Molecular mechanisms of immune regulation

by the receptors CD5 and CD6



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Abstract

T cells are adaptive immune cells that are essential for initiating and regulating immune responses. T cell activation is triggered by stimulation of the T cell receptor. However, sustained T cell activation requires the function of a variety of coreceptors, among them CD5 and CD6. Both receptors have been shown to have activating and inhibitory functions and modulation of CD6 function is dependent on engagement by its ligand CD166. Costimulatory signalling by CD6 involves a phosphorylation-dependent interaction between the C-terminal Y662 residue and the adaptor protein SLP-76. However, the CD6 cytoplasmic region is one of the longest in the immune system and contains phosphorylated residues which suggests that additional signalling molecules can be recruited.

I identified an interaction of CD5 and CD6 with the cytoskeleton via ezrin, radixin and moesin. This interaction has an activating function on Jurkat cell activation and may play a role in receptor localisation.

I also identified the adapter protein GADS as a binding partner of the CD6 Y629 residue and found evidence for bivalent binding of the GADS/SLP-76 complex to the CD6 C terminus. Costimulation by CD6 is dependent on the recruitment of this complex and mutant CD6 that cannot recruit it does not provide costimulation and may even be inhibitory in the absence of its ligand. These results suggest a model in which costimulation by CD6 depends on cooperative binding of the GADS/SLP-76 complex to two C-terminal phosphotyrosine residues in the CD6 cytoplasmic region. Binding of this complex to CD6 may enhance long-term signalling by keeping GADS/SLP-76 at the membrane or it may provide an alternative signalling pathway that enhances TCR signalling.

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Abbreviations

ADAP	Adhesion and degranulation-promoting adapter protein
AICD	Activation-induced cell death
ALCAM	Activated leukocyte cell adhesion molecule
APC	Antigen-presenting cell
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CDR	Complementarity-determining region
CK2	Casein kinase 2
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylendiaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, radixin, moesin
FBS	Foetal bovine serum
FERM	4.1, ezrin, radixin, moesin
GADS	GRB2-related adapter downstream of Shc
GRB2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPK1	Haematopoietic progenitor kinase 1
ICAM-2	Intercellular adhesion molecule 2
Ig	Immunoglobulin
IL-2	Interleukin 2
IP_3	Inositol trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITK	Interleukin 2-inducible T cell kinase
K_D	Equilibrium dissociation constant
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NCK	Non-catalytic region of tyrosine kinase adaptor protein

NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κ light chain-enhancer of activated B cells
NHE-RF	Na^+ H^+ exchange regulatory factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed death 1
PEI	Polyethylenimine
PI3K	Phosphatidylinositol 3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1
PMA	Phorbol myristate acetate
RasGAP	Ras GTPase-activating protein
SH	Src homology
SHP-1	SH2 domain-containing phosphatase 1
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SMAC	Supramolecular activation cluster
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
SRCR	Scavenger receptor cysteine-rich
TCR	T cell receptor
Th	T helper cell
TIRF	Total internal reflection
Treg	Regulatory T cells
TSAd	T cell specific adapter
WASP	Wiskott-Aldrich syndrome protein
WT	Wild type
ZAP-70	Zeta chain-associated protein of 70 kDa

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1 Introduction

The immune system consists of an innate arm and an adaptive arm and an effective immune response requires an interplay between both. The innate immune system senses pathogen-associated molecular patterns (PAMPs) and danger via pattern recognition receptors which triggers immune cell activation and inflammation [1]. However, the innate immune response is often not sufficient for clearing the pathogen from the system and an adaptive immune response is required. Another important function of the innate immune system is to activate the adaptive immune system comprised of a B cell-mediated humoral response and a cellular T cell response.

1.1 T cells

T cells are an essential part of the adaptive immune response. The main type of T cell, the αβ T cell, can be divided into CD4+ helper T cells and CD8+ cytotoxic T cells and both play a crucial role in the immune response.

1.1.1 T cell development

T cells are derived from haematopoietic cells that migrated from the bone marrow to the thymus where they complete development to mature T cells. Developing T cells called thymocytes rearrange their T cell receptor (TCR) gene segments. Thymocytes expressing a functional TCR undergo positive and negative selection which selects TCRs that are not or only weakly self-reactive [2]. Positive selection takes place in the cortex of the thymus where thymocytes encounter cortical thymic epithelial cells expressing major histocompatibility

complex (MHC) molecules and binding of the TCR to MHC provides a pro-survival signal. Negative selection mainly takes place in the thymic medulla where medullary thymic epithelial cells present a large number of autoantigens and T cells that strongly react to these autoantigens undergo apoptosis. Reactivity to foreign antigens is also, at least partially, regulated during thymocyte development. Thymocytes that show low self-reactivity but are not negatively selected escape the thymus and dominate in immune responses in the periphery [3].

1.1.2 T cell differentiation

During activation, CD4+ T cells receive signals that drive transcriptional programmes which ultimately lead to differentiation into more specialised T helper phenotypes. The most studied CD4+ T cell subsets are T helper (Th) 1 cells which are involved in the response against intracellular bacteria, Th2 cells which mediate anti-parasite immunity, Th17 cells which contribute to anti-bacterial responses and regulatory T cells (Tregs) which downregulate immune responses [4]. CD4+ T cells function by secreting cytokines that affect the function of other immune cells and cells not belonging to the immune system. CD8+ T cells are also called cytotoxic T cells due to their function in killing virus-infected or malignant cells by targeted secretion of cytotoxic molecules that induce apoptosis.

1.1.3 Regulation of T cell activation

Due to the important function of T cells in the immune system it is essential that T cell activation is highly regulated to prevent pathogenic T cell effector functions. This is highlighted by the aberrant T cell activation in autoimmune diseases such as multiple sclerosis and the absence of T cell effector mechanisms in cancer [5,6].

Naïve CD4+ and CD8+ T cells travel through secondary lymphatic tissues to search for peptide:MHC specific for the TCR [7]. Signalling through the TCR is the first of three signals T cells require for activation. TCR signalling is enhanced by signalling through costimulatory receptors such as CD28 [8]. Ligands for costimulatory receptors are upregulated during antigen-presenting cell (APC) activation and are expressed at low levels in the absence of inflammation. A third signal required for sustained T cell activation and function is signalling through cytokine receptors [9]. A combination of all three signals leads to T cell activation and ultimately to an immune response to pathogens and cancer. However, the interplay and relative contribution of these signals is a complex topic that requires molecular studies and systems biology approaches to be fully understood.

1.1.4 Costimulation and coinhibition of T cell activation

Full effector function of T cells requires costimulation in addition to the signal through the TCR [8]. This suggests that costimulation enhances TCR signalling but possibly also induces TCR-independent signalling. A key costimulatory receptor that has been extensively studied is the immunoglobulin

(Ig) superfamily member CD28 and its ligands CD80 and CD86 [10-12]. CD28-mediated costimulation acts on early TCR signalling. [13].

Receptors on T cells can also have an inhibitory function. For example, the inhibitory receptor programmed death 1 (PD-1) recruits phosphatases which dephosphorylate molecules involved in TCR signalling [14].

The costimulatory and coinhibitory receptor mentioned here are just well-studied examples among a plethora of coreceptors [8]. Even though many of these coreceptors influence TCR signalling, there is no unifying description of how coreceptors function. Many coreceptors have a clear activating or inhibitory function on T cell activation. CD5 and CD6 are coreceptors with a more complex involvement in T cell activation.

1.2 The coreceptors CD5 and CD6

CD5 and CD6 are related coreceptors expressed by T cells. They have extracellular recognition domains that are unique on αβ T cells and are highly conserved. Their cytoplasmic regions are among the longest in the immune system and are posttranslationally modified making them suited for signalling.

Both receptors are associated with diseases and there are approaches to exploit them for therapy. They are involved in T cell development and function but their mechanism is incompletely understood. My project focuses on molecular mechanisms of regulation of T cell activation by CD5 and CD6.

1.2.1 The scavenger receptor cysteine-rich family of receptors

CD5 and CD6 are members of the scavenger receptor cysteine-rich (SRCR) superfamily [15]. SRCR superfamily members can be grouped into A and B depending on the number of cysteine residues and the number of exons encoding SRCR domains [16]. CD5 and CD6 belong to SRCR group B because they contain 8 cysteine residues per domain with the exception of CD5 domain 2 which contains 6 cysteine residues [17]. Furthermore, each SRCR domain is encoded by one exon whereas SRCR group A domains are encoded by 2 exons. The SRCR family is evolutionarily ancient with the oldest members found in algae [18]. According to the Ensembl database, CD5 and CD6 (Ensembl number ENSG00000110448 and ENSG00000013725, respectively) first emerged in fish, suggesting that the original gene duplication resulting in these two genes occurred in fish [19]. CD5 and CD6 are conserved in mammals with 64 % and 70 % sequence conservation between human and mouse, respectively. Evidence that these two genes emerged from gene duplication includes their similar exon/intron organisation, their proximity on chromosome 11, CD5 being located 82 kb downstream of CD6, the sequence similarities and the linked expression [20].

1.2.2 Distribution in the immune system

CD5 and CD6 are primarily expressed on thymocytes and T cells and are the only SRCR members expressed by αβ T cells [21-23]. CD5 has also been well characterised as being highly expressed on B-1a and regulatory B10 subsets of B cells but all B cells express a low level of CD5 [24,25]. CD6 can also be expressed by subsets of B cells and natural killer cells but if CD6 regulates the

function of these cells is not known [26,27]. For the purpose of my dissertation, I will focus on CD5 and CD6 on αβ T cells because they have been shown to play a role in this cell type.

1.2.3 Extracellular interactions

CD5 and CD6 both contain three extracellular SRCR domains and are glycosylated (Figure 1). CD6 has one well characterised ligand, the Ig superfamily member CD166, also called activated leukocyte cell adhesion molecule (ALCAM) [28]. CD166 is a member of the Ig superfamily and contains two membrane-distal Ig V domains and three Ig C domains. The membrane-distal Ig V domain 1 binds to the CD6 membrane-proximal SRCR domain 3 with an equilibrium dissociation constant (K_D) of 0.4-1 μM, a relatively high affinity for a cell-cell interaction [28,29]. Our group recently solved the structure of CD6 and CD166 which provides further insight into the mode of binding [30]. The structure also showed a kink in the SRCR domain organisation of CD6 which is speculated to help in self-association on the surface of the same T cell. CD166 can form homodimers in trans between two different cells but with a lower affinity than the CD6-CD166 interaction [30].

