always clear what the mode of action of the antibodies are, blocking, crosslinking, modulation or depletion. The experiment was controlled using another nonblocking isotyped matched mAb specific for domain 3 supporting the interpretation that the mode of action of the blocking mAb was disruption of CD6/CD166 interactions (5). We concluded that these data showed that CD6/CD166 interactions had a costimulatory effect on an antigen specific immune response (5). The same CD6 mAb decreased proliferation of human lymphocytes in response to superantigen presented on a CD166 expressing B cell line. The mode of action in this instance was interpreted as delivering an inhibitory signal through crosslinking (38). Resolving conclusively how CD6 function is being perturbed by antibodies is crucial for assessing therapeutic potential. Costimulation by CD6/CD166 interactions marks the interaction as a suitable target for development of blocking immunosuppressive drugs. As the CD6 domain 3 mAb raised against human CD6 cross reacted with rat CD6 (16), it was tested for effects on experimental allergic encephalomyelitis (EAE) in rats but no significant effects were observed (S. J. Simmonds and MHB, unpublished data).

In the *in vitro* experiments described above CD6 mAbs inhibited immune responses. Correlation between the effects of soluble recombinant proteins and antibodies support the interpretation that the mode of action was blocking (5, 14, 43, 49). It is clear that expression of CD6 limits responsiveness of cells (5, 19, 38). The level of expression and the extent of ligand engagement by CD166 and the stimulus will affect the functional outcome. Where there is high expression and limited engagement by CD166, the functional outcome may be negative (5, 38). Mouse CD6 mAbs which are specific for CD6 domain 3 and block CD166 binding in biochemical assays, enhanced allogeneic mixed lymphocyte reactions (1, 5). While these antibodies may be crosslinking and activating it is also possible that the mode of action in this type of experiment is blocking as was shown for a CD5 mAb (18, 50). These data emphasize how important it is to understand regulation of activation and inhibition by CD6 when considering it as a therapeutic target. The data also reveal how caution must be exercised in relating observations made in mouse models to humans.

3.2 Steric hindrance by CD6 domain 1 mAbs

Current therapeutic regimes exploiting CD6 utilise CD6 antibodies which recognize domain 1. Two modes of action have been proposed; depletion and blocking. In the clinic, CD6 domain 1 mAbs have been exploited for depletion of T cells rather than blocking (51). Recently, a CD6 mAb specific for a different epitope on CD6 domain 1 has been licensed in India as an immunosuppressant and there are data to indicate a mode of action other than depleting ((44, 52, 53).

CD6 domain 1 mAbs do not inhibit binding of soluble CD166 to cells but have long been

described as inhibiting T cell responses (43, 46). CD6 domain 1 mAbs only perturb function between cells (12, 13). There has been speculation in the literature on the mode of action of inhibition by CD6 domain 1 mAbs ranging from blocking another interaction (24), inhibiting *cis* interactions (18, 43) and steric hindrance of CD6/CD166 interactions (43, 47). The mode of action was not always definitively proven in functional experiments and there are data which could be interpreted as CD6 domain 1 mAbs crosslinking and activating, crosslinking and inhibiting, blocking an activating signal or blocking an inhibitory signal (54, 55) (Fig. 2). A CD6 domain 1 mAb (T12) inhibited binding of cells transfected with CD6 to cells expressing CD166 showing that steric hindrance could disrupt CD6/CD166 interactions (12). Inhibition by CD6 domain 1 mAbs (UMCD6, MT605 and T12) depends on cell cell interactions (12, 43 , 46, 47). Inhibition by a CD6 domain 1 mAb of CD6/CD166 interactions between cells depended on expression of CD6 containing the ligand binding domain (47). Inhibition by CD6 domain 1 mAbs is dependent on the level of CD6/CD166 engagement (12, 16, 43, 46, 47). A single nucleotide polymorphism increased the relative amount of a form of CD6 lacking the membrane proximal ligand binding domain on the cell with a corresponding loss of ligand engagement and loss of inhibition by a CD6 domain 1 mAb (47). Loss of inhibition by a CD6 domain 1 mAb does not necessarily indicate there is no engagement between CD6 and CD166 as a CD6 domain 3 mAb which blocks soluble and cell bound CD166 binding perturbed responses where no effect of CD6 domain 1 mAb (UMCD6) was detected (16).

Epitope mapping identified R77, a residue at the very top of domain 1 on the opposite side to the CD166 binding face on domain 3, as being important for binding of one (MT605) of the CD6 domain 1 mAbs which perturbs function (15, 43, 46, 47). Binding by another CD6 domain 1 mAb (MEM98) was affected by an E63A mutation but not R77A and *vice versa*, MT605 was not affected by E63A. The epitopes for UMCD6 and MT605 differ ((56, 57), Lee I. Garner and MHB, unpublished data). It appears that CD6 domain1 mAbs specific for different epitopes can sterically hinder CD6/CD166 interactions. There is steric hindrance between UMCD6 and the IOR-T1 or in its humanised form, T1h mAb, currently being tested as an immunosuppressant ((44, 56, 57), Lee I. Garner and MHB, unpublished data). It is conceptually straightforward to visualise how a large antibody binding to the top of CD6 domain 1 would sterically hinder interactions between cells (Fig. 2B). A CD6 domain 1 mAb which is only effective at a sufficient level of engagement between cells may be the optimal immunosuppressive drug for blocking CD6/CD166 interactions which result in costimulation. This would increase the specificity of a drug restricting its effects to interactions between cells expressing high levels of CD6 or CD166.

3.3 Steric hindrance by CD166 mAbs

Mouse mAbs specific for human CD166 domain 1 bind to the opposite face from the CD6 binding face and do not prevent CD166 interactions ((13, 58), Table 1). There are mouse antibodies specific for human CD166 (J4-81, MAB656 R&D Systems) which do not bind domain 1 but perturb CD166 interactions (12, 59). A CD166 mAb (J4-81) inhibited binding of CD6 to cells leading to the identification of CD166 as a ligand for CD6 (12).

Experiments with cells which do not express CD6 have provided evidence that homophilic interactions of CD166 are susceptible to perturbation with the same CD166 mAb (J4-81) with opposite findings (29, 39). The same mAb (J4-81) promoted homophilic interactions by crosslinking CD166 in *trans* between apposing cells (29). The other study showed that both the bivalent IgG and monovalent Fab fragments of J4-81 inhibited CD166 homophilic interactions (39). The discrepancy may be explained by the difference in valency of binding, lower concentrations of antibody were used in the study where the antibody crosslinked (29). Monovalent binding at higher concentrations may be more effective at steric hindrance (39). In some studies CD166 mAbs had no effect at all indicating epitope and/or affinity are crucial (24, 27).

Epitope mapping with domain deletions indicated that the J4-81 mAb recognised the second V domain in CD166 (13). Analysis of binding of J4-81 and MAB656 to recombinant CD166 with and without the N-terminal domain, VVCCC and

VCCC confirmed that both mAbs did not bind the N-terminal domain with MAB656 having the higher affinity (Lee I. Garner and MHB, unpublished data). The blocking effects of CD166 mAbs show CD6/CD166 interactions are susceptible to steric hindrance and reveal the potential difficulty of targeting a homophilic interaction with a bivalent reagent. Others have reported inhibition of CD166 homophilic interactions are effectively inhibited by a bivalent CD166 mAb (AZN-L50) which binds the membrane proximal domains of CD166. Its mode of action is postulated to be prevention of clustering of CD166 at the cell surface (29). The CD166 mAb which blocks CD166 homophilic interactions was less effective than blocking heterophilic interactions with a CD6 domain 1 mAb in inhibiting dendritic cell induced T cell proliferation (43). In addition to the heterophilic interaction being dominant in cell cultures containing dendritic cells and T cells, specifically inhibiting homophilic interactions of CD166 may enhance heterophilic engagement.