Initially CD166 was reported to be expressed by thymic epithelial cells but it is now clear that most immune- and also non-immune cells such as cells of the nervous system express CD166 and it is upregulated by many tumours [31-33].

Several heterotypic ligands for CD5 have been reported including antibody-framework, fungal PAMPs and cell surface proteins but none of these interactions have been confirmed independently [34,35]. There is evidence that CD5 associates with itself on the T cell surface and this is required for CD5

signalling [34,36]. The existence of low affinity ligands of CD5 cannot be excluded but the finding that expression of CD5 did not alter adhesion during T cell-APC interactions suggests that not many contacts were formed by CD5 with molecules on APCs [37]. This is an obvious difference to CD6 which is engaged by CD166 during T cell-APC interactions [38].

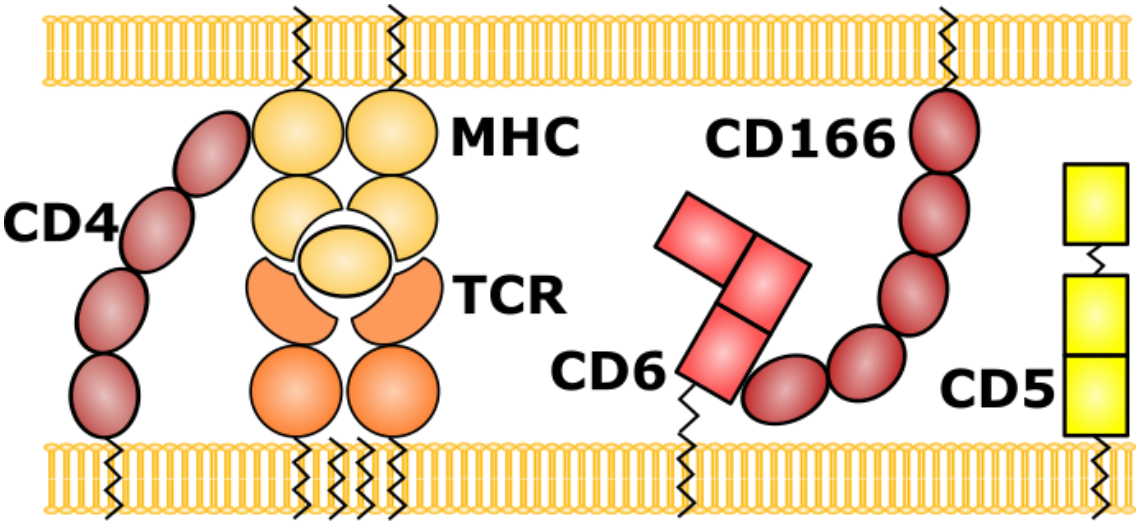


Figure 1. CD5 and CD6 on the T cell surface. *CD5 and CD6 are located on the T cell surface and are closely associated with the TCR. Both receptors contain three extracellular SRCR domains and CD6 has a membrane-proximal stalk region. The ligand for CD6 is the Ig superfamily member CD166 which binds to the membrane-proximal SRCR domain of CD6. Cytoplasmic regions are not shown in this figure. SRCR domains are shown as squares and Ig domains are shown as ovals.*

1.2.4 Influence on T cell development

There is evidence that CD5 and CD6 are involved in the development of T cells. The CD6 ligand CD166 is expressed by thymic epithelial cells and CD5 and CD6 expression is highly regulated during thymocyte development [31,39].

The role of CD5 in T cell development has been studied in more detail. CD5 is already expressed at the pre-TCR stage of thymocyte development and expression increases further during later stages of development [40]. It has been suggested that CD5 functions as a regulator during thymocyte development and CD5 function allows more cells to survive negative selection [41,42]. A possible explanation would be the reduction of TCR signalling during development which would lead to weaker autoreactivity and subsequent escape from negative selection. Loss of the inhibitory effect is seen in CD5 knockout mice which have more strongly reactive T cells [43]. No loss of the inhibitory effect is seen in mice lacking the CD5 extracellular region suggesting that the CD5 cytoplasmic region is still able to inhibit TCR signalling in thymocytes [44]. CD5 expression is also correlated with the affinity of thymocytes to self-peptides [45]. Mandl et al. investigated this further showing that phosphorylation levels of the CD3 ζ chain in thymocytes of TCR transgenic mice correlated with CD5 expression [46]. The thymus is a sterile environment and therefore CD3 ζ chain phosphorylation was a response to self-peptide:MHC. This study also showed that T cells that had a high CD5 expression also dominated in immune responses in the periphery. This domination of $CD5^{hi}$ T cells in immune responses was confirmed by Fulton et al. using mice with a normal TCR repertoire [47]. In this study, murine naïve CD8+ T cells were sorted into $CD5^{lo}$ and $CD5^{hi}$ T cells and transferred into mice. After virus infection, $CD5^{hi}$ T cells showed higher proliferation than $CD5^{lo}$ T cells and

earlier recruitment to the site of infection. CD5 expression is also regulated in mature T cells and seems to be dependent on frequent interactions of T cells with peptide:MHC in the periphery and the resulting low level signalling [48]. However, it has not been shown if CD5 has a function in T cell development or if CD5 expression is just a marker of self-reactivity in the thymus.

In line with the findings that CD5 influences the threshold during negative selection, Raman and colleagues proposed that CD5 protects T cells from activation-induced cell death (AICD) [49]. CD5 protected self-reactive T cells from AICD in experimental autoimmune encephalomyelitis (EAE) mice, a mouse model of multiple sclerosis, exacerbating EAE symptoms in mice [49]. Knocking out CD5 increased AICD in T cells specific for the myelin oligodendrocyte glycoprotein reducing disease severity. Expression levels of CD5 in human tumour-infiltrating T cells also showed a correlation with the protection from AICD [50]. This study also suggested a mechanism for AICD protection by CD5. Incubating cells with a CD5 antibody that was claimed but not proven to be blocking ligand engagement increased the expression of FasL which is involved in AICD. CD5 did not affect FasL mRNA levels but affected surface FasL levels indicating a mechanism in which CD5 reduces transport of FasL to the plasma membrane. These studies do not investigate the molecular mechanism of how CD5 influences AICD. It is also unclear from these studies whether CD5 influences TCR signalling directly or signals independently.

1.2.5 Involvement in T cell differentiation

Some studies also suggested an involvement of CD5 and CD6 in T cell differentiation to T cell subsets and in the function of these subsets. CD5 reduces the number of Tregs, possibly by inhibiting the thymocyte signalling required for thymic Treg development [51]. In addition, CD5 expression also inhibits the suppressive function of Tregs isolated from mice [52]. In contrast, Tregs were initially reported to express a high level of CD5 which was used as a marker for Tregs in early studies [53]. In fact, Sakaguchi et al. have shown that transferring $CD5^{hi}$ T cells from mice with various autoimmune diseases into T cell-deficient recipient mice did not cause autoimmunity but $CD5^{lo}$ T cells induced symptoms of autoimmune diseases [53]. Henderson et al. found that CD5 promotes differentiation of naïve T cells into Tregs in the periphery [54]. Differentiation of $CD5^{-/-}$ naïve T cells into Treg cells was reduced by the action of pro-inflammatory cytokines whereas CD5 expression protected naïve T cells from these cytokines. In this study, it was also suggested that CD5 blocks the activation of the mammalian target of rapamycin (mTOR) downstream of cytokine receptor signalling which then protects the Treg phenotype from the action of cytokines. Yan et al. examined the phosphorylation level or extracellular signal-regulated kinase (ERK) in conventional T cells and Tregs after cross-linking CD3 and CD28 with antibodies [55]. This study showed that when sorting mouse CD4+, CD8+ or Treg cells according to their CD5 expression there is an association of pERK levels with CD5 expression and higher CD5 expression inhibited ERK phosphorylation. Human natural Tregs which have strong suppressor function have no or very low CD6 expression and therefore CD6 is unlikely to affect Treg function [56].

Even though these studies suggest an involvement of CD5 in Treg biology, it is not clear how CD5 regulates Treg differentiation and function. A potential explanation could be the effect of CD5 on the threshold of T cell activation because it has been shown that TCR signalling is required for the Treg phenotype and its suppressive function [57,58].

CD5 and CD6 may also be involved in the differentiation and function of effector T cells. Crosslinking CD5 with antibodies induced elevated differentiation of naïve T cells into the Th17 phenotype and suppressed Th1 differentiation [59,60]. However, in this study, CD5 was only a minor contributor to the differentiation into Th17 cells and these T cells still required other differentiation signals for full lineage commitment. Stimulation of CD6 with CD166 increased the production of interferon γ, suggesting that CD6 signalling enhances Th1 differentiation [61].

1.2.6 Disease associations of CD5 and CD6

CD5 and CD6 are associated with a variety of diseases such as autoimmunity and leukaemia. CD5 is a marker for chronic lymphocytic leukaemia and increased CD5 expression correlates with the malignant phenotype of this cancer [62,63]. A CD5 single nucleotide polymorphism (SNP) is also associated with the autoimmune disease systemic lupus erythematosus [64]. This SNP is located in the cytoplasmic region of CD5 and is associated with stronger T cell activation. However, this study does not examine the level of expression of the investigated haplotypes and differences on T cell activation may just be differences in expression.

Genome-wide association studies also link CD6 SNPs to autoimmune diseases such as psoriasis, rheumatoid arthritis and multiple sclerosis [65,66]. These SNPs affect CD6 expression levels with lower expression resulting in higher susceptibility to autoimmunity [67]. Kofler et al. have identified a multiple sclerosis risk allele that reduces the expression of full length CD6 in CD4+ T cells leading to a higher proportion of CD6 lacking the SRCR domain 3 and therefore lacking the CD166 binding site [68]. This change in the ratio of full length CD6 to CD6 lacking the CD166 binding site is associated with lower T cell activation. If TCR signalling is reduced, self-reactive thymocytes may survive negative selection in the thymus and cause autoimmunity in the periphery.