3.4 CD166 domain 1 blocking mAb

A monovalent single chain CD166 mAb avoids the potential of a conventional bivalent mAb to crosslink rather than block homophilic interactions (Table 1). A human recombinant single chain CD166 Mab isolated from a phage library blocks both human CD6/CD166 and CD166/CD166 interactions in biochemical assays (60). The epitope differed from mouse CD166 mAbs including J4-81 (60). The single chain antibody recognized human, monkey and mouse CD166 whereas J4-81 does not recognise mouse CD166 (60). A mouse antibody specific for human CD166 was reported to have functional effects in mouse (59). In analysis of the specificity of the antibody used (MAB656 R&D Systems) by surface plasmon resonance, we observed binding to recombinant human CD166 but not mouse CD166 even at 1 mg/ml whereas human CD6 bound to both species of CD166 (Lee I. Garner and MHB, unpublished data). These data along with the low affinity of mouse CD6 mAbs ((5) and MHB unpublished data) highlight the absence of mAbs which effectively perturb CD6 and CD166 in mouse models.

The single chain CD166 mAb was selected for its capacity to internalise CD166 with the aim of providing a drug delivery mechanism (60). Its specificity shows that is it possible to produce a blocking CD166 antibody. The efficacy of a single chain antibody may be limited by its affinity. As expected lower concentrations of antibody were more effective at inhibiting the weaker homophilic interaction (60),(14) . This is the only study in which the effects of blocking soluble CD6 or CD166 binding to cells has been compared using one reagent with well defined specificity (60). Importantly it also shows that it is possible to block heterophilic CD6/CD166 and homophilic CD166 interactions with one reagent specific for CD166 (60).

In summary, a high affinity CD166 mAb which effectively blocks homophilic or heterophilic binding which may be useful therapeutically has not yet been reported. The specificity of a recombinant single chain CD166 mAb indicates that production of such a reagent is feasible.

3.5 Perturbing CD6 and CD166 with fusion proteins

Soluble forms of the extracellular regions of CD166 and CD6 inhibit immune responses (14, 43, 49). Theoretically, soluble CD166 blocks heterophilic and homophilic interactions by competitive binding. Blocking of heterophilic CD6/CD166 and homophilic CD166 interactions by soluble CD6 was assumed in the interpretation of effects of soluble CD6 and CD166 (14). This interpretation is supported by our recent data showing competition between heterophilic and homophilic interactions (15). Evidence that soluble CD6-Ig and CD166-Ig block CD166 homophilic interactions has been provided from experiments with interacting cells which each express CD166 and not CD6 (39, 61). Soluble CD166 dimerises through its N-terminal domain but still inhibits immune responses in cell cultures containing antigen presenting cells expressing CD166 and T cells expressing CD6 (14, 43). Fusion proteins of CD166 which form dimers through the Fc region of immunoglobulin are effective in inhibiting responses (43). Cell surface CD6 and CD166 compete for the N-terminal domain of CD166 disrupting homophilic interactions through this domain even when presented as Ig fusion proteins.

In these studies, based on the high expression of CD166 and CD6 on antigen presenting cells and T cells respectively, the primary target of soluble CD6 or CD166 will be the heterophilic CD6/CD166 interaction (14, 43).

The most effective therapeutic reagent may be one which blocks both CD6/CD166 and CD166/CD166 interactions. Soluble CD166 as an Ig fusion protein does bind to cell surface CD166 (29, 60). Effective inhibition by soluble CD6 or CD166 may depend on their capacity to block both interactions (14, 43, 49). Soluble human CD6 was inhibitory in vivo in an innate disease mouse model of LPS induced inflammation (62). The mode of action in this model was not determined. As human CD6 binds mouse CD166 (5) the experiment shows a recombinant form of CD6 with the potential to perturb CD6 and CD166 has effects *in vivo* (62). It has been noted that soluble CD166 is more effective than CD6 in blocking heterophilic interactions between T cells and antigen presenting cells (43). This may be due to differences in specificity or in activity between CD6 and CD166 fusion proteins. In general, we have noted soluble forms of SRCR domain containing proteins are more unstable than IgSF ones (MHB, unpublished observations). The therapeutic efficacy and stability of fusion proteins which perturb CD6 or CD166 have not been systematically studied.

A number of studies have indicated that soluble fusion proteins are more effective than the currently available antibodies in blocking interactions of CD6 and CD166 (5, 14, 26, 43). Soluble CD166 is effective at blocking CD6 because the CD6 competes for binding and splits the dimer of CD166 (15). A rational drug design approach could be adopted by making mutations based on the structure of CD166 (15). A reagent with reduced self association without compromised binding to CD6 may have enhanced potency. The complementary approach would be mutagenesis to specifically increase the affinity for CD6. The challenge here would be to maintain specificity. A rational drug design approach could be adopted to increase the affinity. However expression levels and as discussed, stability, of fusion proteins may be limiting in this approach (MHB, unpublished observations). Optimising antibody reagents is likely to be more productive.

3.6 Implications of single polymorphisms in CD6

The importance of CD6 in regulation of immunity has been highlighted by the association of single polymorphisms (SNP) in CD6 autoimmune disease. The strongest association of a SNP (rs17824933) in CD6 with susceptibility to multiple sclerosis ($P= 3.79X10^{-9}$) is an order of magnitude weaker than CD58 ($P=3.10X10^{-10}$) but it has been reported in different cohorts with albeit weaker levels of association or none at all in a smaller cohort (63). There is evidence for association of CD6 SNPs with autoimmune diseases other than multiple sclerosis (63). The SNP rs17824933 is in an intron of CD6 and reduces expression of CD6. A compensating increase in expression of a form of CD6 which lacks domain 3 ensures the overall expression of CD6 is maintained (47). A CD6 haplotype which has a weaker association of susceptibility to multiple sclerosis ($P=1X10^{-4}$) contains two nonsynonymous SNPs within 100 bp in spliced transcripts (rs11230563, R225W and rs2074225, A257V) which alter the amino acid sequence in CD6 domain 2 and reduce CD6 expression (64). Reduced transcription of the intronic (rs17824933) and exonic (rs11230563, R225W and rs2074225, A257V) nonsynonymous alleles associated with multiple sclerosis was also shown by analysis of allelic specific expression (65).

The effect of the intronic SNP (rs17824933) in CD6 with the strongest association of susceptibility to disease was to reduce ligand engagement while maintaining the threshold of activation set by the level of CD6 (47, 66). It is noteworthy that the correlation of protection by the CD6 haplotype, R225, A257 is not strictly linear in the progression to heterozygous R225, 257V and then 225W, 257V it is the former which is at risk and the latter at mild risk (64). Regulation of activating and inhibitory effects of CD6 is not well enough understood to conclude how alterations in expression of CD6 contribute to autoimmune disease.

4. SIGNAL TRANSDUCTION BY CD6

4.1 Activating and inhibitory effects of CD6

Experiments in which CD6 was crosslinked and triggered or in which interactions of the extracellular region were inhibited by blocking revealed the potential for CD6 to be costimulatory and contribute to survival in response to ligand engagement ((5, 14, 43, 49, 54, 55) and others quoted in these references). In the first systematic analysis of contribution of the CD6 cytoplasmic region to signal transduction in a model system, costimulatory effects of CD6 were revealed by using mAbs to crosslink CD6 and CD3 (67). Dissection of the contributions of regions of the cytoplasmic tail of CD6 to signalling mechanisms, showed that the N-terminal and C-terminal regions were essential for calcium mobilisation and tyrosine phosphorylation respectively (67). In these experiments, the chimeric receptors formed between the extracellular region of mouse and human intracellular region of CD6 used to analyse the different forms were expressed at low levels in a Jurkat cell line and manifested activating effects when crosslinked with antibodies (67). We also observed that chimeric receptors of this kind were expressed at low levels in cell lines (MHB, unpublished data).