1.2.7 Disease associations of CD166

CD166 is expressed on blood-brain barrier (BBB) endothelium and promotes trafficking of CD4+ T cells across the BBB [69]. CD166 on the BBB endothelium and CD6 on T cells colocalise, indicating that the CD6-CD166 interaction is required for trafficking of T cells across the BBB and blocking this interaction may be a new therapeutic approach for multiple sclerosis and similar neuroinflammatory diseases. CD166 is also highly expressed on many tumour cells [70]. Expression of CD166 in tumour cells enhances adhesion due to homophilic interactions and thereby reduce metastasis formation. Evidence for this comes from a truncated version of CD166 that does not bind in a homophilic manner and increases tumour cell mobility [71].

In the light of CD166 being able to activate T cells by ligating CD6, the overexpression of CD166 on tumour cells could be a possible target for T cell-mediated immunity and immunotherapy using CD6 [28].

1.2.8 Targeting CD6 for antibody-mediated therapy

Treatment with CD6 antibodies led to decreased severity of autoimmunity in clinical trials [65]. The CD6 antibody itolizumab is licensed in India for the treatment of plaque Psoriasis [72]. The mechanism of action of itolizumab in patients is unknown. Itolizumab is specific for the SRCR domain 1 which is not the ligand binding domain. It was claimed that steric hindrance might be the mechanism because the CD6 domain 1 monoclonal antibody MT605 only inhibits CD6 costimulation when CD166 is able to bind CD6 [68]. Some CD6 antibodies may also function by depleting T cells. An example is the CD6 domain 1 binding T12 antibody which has been used to prevent graft-versus-host disease that emerged during a severe combined immunodeficiency treatment [73]. Bone marrow grafts that were depleted of T cells using CD6 antibodies have also been used in the treatment of leukaemia [74]. Interestingly, itolizumab does not work by depleting T cells [75]. If itolizumab does not block CD6 ligation or deplete T cells, another explanation could be that CD6 is crosslinked by the antibody which then leads to inhibitory signalling [38,75].

1.3 Costimulation and coinhibition by CD5 and CD6

Initial studies showed that cross-linking CD5 with antibodies induced proliferation and activating signalling in T cells [21,76]. An activating function was

also compatible with *in vivo* results by Axtell et al. who injected EAE mice with adenovirus expressing murine CD5 as an Fc fusion protein [49]. In this study, infected cells released a soluble form of the CD5 extracellular region which interfered with binding of host CD5 to potential ligands which resulted in decreased EAE severity. This is especially remarkable because it suggests a function for the extracellular region of CD5 even though it might lack a high affinity ligand. However, in CD5 knockout mice, thymocytes and splenic T cells are hyperresponsive which indicates an inhibitory role in TCR signalling [43]. An inhibitory role of CD5 was also proposed in human T cells [77]. Tumour-infiltrating T cells were isolated from patient samples and tested for their anti-tumour activity. A panel of surface markers was assessed for correlation with anti-tumour activity and only CD5 expression varied significantly showing an inverse correlation with anti-tumour activity.

The same phenomena of activating and inhibiting effects was observed with CD6. In CD6 knockout mice, the response of T cells to myelin oligodendrocyte glycoprotein which is used in the EAE model was increased, suggesting an inhibitory role of CD6 (Killen N, unpublished observations). When CD6 is expressed but not engaged by a ligand, it appears to inhibit T cell signalling events *in vitro* [78,79]. Blocking the CD6-CD166 interaction with soluble receptor or ligand shows that ligand engagement provides an activating signal that enhances TCR signalling and is in fact required for optimal T cell activation [28,38,79]. CD6 costimulatory function can be mimicked using cross-linking antibodies [38,80]. Antibodies binding to CD6 domain 3 can also block the interaction of CD166 with CD6 without affecting the CD166 homophilic interaction

[79]. However, it cannot be excluded that these antibodies crosslink CD6 and trigger inhibitory signalling [78].

These reports suggest an inhibitory role for CD5 and CD6 to prevent spontaneous activation of T cells and an activating role during TCR signalling, especially for CD6 when it is ligated. The signalling pathways are only incompletely understood but it can be conceived that inhibitory and activating pathways are active to different degrees and the net signalling effect determines the functional outcome of these receptors.

1.3.1 The immunological synapse

The organisation of membrane-associated proteins in T cells is highly regulated. Therefore, the location of CD5 and CD6 in the immunological synapse may reveal information on their function. Colocalisation with the TCR is observed for activating receptors and segregation from the TCR for inhibitory molecules [81]. Fluorescence microscopy showed that proteins are located in supramolecular activation clusters (SMACs) when the T cell is engaged by an APC [82]. Activating signalling molecules such as the TCR and CD3 concentrate in the central SMAC. Adhesion molecules form a peripheral SMAC around the central SMAC. Inhibitory molecules such as the phosphatase CD45 migrate towards a distal SMAC [83]. However, phosphorylation of signalling molecules after TCR triggering happens earlier than synapse formation suggesting that studies of early membrane organisation is required [84]. Indeed, total internal reflection (TIRF) microscopy of cells on supported lipid bilayers showed that receptors and signalling molecules form microclusters within a few seconds after

TCR triggering [85]. These clusters are hot spots for signalling and migrate to the centre of the synapse in an actin polymerisation-dependent manner. TIRF microscopy of microclusters also challenged the view of the central SMAC as being the area of the T cell-APC contact site that mediates signalling. When microclusters rich in TCR, CD3 and signalling molecules reach the centre of the synapse they fuse, become enriched in CD45 and are ubiquitinated and thereby targeted for degradation [86]. This discovery reinforces the notion that TCR signalling takes place in migrating microclusters and is terminated in the central SMAC.

CD5 and CD6 were shown to associate with the TCR, CD3, CD2 and CD4 or CD8 on the surface of the same T cells [87-90]. These associations indicate that these receptors are all located in the same microclusters on the T cell surface [89]. This is also supported by the finding that SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), a downstream signalling molecule of CD6, is located to microclusters [79,91]. However, most of these studies used co-immunoprecipitation and microscopy to identify physical interactions. It is important to bear in mind that co-immunoprecipitation does not provide information whether the interaction is direct or if the proteins are part of a larger complex [92]. Colocalisation seen using confocal microscopy and TIRF reveals that these receptors are in proximity but they may just localise to the immunological synapse. A physical association of these receptors cannot be excluded but further work is required to confirm this and establish if this is relevant for receptor function.

Fluorescence-labelled CD5 colocalised with CD3 in the immunological synapse and migrates with similar kinetics after antigen stimulation [37]. Even

though CD5 colocalised with CD3 similarly to activating molecules on T cells, it inhibited tyrosine phosphorylation and Ca^{2+} influx in T cells and therefore behaved like an inhibitory receptor.

Castro et al. demonstrated that alternative splicing of the CD166-binding domain 3 of CD6 alters membrane localisation of CD6 [93]. This finding suggests that the ligand may be required for colocalisation of CD6 with the TCR and TCR-associated signalling molecules [93].

1.3.2 Mechanisms of T cell triggering

The αβ TCR consists of an α and a β chain that both contain two Ig-like domains [94]. The membrane-distal Ig domains possess three complementarity-determining regions (CDRs) that emerge during TCR gene rearrangements and provide specificity to peptide:MHC complexes. CDR 1 and 2 of each chain interact with the MHC whereas CDR 3 mainly interacts with the presented peptide [94]. This interaction is stabilised by binding of CD4 or CD8 to a conserved region of MHC II or MHC I, respectively. The cytoplasmic tails of the TCR do not contain catalytic domains or signalling motifs but the TCR interacts with the CD3 complex through the transmembrane domains. CD3 is a complex of the γε and δε heterodimers which possess one immunoreceptor tyrosine-based activation motif (ITAM) for each subunit and a ζζ homodimer with three ITAMs per subunit [95].

Many hypotheses of how TCR ligand engagement is transduced across the membrane to induce phosphorylation of tyrosine residues in ITAMs have been proposed [96]. Potential triggering mechanisms include receptor

dimerisation, conformational changes due to forces acting on the TCR and the segregation of phosphatases with large extracellular domains such as CD45 which results in a local shift of the kinase/phosphatase balance in favour of the kinases, termed the kinetic segregation model [97]. According to the kinetic segregation model, proteins with large extracellular domains do not colocalise with the TCR on T cells and are probably inhibitory and proteins or receptor-ligand pairs with similar dimensions as the TCR-peptide:MHC complex colocalise with the TCR and may enhance TCR signalling. The distance between the T cell membrane to an APC membrane during antigen presentation is about 13 nm which is approximately the size of the TCR-peptide:MHC complex [98]. The size of the CD6 SRCR domain 1-3 crystal structure lacking the stalk region (Figure 1) is 78.1 Å and the size of the CD166 structure is estimated to be 90.4 Å [30]. However, since CD166 interacts with the membrane-proximal domain of CD6, the total dimension of the CD6-CD166 complex can be estimated as approximately the size of the TCR-peptide:MHC complex. This means the CD6-CD166 complex is unlikely to be excluded from TCR-peptide:MHC complexes.

1.4 T cell receptor signalling

TCR signalling is a complex pathway consisting of many molecules, branches and positive and negative feedback loops [99]. The main steps are shown in Figure 2 with a focus on early signalling events. During TCR triggering, tyrosine residues in ITAM motifs of CD3 are phosphorylated by CD4 or CD8-bound and coreceptor-independent forms of the kinase lymphocyte-specific protein tyrosine kinase (LCK) [100]. Phosphorylated ITAMs recruit the kinase

zeta chain-associated protein of 70 kDa (ZAP-70) which binds to the two phosphorylated tyrosine residues in each ITAM with its tandem Src homology (SH) 2 domains [101]. Recruited ZAP-70 is phosphorylated by LCK and then phosphorylates proteins further downstream on tyrosine residues [102]. One of the ZAP-70 substrates is the linker for activation of T cells (LAT), an adapter molecule with a transmembrane domain but no extracellular domain and several cytoplasmic tyrosine residues that are phosphorylated by ZAP-70 [103]. LAT is a central hub where signalling diversifies into several pathways [104]. Interestingly, LAT knockout mice still possess naïve T cells even though the number is significantly reduced [105]. This suggests that LAT plays an important role in T cell development but other pathways may be used as well. CD6 is discussed as an alternative scaffold protein because it can recruit proteins that are also recruited to LAT such as SLP-76 [79,106]. The relevance of the SLP-76-CD6 interaction is discussed in section 1.4.2.

One pathway requires the recruitment of the adapter molecule growth factor receptor-bound protein 2 (GRB2) to LAT which then leads to the activation of the mitogen-activated protein kinase (MAPK) pathway and ultimately the transcription factors AP-1 and ATF2 [107].