The potential for CD6 to be inhibitory was revealed in experiments in which CD6 was overexpressed in cell lines (5, 19, 38). In contrast to the chimeric mouse/human CD6 receptors in human Jurkat T cells, human CD6 in its entirety was quite stable at the cell surface in cell lines (5, 67). Cell lines expressing high levels of CD6 were more resistant to activation or apoptosis (5, 19, 38). The inhibitory effect of the CD6 was dependent on its cytoplasmic region (38). Cells expressing high level of CD6 were resistant to antigen specific stimulation or CD3 mAb crosslinking as assessed by reduced IL-2 production or calcium mobilisation (5), (38) .

The inhibitory effect of CD6 was demonstrated in primary human T cells, firstly when the level of CD6 was increased by expressing rat CD6 which resulted in reduced calcium mobilisation in response to stimulation (38). Secondly, a reduction in the expression of endogenous levels of CD6 in primary human T cells enhanced responses showing that physiological levels of CD6 exert a restraint on the threshold of activation (38). Inhibition of calcium mobilisation by a form of CD6 lacking the ligand binding domain transfected into primary cells supported the hypothesis that inhibitory effects of CD6 occur without engagement in *trans* by CD166 (38). Inhibition by high expression of CD6 indicates that the level of ligand engagement will influence the overall net outcome of CD6 signalling which might not necessarily be activating. The balance between activating and inhibitory signals will determine the overall net effect of CD6 on a response.

It is important to understand how activating and inhibitory effects of CD6 are regulated if perturbation of engagement of CD6 is to be tested as a therapeutic strategy in autoimmune disease. The cytoplasmic region of CD6, to our knowledge, is the longest cytoplasmic region of a lymphocyte receptor which does not have enzymatic activity. In human, the cytoplasmic region contains 244 amino acids. The tail contains a number of tyrosine and serine residues and phosphorylation of several of these have been shown to regulate signal transduction through CD6 (5, 67-69) (Fig. 3). The tyrosine residues in CD6 do not conform to any consensus sequences for immunoreceptor activation, inhibitory or switch motifs. Dissection of the contribution of specific regions or residues in the tail revealed that different sites on CD6 regulated activation and inhibition in an immune response (5, 67). Tyrosine phosphorylation of CD6 will be regulated by the balance of kinases and phosphatases and in common with leukocyte receptors with noncatalytic intracellular regions, these enzymes will interact directly with CD6 to regulate proximal signalling (8). However these interactions do not define the specific niche CD6 fills.

4.2 Tyrosine phosphorylation dependent activating interactions of CD6: SLP-76

Deletion mutants and subsequently a single point mutation identified the C-terminal tyrosine residue as important for phosphorylation of CD6 (67) (Fig. 3). Phosphorylation of the C-terminal tyrosine was shown to be important for costimulation by CD6 as a single point mutation inhibited cytokine production in cell lines (5). The C-terminal tyrosine, (Y662) motif was shown to bind the SH2 domain of SLP-76 (Fig. 3) in a phosphorylation dependent manner with an affinity (K_D~0.5 μM at physiological temperature) similar to another interaction of a protein containing a single SH2

domain, SHIP and a tyrosine phosphorylated motif in FcRγIIb, an interaction shown to be functionally important indicating the SLP-76/CD6 interaction is also physiologically relevant (5). The CD6 motif matches the consensus binding site for the adaptor protein SLP-76 in two other proteins described as interacting directly with the SH2 domain, the adaptor protein, ADAP and the serine/threonine kinase, HPK1 ((70, 71) and reviewed in (72)). SLP-76 SH2 domain interactions are important for the intracellular signalling complexes which assemble after T cell triggering (72, 73). All three sites in ADAP which bind the SLP76 SH2 domain with varying affinities contribute to formation of the signalling complexes which regulate T cell activation (72). Competition between the SLP-76 SH2 domain binding proteins, ADAP and HPK1 has been shown (74). Interactions of these proteins may not necessarily be mutually exclusive in a complex as SLP-76 has the potential to form higher order oligomers through its N-terminal sterile α motif (SAM) domain (75). Studies in Jurkat cells have not identified a role for the association between SLP-76 and CD6 as CD6 is either absent or expressed at a low level.