Phosphorylated tyrosine residues on LAT can also recruit the SH2 domain of GRB2-related adapter protein downstream of Shc (GADS) [108]. GADS links the adapter protein SLP-76 to LAT by binding a proline-rich region in SLP-76 with its SH3 domain [109]. SLP-76 is a platform for further downstream signalling essential for TCR signalling (Figure 2) [110].

The SLP-76 N-terminus contains a sterile α motif which mediates SLP-76 dimerisation [111]. In addition, three tyrosine residues close to the N-terminus can be phosphorylated and recruit signalling molecules: VAV1, non-catalytic region of tyrosine kinase adaptor protein (NCK) and Interleukin 2-inducible T cell kinase (ITK) [110]. VAV1 is a guanine nucleotide exchange factor for Rho family GTPases and is important for the activation of several transcription factors [112]. NCK is an adapter protein involved in VAV-mediated activation of the Wiskott-Aldrich syndrome protein (WASP) which is involved in cytoskeletal rearrangements [113]. ITK phosphorylates phospholipase Cγ1 (PLCγ1) which activates its catalytic activity [114].

The proline-rich region of SLP-76 located in the centre of SLP-76 recruits the SH3 domain of PLCγ1 which is also bound to LAT through its SH2 domain (Figure 2) [115]. PLCγ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in the generation of inositol trisphosphate (IP_3) which binds to its receptor in the endoplasmic reticulum (ER) membrane. This induces a Ca^{2+} flux into T cells by depleting ER Ca^{2+} and ultimately leads to the dephosphorylation and nuclear localisation of the transcription factor nuclear factor of activated T cells (NFAT) [116]. PIP_2 hydrolysis also generates diacylglycerol (DAG) which activates protein kinase C (PKC) θ signalling leading to the activation of the transcription factor nuclear factor κ light chain-enhancer of activated B cells (NF-κB) [117].

The SH2 domain of SLP-76 is located at the C terminus and can recruit the adhesion and degranulation-promoting adapter protein (ADAP) which is involved in integrin inside-out signalling and the haematopoietic progenitor kinase 1 (HPK1), a negative regulator of TCR signalling [118,119]. SLP-76 can

also be recruited to CD6 by binding to the phosphorylated tyrosine 662 residue of CD6 with its SH2 domain [79]. The SH2 domain interaction partners may compete for binding to the SLP-76 SH2 domain which has been shown for ADAP and HPK1 [120].

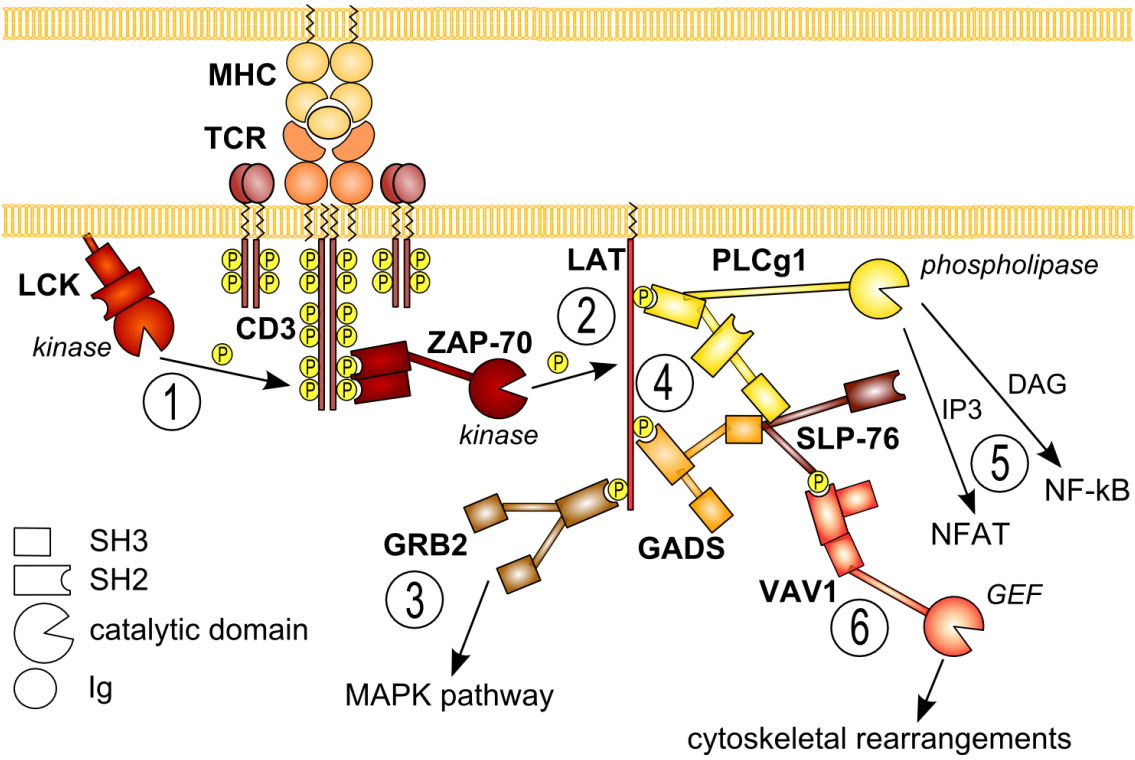


Figure 2. TCR signalling. ***(1)*** *When the TCR is ligated by peptide:MHC the kinase LCK phosphorylates ITAM motifs in the CD3 chains. ZAP-70 is recruited to phosphorylated CD3. Recruited ZAP-70 is also phosphorylated by LCK.* ***(2)*** *ZAP-70 phosphorylates downstream targets such as LAT which recruits more interaction partners.* ***(3)*** *GRB2 is recruited to LAT and then activates the MAPK pathway.* ***(4)*** *GADS links the adapter SLP-76 to LAT. PLCγ1 binds to SLP-76 and LAT and is activated by phosphorylation.* ***(5)*** *Active PLCγ1 hydrolyses* PIP_2 *to*

produce IP_3 and DAG which activate the transcription factors NFAT and NF-κB, respectively. ***(6)*** *SLP-76 recruits VAV1 which regulates cytoskeletal rearrangements. Protein names are shown in bold and enzyme functions are shown in italic.*

1.4.1 Molecular interactions with the cytoplasmic region of CD5

CD5 and CD6 possess long cytoplasmic regions with several residues that may recruit signalling proteins (Figure 3). The human CD5 cytoplasmic region contains 93 amino acids and possesses 3 tyrosine residues. Two tyrosine residues are known to be phosphorylated (Y453 and Y487) after T cell stimulation with anti-CD3 antibodies, possibly by the tyrosine kinase LCK [121,122]. It was claimed that a fourth tyrosine is part of a motif similar to the immunoreceptor tyrosine-based inhibitory motif (ITIM), but since this tyrosine may not be phosphorylated and may be buried in the membrane, it is doubtful that this motif is functional [121,123]. The phosphatase SH2 domain-containing phosphatase 1 (SHP-1) was reported to interact with the CD5 ITIM but this association was found using co-immunoprecipitation which works in a membrane-free system that exposes the ITIM [123,124]. Y453 and Y465 lie within a motif similar to an ITAM (YSQPPRNSRLSAYPAL which is related to the ITAM consensus YxxL/Ix$_{(6-8)}$YxxL/I), but since a doubly-phosphorylated peptide containing Y453 and Y465 does not bind ZAP-70 (Lacey E and Brown MH, unpublished observations), this motif might not be functional or at least does not behave like an ITAM [121]. However, there is some evidence that mutating Y465 has an inhibiting function on interleukin 2 (IL-2) production and survival, suggesting that this residue is relevant for CD5 function (Lacey E, unpublished observations and

[125]). Several signalling molecules have been reported to be associated with the intracellular tail of CD5 as assessed by immunoprecipitation experiments. Dennehy et al. reported that phosphorylated CD5 associates with the regulatory subunit of phosphatidylinositol 3 kinase (PI3K) and recruits the PI3K catalytic subunit because the recruited protein exhibits lipid kinase activity [126]. The same group also suggested Ras GTPase-activating protein (RasGAP) and the E3 ubiquitin ligase c-cbl to be interaction partners of CD5 [127]. CD5 was shown to be ubiquitinated by c-cbl and therefore targeted for proteasomal degradation in a model of suboptimal TCR signalling [128]. ZAP-70 also co-precipitated with CD5 but only in activated cells indicating that only phosphorylated ZAP-70 associates with CD5 [129]. As mentioned before, co-immunoprecipitation does not show a physical interaction and these identified interactions were not confirmed independently or further characterised biochemically. It is possible that in these studies larger insoluble membrane fractions containing CD5 but also other receptors were isolated and these signalling molecules bound to other receptors.

In addition, CD5 is constitutively phosphorylated on C-terminal serine residues in an SDSDY sequence (residues 483-487) and in a yeast two-hybrid system, Casein kinase 2 (CK2) was identified as the serine/threonine kinase responsible for this *in vitro* [130]. Lozano and colleagues proposed that CK2 also binds to the phosphorylated serine residues in the CD5 SDSDY sequence and interactions with this site have functional consequences on T cell differentiation and survival [59]. EAE mice with a deletion of SDSD in CD5 showed reduced EAE symptoms and T cells were more susceptible to AICD [131].

The CD5-CK2 interaction has also been identified by Raman et al. using co-immunoprecipitation [132]. Both groups did not compare the CD5 immunoprecipitation with another receptor or mutant CD5 as control and therefore CK2 might not bind specifically. Nevertheless, these findings stress the importance of the SDSDY sequence in the function of CD5.

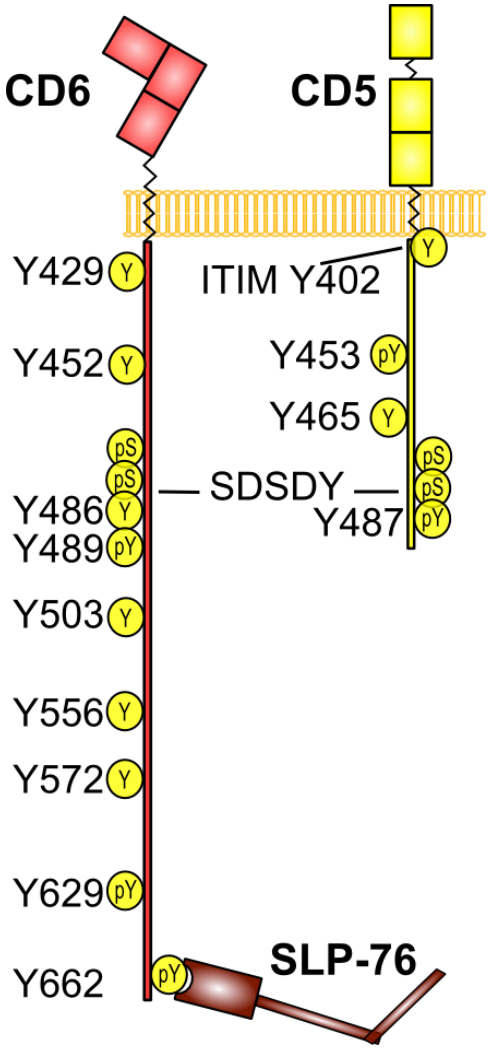


Figure 3. CD5 and CD6 cytoplasmic regions. *Both proteins possess tyrosine and serine residues (Y and S, respectively), some of which have been shown to be phosphorylated (Indicated by "p"). CD5 Y402 lies within an ITIM motif that*

exerts inhibitory function. The SDSDY sequence can be serine and tyrosine phosphorylated and is located at the same distance from the membrane in CD5 and CD6. Despite the long cytoplasmic region of CD6, only Y662 is known to directly recruit a protein, SLP-76.

1.4.2 Molecular interactions with the cytoplasmic region of CD6

Human CD6 possesses the longest cytoplasmic region without catalytic activity in the immune system with 244 amino acids (Figure 3) [81]. It contains 9 intracellular tyrosine residues and an SDSDY sequence (residues 482-486) but which tyrosine residues are phosphorylated *in vivo* has not been examined in detail [133]. CD6 tyrosine phosphorylation occurs in response to TCR triggering [134]. One of the phosphorylated tyrosine residues is CD6 Y629 [133].

There is evidence that the serine residues of SDSDY are phosphorylated [133,135]. Interestingly, the CD6 SDSDY sequence is conserved in mammals with minor changes in some mammals such as rat which possesses a glutamic acid instead of the first aspartic acid which means the charge is conserved. The SDSDY sequence is located at the same distance from the membrane in CD5 and CD6. The influence of serine phosphorylation on receptors and receptor-proximal signalling in the immune system is not well known. However, phosphoserine can bind to phosphoserine-binding domains and therefore might recruit proteins to the SDSDY motif. An intriguing example for the importance of phosphoserine is the B cell receptor α chain which can be phosphorylated at serine 197 and this phosphorylation inhibits B cell receptor signalling [136]. Another example of serine phosphorylation in immune cell signalling is the

targeting of SLP-76 for proteasomal degradation after serine 376 phosphorylation [137]. This phosphoserine recruits 14-3-3 proteins which mediates the recruitment of an E3 ubiquitin ligase and subsequent polyubiquitination.

The finding that SLP-76 function is also regulated by serine phosphorylation is particularly intriguing because CD6 signals through SLP-76, the only confirmed binding partner of the CD6 cytoplasmic domain [79,106]. The SH2 domain of SLP-76 binds to the phosphorylated tyrosine 662 residue with a K_D of 0.5 µM. Mutation of tyrosine 662 resulted in a reduced production of IL-2 in an antigen-presenting cell-T cell coculture system [79]. This raises the question of how this interaction affects TCR signalling since SLP-76 is recruited to LAT via GADS binding to the proline-rich region of SLP-76. LAT is downstream of the TCR and therefore CD6 might act in synergy with TCR signalling on SLP-76 phosphorylation or have a separate effect, for example keeping SLP-76 at the cell surface [91]. This could explain the activating mechanism of CD6 because endocytosis events are frequently observed at the central supramolecular activation cluster of the immunological synapse and are associated with signal termination [138]. Using a proteomics approach, Roncagalli et al. proposed that CD6 may also function as an alternative to LAT [106]. In the absence of LAT, CD6 can recruit signalling molecules similar to LAT and could therefore be an alternative or evolutionary older scaffold protein. However, T cell activation is impaired in the absence of LAT indicating that CD6 cannot fully replace LAT in TCR signalling but may function in synergy [106]. Y662 can also recruit syntenin-1, a cytoskeleton-associated protein which also interacts with CD166 but it is unknown whether SLP-76 and syntenin-1 compete for Y662 [139,140].

Interestingly, the effect of mutating tyrosine 662 to phenylalanine on T cell activation can be partially reversed by mutating tyrosine 489 [79]. This indicates that CD6 tyrosine 489 provides a negative signal in TCR signalling. Tyrosine 489 is only three amino acids downstream of the SDSDY sequence which has been shown to have an inhibitory function in CD5 [59]. This means tyrosine 489 could function together with this sequence as a larger SDSDYEHY sequence.

1.5 Open questions in CD5 and CD6 biology

Neither *in vitro* nor *in vivo* studies show when CD5 and CD6 function as activating or inhibitory receptors and what the precise role of these receptors is. Molecular interactions show activating and inhibitory binding partners but considering the size of the cytoplasmic domains and the lack of independently confirmed interactions, it is clear that not all relevant interactions have been identified.

1.6 Aims of this thesis

Understanding molecular interactions of CD5 and CD6 is key to gain insight into how these receptors function in T cells. Knowledge of molecular mechanisms also provides potential new avenues of how to target these receptors for autoimmunity or cancer therapy.

Therefore I aim to identify novel molecular interactions with the cytoplasmic tails of CD5 and CD6 focusing on the SDSDY sequence shared by CD5 and CD6 and the Y629 residue of CD6 because there is evidence that these residues can be phosphorylated and have a functional importance. I shall investigate biochemical and biophysical features of these newly identified interactions to reveal their exact niche in T cell signalling.

In addition, functional consequences of molecular interactions on cell lines and primary T cells are investigated to reveal the contribution of CD5 and CD6 to regulation of T cell activation.

2 Materials and methods

2.1 Molecular biology

2.1.1 Vectors

The pET49b(+) containing a glutathione S-transferase (GST) tag and a Kanamycin resistance gene was provided by Toshio Hakoshima, Nara Institute of Science and Technology [141]. The pOPINF vector containing a his tag and an Ampicillin resistance gene was provided by the Oxford Protein Production Facility. The pTrc HisA vector containing a his tag and an Ampicillin resistance was provided by Thermo Fisher.

The pHR-SIN-EGFP vector containing an Ampicillin resistance (Figure 4) encoding wild type (WT) human CD6 or the Y662F mutant fused to enhanced green fluorescent protein (EGFP) at its C terminus was provided by Marion H. Brown (University of Oxford) [122]. Expression by this vector is controlled by the spleen focus-forming virus promoter.

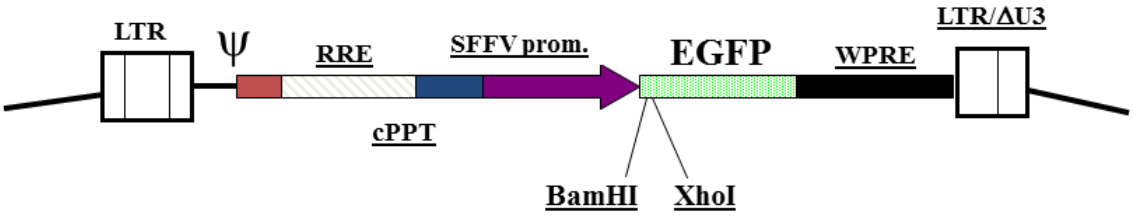


Figure 4. Components of the self-inactivating (SIN) 3rd generation lentiviral pHR-SIN-EGFP vector. *LTR: long terminal repeats that flank DNA that is integrated into the host genome. Ψ: Target site for packaging of RNA into capsids. RRE: Rev response element that is responsible for binding of Rev which mediates export of viral RNA out of the nucleus. cPPT: polypurine tract which enhances integration into the host genome. SFFV prom: Spleen focus-forming virus promoter. WPRE: Woodchuck element that enhances expression via the formation of a tertiary structure of its transcript. Inserts without stop codon cut with BamHI and SalI were cloned into pHR cut with BamHI and XhoI (destroying the XhoI site) and are fusion proteins with EGFP at their C-terminus.*

2.1.2 Bacterial strains

Vectors were transformed into the competent MC1061 *E. coli* strain (in house competent cell production) for DNA amplification.

All proteins were expressed in competent Rosetta™ 2(DE3)pLysS cells (Novagen). This bacterial strain is based on BL21 *E. coli* which express a lower amount of proteases to enhance protein expression. The Rosetta strain encodes additional tRNAs which are commonly used in eukaryotic proteins. These bacteria possess a T7 RNA polymerase gene under control of the lac operon indicated by DE3. The pLysS plasmid encodes a T7 phage lysozyme which

reduced the expression of the T7 RNA polymerase unless the lac operon is activated.

2.1.3 Transformation and DNA production

Competent bacteria cells were transformed with 1 ng DNA by heatshocking them for 1 min at 42° C and then plating them out for 18 h containing antibiotics.

DNA was amplified using the MC1061 strain and purified using a plasmid midi kit (Qiagen) according to the manufacturer's instructions. DNA sequences were confirmed by Sanger sequencing conducted by Source Bioscience.

2.1.4 Mutagenesis

Cytoplasmic residues of CD6 were mutated using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and 1x G5 High GC Enhancer (New England Biolabs) with the primers shown in Table 1 according to the manufacturer's instructions. For all mutagenesis reactions, the following PCR programme was used on a Tgradient thermocycler (Biometra): 10 s at 98° C denaturation, 30 s at a primer-specific annealing temperature (61° C for CD6 SDSDF, CD6 ADADY and CD6 ADADF and 66° C for CD6 Y629F) and 420 s at 72° C elongation for 25 cycles.

Table 1. CD6 cytoplasmic mutants and mutagenesis primers

Mutation	5'→3' forward	5'→3' reverse
WT	Construct provided by Marion H. Brown	
SDSDF	AGACTTTGAGCACTATGA CTTCAGC	GAGTCCGAGCCAGAGTCTGA GTCCTC
ADADY	AGACTATGAGCACTATGA CTTCAGC	GCGTCCGCGCCAGAGTCTGA GTCCTC
ADADF	AGACTTTGAGCACTATGA CTTCAGC	GCGTCCGCGCCAGAGTCTGA GTCCTC
Y629F	CGGGGAGTGGTTCCAGA ACTTCC	GATGAGGTGCTGGAGCTG
Y662F	Construct provided by Marion H. Brown	
Y629F Y662F	Mutagenesis of the Y662F construct using the Y629F mutagenesis primers	

The PCR product was then phosphorylated and circularised and the template was degraded with the KLD reagent of the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and the DNA was transformed into NEB5alpha competent bacteria (New England Biolabs).