Recruitment to the membrane was shown to be crucial for the activity of SLP-76 (72). CD6 is the only receptor described as directly binding SLP-76 (5, 76). SLP-76 is not reliant on CD6 for function but the effect of the C-terminal tyrosine mutation indicates the converse is true: signal transduction by CD6 in T cells is dependent on SLP-76 (5, 67). Both CD6/CD166 interactions and SLP-76 containing microclusters have been implicated in maintaining immune responses consistent with a specific niche for the CD6/SLP-76 interaction in keeping SLP-76 at the membrane (43, 77).

The CD6/SLP-76 interaction is conserved between human and mouse, the crucial SH2 domain binding amino acids in the C-terminal tyrosine motifs being identical, DYDDI (5, 76). Human CD6 coprecipitated with mouse SLP-76 (5). CD6 was identified as a significant binding partner for SLP-76 in primary mouse cells in a screen to identify the components of signalling complexes (76). A model is proposed in which CD6 and LAT act as scaffolds incorporating SLP-76 (76). CD6 can signal independently of LAT but is much less effective consistent with CD6 primarily acting as an accessory molecule in TCR signalling (76).

Before proceeding to discuss inhibitory signalling effects of CD6, it should be noted that SLP-76 has also been observed to be associated with inhibitory effects (78).

4.3 Other Tyrosine phosphorylation dependent activating interactions of CD6

A form of CD6 lacking the exon containing Y629 (Fig. 3) was not phosphorylated in response to CD3 mAb crosslinking yet the single point mutant, Y629F in the full length CD6 showed no reduction in tyrosine phosphorylation of CD6 (67). The contribution of Y629 phosphorylation was revealed by the double mutant Y629F and Y662F (67). Cooperativity between the effects of the two distal tyrosine residues is consistent with them both interacting with components of the same signalling complex and both being important for the activating effects of CD6.

4.4 Inhibitory interactions of CD6

Dissection of relative contributions by the different regions of the CD6 cytoplasmic region to signalling in the cell line model using chimeric CD6 receptors expressed at a low level showed that when the exon containing the conserved sequence, SDSDY (Fig. 3), conserved between CD5 and CD6 was missing, the cells mobilised calcium in response to crosslinking of CD6 alone suggesting release from an inhibitory interaction which limits responsiveness (5, 67). Deletion of SDS from this motif in CD5 results in altered signalling (79). The net effect of this deletion in CD5 depended on whether the cells being tested were naive or antigen experienced. In antigen experienced cells where expression of CD5 is increased compared with naïve T cells, the deletion increases immune responses consistent with release from an interaction which has inhibitory effects (79). The opposite was observed for naïve T cells (79). The dual effects of the same motif may contribute to explaining why both activating and inhibitory effects of CD6 are observed depending on the response being investigated. The dual effects of the SDSDY motif may be due to different direct binding partners under different conditions or be a consequence of indirect interactions which alter.

4.5 The role of serine/threonine phosphorylation

CD6 contains serine and threonine residues which are variably constitutively and inducibly phosphorylated (68, 69). A role for phosphorylation of the SDSDY motif has been demonstrated for the two serine residues in CD6 (68). There is precedent for an inhibitory role for serine phosphorylation in T cell activation through interactions with 14-3-3 proteins (80). This inhibitory loop involves SLP-76 and haematopoietic progenitor kinase 1 (HPK1) (70, 80). HPK1 phosphorylates serine and threonine residues in SLP-76 (70). Tyrosine motifs in CD6 and HPK1 are specific for the SH2 domain of SLP-76. Given the direct interaction between CD6 and SLP-76 and indirectly with HPK1, it is tempting to speculate that HPK1 regulates CD6 function. HPK1 regulates disassembly of SLP-76 clusters in T cell activation (81). Based on data with CD5, it has been proposed that casein kinase II (CKII) I is the relevant serine kinase for phosphorylating serine residues CD6 (68). CKII is largely a nuclear enzyme without a well established role in regulating membrane receptors.