2.2 Biochemistry

2.2.1 Proteins and peptides

All the recombinant human proteins, the source of the sequence, the vector, the size exclusion column and the buffer used for size exclusion and surface plasmon resonance (SPR) are shown in Table 2. The buffer used for GADS SH2, GRB2 SH2 and T cell-specific adapter (TSAd) SH2 is from hereon called cytoplasmic buffer because it resembles the cytoplasmic environment.

Table 2. Recombinant proteins, size exclusion column and buffer

Name	Protein	Vector	Size exclusion column (GE Healthcare)	Buffer
Radixin FERM	radixin 1-310 GST	pET49b(+)	Superdex 200 10/300 GL	300 mM NaCl 1 mM DTT* 20 mM MES** pH 6.8
GADS SH2	GADS 58-149 his	pOPINF	Superdex 75 10/300 GL	0.15 M KCl 3 mM EDTA*** 0.005 % Tween 10 mM HEPES**** pH 7.4
GRB2 SH2	GRB2 60-152 his	pOPINF	Superdex 75 10/300 GL	0.15 M KCl 3 mM EDTA 0.005 % Tween 10 mM HEPES pH 7.4
TSAd SH2	TSAd 95-186 his	pOPINF	Superdex 75 pg HiLoad 16/600	0.15 M KCl 3 mM EDTA 0.005 % Tween 10 mM HEPES pH 7.4
SLP-76 SH2	SLP-76 422-530 his	pTrc HisA	Superdex 75 10/300 GL	0.15 M KCl 3 mM EDTA 0.005 % Tween 10 mM HEPES pH 7.4

* Dithiothreitol

** 2-(N-morpholino)ethanesulfonic acid

*** ethylendiaminetetraacetic acid

**** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Table 3 shows the peptides used in this project, their sequences and their applications.

Table 3. Peptides used for biochemical studies

Peptide	Sequence	Application
CD5 Y1	Biotin-HVDNEYSQPPRNS	Pulldown
CD5 SDSDY	Biotin-PDNSSDSDYDLHGAQRL	SPR
CD5 pSDpSDpY	Biotin-PDNSpSDpSDpYDLHGAQRL	Pulldown SPR
CD6 SDSDY	Biotin-GSDSDYEHYDFSAQ	Pulldown SPR
CD6 pSDpSDpY	Biotin-GpSDpSDpYEHYDFSAQ	Pulldown SPR
CD6 Y629	Biotin-SSGEWYQNFQP	Pulldown SPR
CD6 pY629	Biotin-SSGEWpYQNFQP	Pulldown SPR
CD6 pY662	Biotin-PDSTDNDDpYDDISAA	SPR
CD6 pY629 pY662	Biotin-STSSGEWpYQNFQPPPQPPSEEQF GCPGSPSPQPDSTDNDDpYDDISAA	SPR
CD6 pY629 Y662	Biotin-STSSGEWpYQNFQPPPQPPSEEQF GCPGSPSPQPDSTDNDDYDDISAA	SPR
CD6 Y629 pY662	Biotin-STSSGEWYQNFQPPPQPPSEEQF GCPGSPSPQPDSTDNDDpYDDISAA	SPR
CD28 pYMNM	Biotin-LLHSDpYMNMTP	SPR
ICAM-2	Biotin-HLRQQRMGTYGVRAAWRRLPQAFRP	SPR

2.2.2 Radixin FERM expression

One colony of radixin 4.1 protein, ezrin, radixin and moesin (FERM) domain-transformed bacteria was transferred into a starter culture with LB broth and 50 µg/ml Kanamycin and incubated for 18 h. The starter culture was diluted 1:100 in 500 ml LB broth and 50 µg/ml Kanamycin. The culture was grown at 37° C at 200 rpm to an optical density of 0.6, cooled to room temperature and then 0.5 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) was added and the culture was incubated at room temperature for 4 h at 150 rpm. IPTG is a strong inducer of the lac operon and therefore induces expression of radixin FERM [142]. The bacteria were centrifuged at 3500 g for 15 min at 4° C and the pellets were frozen at -20° C.

2.2.3 Radixin FERM purification

Bacterial pellets were resuspended with 1 % Triton X-100, 1 mM DTT (Sigma-Aldrich) and 1:100 protease inhibitor cocktail (Sigma-Aldrich) in phosphate buffered saline (PBS) under sonication, centrifuged 14,000 g for 20 min at 4° C and filtered through a 0.22 µm Millex-GP filter (Merck). Glutathione Sepharose 4B (GE Healthcare) was washed with PBS and incubated with the lysate for 1 h at 4° C in an Econo column to allow the GST-tagged protein to bind to the sepharose. The flowthrough was removed and the beads were washed 4 times with PBS.

2.2.4 Cleavage of the GST tag with the HRV 3C protease

The HRV C3 protease (Novagen) recognises the sequence LEVLFQGP between the GST tag and radixin FERM and cleaves before the GP residues,

leaving these two amino acids on the protein [143]. HRV C3 catalysis is not impaired by the presence of DTT or incubation at 4° C and was therefore chosen as the optimal reagent to cleave the GST tag. GST-tagged radixin FERM bound to Glutathione Sepharose 4B was incubated with 100 units HRV C3 and 1 mM DTT in 150 mM NaCl in 50 mM Tris-HCl, pH 7.5 for 18 h at 4° C. The FERM domain was then eluted from the Econo column and further purified using size exclusion chromatography directly after elution.

2.2.5 His-tagged protein expression

For protein expression, one colony of transformed bacteria was transferred into a starter culture with autoinduction medium (2YT broth base including trace elements, Formedium) with 1 % (w/v) glucose and 100 µg/ml Ampicillin and incubated for 18 h. The starter culture was diluted 1:100 in autoinduction medium and 100 µg/ml Ampicillin and incubated 4 h at 37° C to allow the bacteria to deplete the glucose in the autoinduction medium. The bacteria were incubated for 20 h at 25° C for protein expression which is activated by the lactose in the autoinduction medium. The bacteria were centrifuged at 3500 g for 15 min at 4° C and the pellets were frozen at -20° C.

2.2.6 His-tagged protein purification

Bacterial pellets were resuspended with 1 % Triton X-100, 1 mM DTT and 1:100 protease inhibitor cocktail (Sigma-Aldrich) in PBS under sonication, centrifuged 14,000 g for 20 min at 4° C and filtered through a 0.22 µm filter. The lysate was diluted to 50 ml and imidazole (Sigma-Aldrich) was added to a final concentration of 20 mM to reduce nonspecific binding during Ni affinity

purification. His-tagged proteins were purified on an Äkta pure chromatography system (GE Healthcare) with a cOmplete™ His-Tag purification column (Sigma-Aldrich) using 20 mM imidazole in PBS as running buffer and a flow speed of 1 ml/min. Proteins were eluted with 250 mM imidazole in PBS in fractions of 500 µl. Proteins were then dialysed against PBS.

2.2.7 Size exclusion chromatography

Purified proteins were centrifuged at 21,000 g for 10 min at 4º C in a Mikro 200R centrifuge (Hettich) and filtered through a 0.22 µm Millex-GP filter (Merck) prior to size exclusion chromatography to remove aggregated protein.

500 µl (10/300 columns) or 4 ml (16/600 column) protein solution was injected via an injection loop into the size exclusion column indicated in Table 2 on an Äkta pure chromatography system (GE Healthcare). Size exclusion chromatography was performed at 500 µl**/**min flow speed and 500 µl fractions were collected using the buffer indicated in Table 2.

To test the purity of the purified proteins, samples were loaded onto NuPAGE 4-12 % Bis-Tris protein gels (Thermo Fisher) for electrophoretic separation and proteins were stained with 1.25 % Coomassie Brilliant Blue R-250 dye (Thermo Fisher).

2.2.8 Surface plasmon resonance with BIAcore 3000

SPR can be used to obtain affinity and kinetic information of molecular interactions [144]. Material bound to the SPR chip changes the refractive index of the chip surface. Incident light is reflected by the SPR chip and the reflection

angle depends on the refractive index. The reflection angle, measured in response units, is directly proportional to material bound to the chip surface allowing accurate measurements of binding and dissociation of molecules. Therefore, SPR was used to confirm associations detected by pulldowns as being direct.

For SPR equilibrium binding, a BIAcore 3000 with CM5 chips (GE Healthcare) was used. To immobilise biotinylated peptides, streptavidin was bound to the chip surface using an amine coupling kit (GE Healthcare) according to the manufacturer's instructions. 25 response units of peptides were immobilised. Eight serial dilutions of recombinant proteins in the respective running buffers were injected over the chip at 15º C, 25º C or 37º C and the response units at the end of each injection were measured in comparison to the baseline response units before injection. Protein concentrations were determined by measuring their absorbance at 280 nm. Equilibrium binding was analysed using GraphPad Prism 6.

Peptides used to measure binding to CD5 and CD6 SDSDY were CD5 SDSDY, CD5 pSDpSDpY, CD6 SDSDY, CD6 pSDpSDpY and intercellular adhesion molecule 2 (ICAM-2) as positive control [145]. Peptides used to measure binding to CD6 Y629 were CD6 Y629, CD6 pY629 and CD28 pYMN as positive control [146]. Peptides used to measure GADS SH2 and SLP-76 SH2 specificity were CD6 pY629 pY662, CD6 pY629 Y662 and CD6 Y629 pY662.

2.2.9 Peptide pulldown

To identify potential binding partners of CD6 cytoplasmic residues, proteins were pulled down using synthetic peptides and lysates of the human Jurkat T cell leukaemia line.

Jurkat cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with L-Glutamine (Sigma-Aldrich), 10 % foetal bovine serum (FBS, Sigma-Aldrich) and 1 % Penicillin/Streptomycin (Life Technologies) at a density of $1x10^6$ cells/ml at 37° C and 10 % CO_2 in a humidified incubator. Cells used in CD6 pY629 pulldowns were treated with 500 ng/ml ionomycin and 50 ng/ml phorbol myristate acetate (PMA) for 24 h to induce expression of potential CD6 pY629 binding partners. $2x10^8$ cells per sample were washed twice with PBS and centrifuged at 3500 g for 20 min at 4° C. Pellets were lysed in PBS with 1 % Triton X-100 (Sigma-Aldrich), 1:100 mammalian protease inhibitor cocktail (Sigma-Aldrich) and 1 mM of the phosphatase inhibitor sodium orthovanadate (Sigma-Aldrich) for 60 min at 4° C under rotation.

The lysates were then centrifuged at 14,000 g for 30 min at 4° C and passed through a 0.22 µm Hydrophilic Polyvinylidene Fluoride Filter (Merck). Dynabeads M-280 Streptavidin (Life Technologies) were washed twice with 0.1 % Triton X-100 in PBS using a Dynal MPC-E magnetic particle concentrator (Life Technologies) and 250 µl of Dynabeads M-280 Streptavidin per sample were added to the samples and the suspensions were rotated at 4° C for 1 h to pre-clear the lysate. 250 µl of beads per sample were washed twice with PBS and coated with 50 µg biotinylated peptide under rotation for 1 h at room temperature. Peptide-coated beads were then washed with PBS and transferred to new tubes for every wash. Pre-clear beads were removed and peptide-coated

beads were added to the lysates and rotated for 18 h at 4° C. Beads were washed 3 times with 0.1 % Triton X-100 in PBS and transferred to new tubes for every wash. To elute bound proteins, beads were resuspended in 0.1 M glycine pH 2.5 for 1 h at 70° C under frequent agitation and the supernatant was concentrated in a Vivacon 500 filter (Vivaproducts) at 18,000 g at room temperature [147].

2.2.10 Tandem Mass Spectrometry

The concentrated proteins in the filter from section 2.2.9 were washed 5 times with PBS and incubated for 1 h at room temperature with 8 M urea for protein denaturation. Then 10 mM DTT was added and incubated 1 h at room temperature to reduce proteins. The urea and DTT were removed by spinning the filter at 18,000 g at room temperature and then the proteins were alkylated with 2 mg/ml iodacetamide (Sigma-Aldrich) for 1 h at room temperature to prevent reformation of disulphide bonds by alkylation and washed twice with PBS. Then the proteins were washed twice with 25 mM NH_4HCO_3 and digested with 1 µg trypsin in PBS for 20 h at 37° C under agitation. Trypsin was removed by centrifugation and the peptides were eluted with 0.1 % formic acid in H_2O, 0.1 % formic acid in 50 % acetonitrile and then 0.1 % formic acid in 100 % acetonitrile with centrifugation steps between each elution. The eluted peptides were concentrated in a vacuum concentrator and resuspended in 0.1 % formic acid in H_2O. The resuspended peptides were loaded onto a C18 column (Sigma-Aldrich) to desalt the solution and eluted once with 0.1 % formic acid in 50 % acetonitrile and twice with 0.1 % formic acid and 100 % acetonitrile into the same tube and then concentrated with a vacuum concentrator.

The peptides were resuspended in 0.1 % formic acid in 5 % acetonitrile under sonication for 10 minutes and then directly injected into the Ultimate 3000 RSLCnano high performance liquid chromatography (Dionex)-tandem mass spectrometry Q Exactive Orbitrap machine using a 25 cm by 75 µm inner diameter picotip analytical column (New Objective) in the central proteomics facilities at the Sir William Dunn School of Pathology, University of Oxford. The liquid chromatography used a flow rate of 300 nl/min and 0.1 % formic acid as buffer A and 0.1 % formic acid in 80 % acetonitrile as buffer B and a gradient that increased from 5 % to 30 % buffer B in 100 min and then from 30 % to 50 % buffer B in 10 min.

The resulting spectra were then analysed using the Mascot search engine [148]. Mascot provides data on the number of unique peptides found for each proteins and the percentage of sequence of each protein covered by the identified peptides.

2.2.11 Co-immunoprecipitation

CD6 co-immunoprecipitation was conducted to confirm that identified interactions also occurred in cells with the whole receptor and not just peptides.

The Jurkat 1G4 T cell line transfected with the 1G4 TCR and the CD8 co-receptor was provided by Oreste Acuto (University of Oxford) [149]. Jurkat 1G4 cells were treated with 100 µM Na_3VO_4 (Sigma-Aldrich) for 30 min at 37° C and 10 % CO_2 in a tissue culture incubator. Na_3VO_4 is a tyrosine phosphatase inhibitor that increases phosphorylation-dependent interactions. Cells were washed twice with PBS and lysed in PBS with 1 % Triton X-100 (Sigma-Aldrich), 1:20

mammalian protease inhibitor cocktail (Sigma-Aldrich) and 1 mM Na_3VO_4 for 30 min at room temperature. Insoluble debris was removed by centrifugation at 16,000 g at 4° C for 30 min and filtering through a 0.22 µm Hydrophilic Polyvinylidene Fluoride filter (Merck). Lysate from $3x10^6$ cells was incubated with 5 µg MEM-98 CD6 antibody (Abcam) for 18 h at 4° C under rotation. Pierce© Protein A/G Magnetic Beads (Thermo Fisher) were washed twice with 1 % Triton X-100 in PBS and incubated with the lysate with MEM-98 for 1 h at room temperature to allow binding of the antibody to the beads. The supernatant was removed and the beads were washed twice with 1 % Triton X-100 in PBS and transferred to new tubes during the second wash. Proteins were eluted from the beads using Laemmli buffer (2 % Sodium dodecyl sulfate, 10 % glycerol and 0.002 % bromophenol in 125 mM Tris pH 6.8) at 100° C for 5 min.

2.2.12 Western blot

Samples were reduced by adding 10 % 2-mercaptoethanol and loaded onto NuPAGE 4-12 % Bis-Tris protein gels (Thermo Fisher) for electrophoretic separation. Samples were transferred to Odyssey® Nitrocellulose Membranes (LI-COR Biosciences) and probed with antibodies shown in Table 4. Membranes were read in a LICOR Odyssey Sa Imaging System (LI-COR Biosciences).

Table 4. Antibodies used for western blots

Target	Clone	antibody species/ conjugate	Concentration/ dilution	Supplier
EGFP	polyclonal	rabbit	1:500	provided by Gillian Griffiths
SLP-76	AS55	mouse	1 µg/ml	Sigma-Aldrich
GADS	polyclonal	rabbit	1:2000	Thermo Fisher
GRB2	polyclonal	rabbit	1:1000	Cell Signaling Technologies
TSAd	polyclonal	sheep	1 µg/ml	R&D Systems
sheep IgG	polyclonal	rabbit Dylight 680	30 µg/ml	Thermo Fisher
mouse IgG	polyclonal	goat Dylight 800	30 µg/ml	Thermo Fisher
rabbit IgG	polyclonal	goat Dylight 800	30 µg/ml	Thermo Fisher

2.3 Cellular biology

All cells were grown in DMEM with L-Glutamine (Sigma-Aldrich), 10 % FBS (Sigma-Aldrich) and 1 % Penicillin/Streptomycin (Life Technologies), from hereon called medium.

2.3.1 Lentiviral transduction into Jurkat 1G4 T cells

The packaging vectors pCMVR-dR8.91 (containing gag, pol, tat and rev genes) and pMD2.G (containing the VSV G envelope gene) (5.26 µg each) and the expression vector (10.52 µg) were mixed with 210 µg linear polyethylenimine (PEI, Sigma-Aldrich) in 1.3 ml serum-free DMEM (Sigma-Aldrich) and incubated for 45 min at room temperature. PEI plus DNA were then added to human

embryonic kidney (HEK) 293T cells grown in 10 ml medium. Cells were then incubated for 5 h at 37° C and 10 % CO_2 in a tissue culture incubator to allow transfection of HEK 293T cells. The PEI-containing medium was then removed and replaced with medium and incubated for 48 h to allow virus production. Viruses were harvested, centrifuged for 5 min at 500 g and 4° C and filtered through a 0.22 µm Millex-GP filter (Merck) to remove cell debris. The virus-containing suspension was incubated with the Lenti-Pac™ Lentivirus Concentration Solution (GeneCopoeia) in a 5:1 v/v ratio for 18 h at 4° C to precipitate the virus. The virus was then centrifuged for 25 min at 2800 g and 4° C and the pellets resuspended in medium at 5 % of the original volume. $1x10^6$ Jurkat 1G4 cells in medium were then transduced with the concentrated virus at a final volume of 1 ml.

2.3.2 CD4+ T cell isolation from blood

Leukocyte cones were provided by NHS Blood & Transplant. Blood was diluted 1 in 10 in PBS and 30 ml of diluted blood was layered over 15 ml of Ficoll-Paque (GE Healthcare). Tubes were centrifuged at 800 g for 30 min at room temperature without brakes to isolate peripheral blood mononuclear cells (PBMCs) from other blood components due to different density. The leukocyte layer was separated and washed twice with PBS.

CD4+ T cells were isolated using a Dynabeads Untouched human CD4 T cells kit (Thermo Fisher). $2x10^7$ cells were incubated with 40 µl antibody mix for 20 min at 4° C to allow binding of antibodies to other PBMCs but not CD4+ T cells. After washing the cells, PBMCs were incubated with 200 µl Dynabeads

for 15 min at room temperature to allow binding of Dynabeads to antibody-coated cells. The cell suspension was then loaded onto a Dynamag-15 magnet (Thermo Fisher) and the supernatant containing CD4+ T cells was removed. CD4+ T cells were then cultured at $4x10^6$ cells per ml in medium at 37° C and 10 % CO_2 for 18 h or until used.

2.3.3 Lentiviral transduction into primary T cells

To test the function of CD6 mutants in primary T cells, chimeric CD6 that contains rat CD6 SRCR domain 1 and a part of domain 2 up to a conserved endogenous HindIII site at base pair position 702 followed by the remainder of human CD6 was used (provided by Marion H. Brown). This allows antibody crosslinking of CD6 rat domain 1 without crosslinking endogenous CD6. Lentiviruses were produced as described in section 2.3.1.