4.6 Cytoskeletal Interactions

Cytoskeletal interactions were first reported for CD166. CD166 homophilic interactions were reported to be stabilised through attachment of the short cytoplasmic tail of CD166 to the actin cytoskeleton (82). Continuation of these studies revealed that interactions of CD166 with the cytoskeleton stabilised both CD166 homophilic and heterophilic interactions with CD6 (28, 41, 82, 83). Stabilising links between CD166 and the cytoskeleton depended on two cytoskeletal proteins, syntenin-1 and ezrin (83). Syntenin-1 is an adaptor protein that has two PDZ domains. Mutation of two threonine residues in the cytoplasmic tail of CD166 reduced the interaction with syntenin-1, the most distal threonine in the motif KTEA being the likely candidate containing a consensus sequence for a class III PDZ domain binding motif (D/E-X-Ø) (83, 84). Differential dependence on syntenin-1 and ezrin revealed by analysis of the mutant CD166 suggests the two proteins bind to different regions of the CD166 cytoplasmic tail (83).

There are data showing a role for extracellular and intracellular interactions of CD6 in lateral movement in response to T cell activation. Recruitment of CD6 by CD166 to cell contact sites can occur independently of the cytoplasmic tail of CD6 (16). Observation using total internal reflection microscopy revealed a striking effect of CD6 on cell spreading (85). Actin filaments were observed when CD6 was captured by a surface containing immobilized CD6 mAb (85). Based on preliminary data it was speculated that CD6 mediates an active process in the actin reorganisation (85). Potential for an interaction between CD6 and syntenin-1 was identified in a yeast two hybrid screen (86). Class II (ISA) and III (DDA) PDZ domain binding motifs are present in the amino acid residues (YDDISAA) implicated in binding syntenin-1 at the C-terminus of CD6 (84, 86) . The class II site was shown to be important and the association was also regulated by the tyrosine residue (86). Colocalisation studies showed that the distribution of syntenin-1 and CD6 were compatible with a direct interaction (86). An interaction between CD6 and syntenin-1 suggests there is a conserved mechanism of stabilising adhesion between apposing cells in CD166/C166 and CD166/CD6 interactions (86). The relative importance of an interaction between CD6 and syntenin-1 compared with SLP-76 at the same site is not yet clear. Over expression of syntenein-1 in metastatic cells and a complicated connection between CD166 expression and cancer cells indicate it is important to understand how the interaction between CD166 and syntein-1 affects function when considering targeting CD6 and CD166 therapeutically (31, 84). As PDZ domains are potential drug targets this is a potential alternative avenue of modulating CD6 and CD166 interactions (87).

5. THERAPEUTIC POTENTIAL OF PERTURBING CD6 SIGNALLING.

The long cytoplasmic tail of CD6 has the potential to provide scaffolding for multiple intracellular proteins. The overall function is connected with stabilisation of interactions at the plasma membrane and facilitating signal integration. The extracellular and intracellular regions have well defined specificity for CD166 and SLP-76. The data indicate that in an immune response where CD6 has an overall costimulatory effect, these interactions are likely to be the primary interactions

affected by therapeutic intervention. The current drug therapy specific for CD6 is aimed at targetting extracellular engagement but there is no good evidence *in vivo* that this is an effective strategy. Agonistic reagents which perturb CD6 signalling are likely to be more useful and need to be systematically analysed. There are data to indicate triggering through CD6 can result in activation (43) and inhibition (38). The immunosuppressive effects of the therapeutic antibody could be a result of induction of inhibitory signalling through CD6. Systematic analysis, an understanding of mechanism and optimisation of reagents which perturb CD6 function is needed to assess its future as a therapeutic target.

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TABLE 1. Antibodies used to perturb the function of CD6 and CD166. Relevant characteristics of antibodies mentioned in this review.

Name	Specificity	isotype	Notes	References
OX126	Human CD6 domain 3	Mouse IgG1	Raised against recombinant CD6 domain 3 and crossreacts with rat CD6. Blocks soluble CD166 binding to CD6. Blocks interactions between cells	(5)
T12	Human CD6 domain 1	mouse IgM	Blocks interactions between cells. Used clinically to deplete T cells.	(12) and reviewed in (51)
UMCD6	Human CD6 domain 1	Mouse IgG1	Higher affinity for CD6 than MT605. Competes with iort1. Blocks interactions between cells.	(57)
MT605	Human CD6 domain 1	Mouse IgG1	Lower affinity for recombinant CD6 than UMCD6. Partially competes with iort1. Blocks interactions between cells.	(57)
Iort1/T1h	Human CD6 domain 1	Mouse IgG2a/human IgG1	Competes with UMCD6 and partially with MT605. Blocks interactions between cells. Used clinically as an immunosuppressant.	(57)
J4-81	Human CD166 V2. Bound VV but not V1 or VCCC.	Mouse IgG1	Partially inhibits soluble CD6 binding to cells. Can both promote and inhibit CD166 homophilic interactions presumably depending on valency	(13, 29, 39)
AZN-L50	Human CD166 C2C3	Mouse IgG2a	Inhibits CD166 homophilic *trans* interactions proposed to be by inhibiting CD166 homophilic *cis* associations	(29)
MAB656	Human CD166 VCCC	Mouse IgG1	Inhibitory effects reported in humans and mice but does not bind to recombinant mouse CD166	(59), Lee I. Garner, unpublished data
I/F8	Human CD166 domain 1	Human single chain antibody fragment	Blocks CD6/CD166 and CD166/CD166 interactions and binds monkey and murine CD166	(60)

FIGURE LEGENDS

FIGURE 1. Cartoon showing CD6 interacting with CD166 at the cell surface. The membrane proximal scavenger receptor cysteine rich domain (Sc) of CD6 binds the N-terminal V-like immunoglobulin superfamily (IgSF) domain of CD166. CD6 and CD5 both contain three scavenger receptor cysteine rich domains in their extracellular regions.

FIGURE 2. Cartoon showing how CD6 function has been postulated to be perturbed by a CD6 mAb. (A) CD6 binds CD166. (B) A CD6 mAb sterically hinders CD6/CD166 interactions inhibiting costimulation. (C) A CD6 mAb crosslinks CD6 and induces signalling which maybe activating or inhibitory.

FIGURE 3. Cartoon highlighting residues which do, or are likely to, regulate CD6 signalling. The SH2 domain (open rectangle) of the intracellular adaptor, SLP-76 binds phosphorylated Y662 in the C-terminal tyrosine motif of the cytoplasmic region of CD6. Y629 and Y662 are important for phosphorylation of CD6. SDSDY present in CD5 and CD6 and regulates CD5 function.

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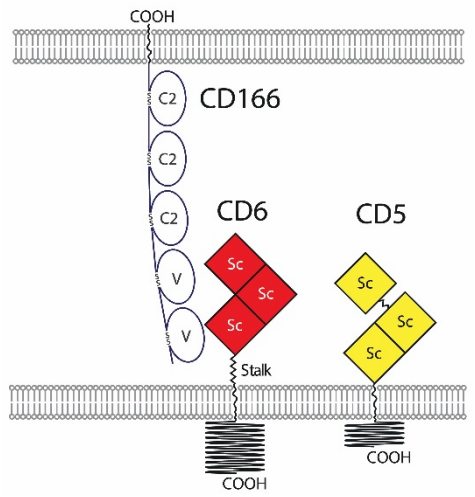


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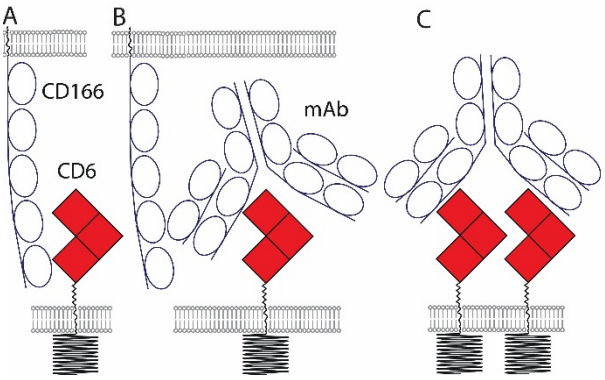


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