T cells were activated with human T-activator CD3/CD28 Dynabeads® (Thermo Fisher) with a ratio of 3 beads per cell. Concentrated virus was added 1 day after addition of beads. T cells were incubated with beads and virus in the presence of 100 units/ml human IL-2 (Thermo Fisher) for another 4 days at 37° C and 10 % CO_2. The beads were removed using a DynaMag-15 magnet (Thermo Fisher) and cells were analysed for surface marker expression and used in functional experiments. Functional experiments with primary T cells were conducted 9-11 days after transduction.

2.3.4 Enrichment of CD6-transduced Jurkat 1G4 cells

The transduction efficiency differed between CD6 mutant constructs and therefore an enrichment of transduced cells was performed before functional experiments. Cells were coated with tissue culture supernatant of the T12.1 monoclonal CD6 antibody (ATCC, produced in house) at 4° C for 10 min in 50 ml Falcon tubes. Pierce© Protein A/G Magnetic Beads (Thermo Fisher) were washed with PBS and 25 µl bead suspension per $1x10^7$ cells was added to the cells and incubated at 4° C for 15 min. Beads and cells were washed with cold PBS and cells coated with beads were isolated using a DynaMag-15 magnet (Thermo Fisher) with three washes with cold PBS before elution with medium. Beads were removed by dilution through continuous culturing of the cells.

2.3.5 Single cell functional assays

Jurkat 1G4 cells transduced with WT or mutant CD6-EGFP or primary T cells transduced with WT or mutant human CD6 with domain 1 being replaced by rat domain 1 and fused to EGFP were used for functional experiments.

96 well round bottom tissue culture plates (Corning) were coated with 0.5, 1 or 2 µg/ml CD3 UCHT1 antibody (eBioscience) in PBS or just PBS as control for 18 h at 4° C. The liquid is aspirated and 2.5 µg/ml human CD6 antibody T12.1 (purified in house), 5 µg/ml rat CD6 domain 1 OX52 antibody (purified in house) [150] or PBS was added and incubated for 18 h at 4° C. $2x10^5$ transduced Jurkat 1G4 cells or primary T cells per well were added after aspiration of the liquid and incubated at 37° C and 10 % CO_2 for 18 h. Cells were then analysed for markers of T cell activation.

2.3.6 Crosslinking of rat CD6 domain 1 by OX52 in hybridoma

Hybridoma cells were used to test whether chimeric CD6 expressing rat domain 1 can be specifically crosslinked for functional studies. Murine 2B4 Saito hybridoma cells [151] transduced with the retroviral vector pFB-Neo with chimeric CD6 that contains rat CD6 SRCR domain 1 and a part of domain 2 up to an endogenous HindIII site at base pair position 702 followed by the remainder of the extracellular and transmembrane regions of human CD6 and the cytoplasmic region of mouse CD3 ζ were provided by Marion H. Brown. 96 well round bottom tissue culture plates (Corning) were coated with 1 or 5 µg/ml OX52 rat monoclonal CD6 domain 1 antibody (ECACC catalogue entry 87081805) in PBS or just PBS as control for 18 h at 4° C [152]. The antibody solution was aspirated and $1x10^5$ cells per well were added and incubated at 37° C and 10 % CO_2 for 18 h. The tissue culture plates were centrifuged at 500 g for 5 min at room temperature and the supernatants were analysed for the presence of mouse IL-2.

2.3.7 Activation marker expression

Cells were stained with 1:100 anti-human CD69 APC (Thermo Fisher) for 30 min at 4° C and washed twice with 0.25 % bovine serum albumin (BSA, Sigma-Aldrich) and 0.1 % NaN_3 in PBS. The cells were fixed in 2 % paraformaldehyde and read on a FACSCalibur™ (BD Biosciences) using the 96 well plate reader. Flow cytometry data was analysed with FlowJo™ (FlowJo, LLC). Cells were gated on a narrow level of EGFP signal and only CD69 expression of cells within this gate was considered to ensure similar CD6 expression levels.

2.3.8 Cytoplasmic cytokine flow cytometry

Cells were stimulated with plate-bound antibodies for 18 h as described in 2.3.5. Cells were stimulated in the presence of 10 µg/ml Brefeldin A (Sigma-Aldrich) which disrupts the function of the Golgi apparatus and therefore blocks cytokine secretion [153]. Cells were fixed and permeabilised by fixation buffer (BD Biosciences) for 20 min at room temperature and stained with 5 µg/ml APC-conjugated IL-2 antibody (Biolegend) diluted in Perm/wash buffer (BD Biosciences) for 30 min at room temperature. Cells were resuspended in Perm/wash buffer and read on a FACSCalibur™ (BD Biosciences).

2.3.9 T cell phenotyping

$5x10^5$ cells were stained in 96 well round bottom tissue culture plates (Corning). 0.25 % BSA and 0.1 % NaN_3 in PBS was used for all washes and dilutions. T cells were stained with primary and secondary antibodies (Table 5) for 30 min at 4° C with washes in between and at the end before fixing the cells with 2 % paraformaldehyde. The stained cells were read on a FACSCalibur™ (BD Biosciences). Expression levels were analysed with FlowJo™ (FlowJo, LLC).

Table 5. Antibodies used for phenotyping

Target	Clone	antibody species/ conjugate	Concentration/ dilution	Supplier
hCD6 d1	T12.1	mouse IgG1	neat tissue culture supernatant	ATCC (Produced In house)
hCD6 d3	OX124	mouse IgG1	neat tissue culture supernatant	Produced In house [79]
rCD6 d1	OX52	mouse IgG2a	neat tissue culture supernatant	Produced In house [150,152]
TCR	IP26	mouse IgG1 biotin	5 µg/ml	eBioscience
CD3	UCHT1	mouse IgG1 biotin	5 µg/ml	eBioscience
Isotype	unknown	mouse IgG1κ	5 µg/ml	Thermo Fisher
Isotype	unknown	mouse IgG2a	5 µg/ml	Thermo Fisher
Isotype	unknown	mouse IgG1κ biotin	5 µg/ml	AbD Serotec
mouse IgG	polyclonal	goat Dylight 650	5 µg/ml	Abcam
biotin	no antibody	Streptavidin APC	5 µg/ml	BD Biosciences

2.3.10 Enzyme-linked immunosorbent assay

Secreted antibodies were detected using the Enzyme-linked immunosorbent assay (ELISA). Nunc maxisorp plates (eBioscience) were coated with 1 µg/ml mouse IL-2 capture antibody (BD Biosciences) in PBS at 4° C for 18 h. The plates were blocked with 1 % BSA in PBS for 1 h at room temperature and then washed 3 times with 0.05 % Tween in PBS. Supernatants or recombinant mouse IL-2 were added and incubated for 2 h at room temperature.

After washing 3 times, biotinylated mouse IL-2 antibody (0.5 µg/ml) and ExtrAvidin Peroxidase (1:2000, Sigma-Aldrich) was added and incubated 1 h at room temperature. The plates were washed 6 times and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added and incubated until colour developed. The reaction was stopped with 1 M H_2SO_4 and the colour development was quantified using an Emax ELISA plate reader (Molecular Devices). The recombinant IL-2 standards were used to interpolate IL-2 concentrations in samples.

3 Results

3.1 CD5 and CD6 SDSDY interacts with ERM proteins suggesting a cytoskeletal connection

3.1.1 CD5 and CD6 SDSDY is associated with ERM proteins

3.1.1.1 The SDSDY sequence recruits cytoskeletal proteins

The SDSDY sequence in CD5 has been shown to play a role in CD5 function [131]. To find out whether the reported interaction of SDSDY with CK2 can be confirmed [130] or whether other interaction partners can be found, a screen for SDSDY interaction partners was conducted. Magnetic beads coated with serine- and tyrosine phosphorylated CD5 or CD6 pSDpSDpY peptides were used as bait to isolate SDSDY binding partners in Jurkat cell lysates. Potential interaction partners were then analysed using tandem mass spectrometry. Table 6 shows hits of three CD5 pSDpSDpY and one CD6 pSDpSDpY pulldown experiments. Hits were only shown when they were detected in all 3 CD5 pulldowns or were closely related to proteins that were detected in all 3 CD5 pulldowns. The number of unique peptides and sequence coverage of the peptides identified by mass spectrometry indicated that there was a true enrichment of these proteins by the peptide pulldown.

Table 6. CD5 and CD6 pulldown mass spectrometry results

	Number of unique peptides*		% sequence coverage***	
Protein	**CD5**	**CD6**	**CD5**	**CD6**
Ezrin	24, 24, 27**	9	42, 44, 46	24
Moesin	25, 35, 0	10	49, 54, 0	32
Radixin	13, 7, 0	0	25, 31, 0	0
NHE-RF2	6, 8, 0	2	18, 33, 0	8
NHE-RF1	11, 13, 7	0	44, 50, 25	0

*Peptides only found in the target protein
**each number represents one independent experiment
***protein sequence covered by the identified peptides

The top hits revealed by mass spectrometry were ezrin, radixin, moesin (ERM) and Na^+ H^+ exchange regulatory factors (NHE-RF) (Table 6). ERM proteins are FERM domain-containing proteins that link plasma membrane receptors to the actin cytoskeleton [154]. NHE-RF 1 and 2 are ERM interaction partners that strongly associate with ERM proteins [155,156].

These pulldown hits suggested a connection of CD5 and CD6 with the actin cytoskeleton. Pulldowns do not provide information on direct interactions and therefore it could be NHE-RF or ERM proteins that bound to the SDSDY sequence since both have been shown to interact with plasma membrane receptors [157]. Thus, further biochemical characterisation of the CD5 and CD6 SDSDY-mediated interactions was required.

3.1.1.2 ERM proteins directly bind to CD5 and CD6 pSDpSDpY

The FERM domain of ERM proteins binds to the cytoplasmic region of many receptors and was therefore the first choice to investigate the direct binding partner of CD5 and CD6 pSDpSDpY. It is difficult to predict whether the FERM domain binds to the pSDpSDpY sequence because it can bind different motifs and most of these motifs have positive charges unlike the negatively charged pSDpSDpY sequence [158]. SPR was used to analyse binding of the radixin FERM domain to CD5 and CD6 pSDpSDpY, the unphosphorylated SDSDY peptide and the FERM-binding motif of ICAM-2 as positive control [158]. The radixin FERM domain was expressed in bacteria as GST-tagged protein. The GST tag of Glutathione Sepharose-purified FERM-GST protein was cleaved and the FERM domain was further purified using size exclusion chromatography. Most of the purified protein aggregated but a peak at the expected elution volume contained radixin FERM with high purity (Figure 5).

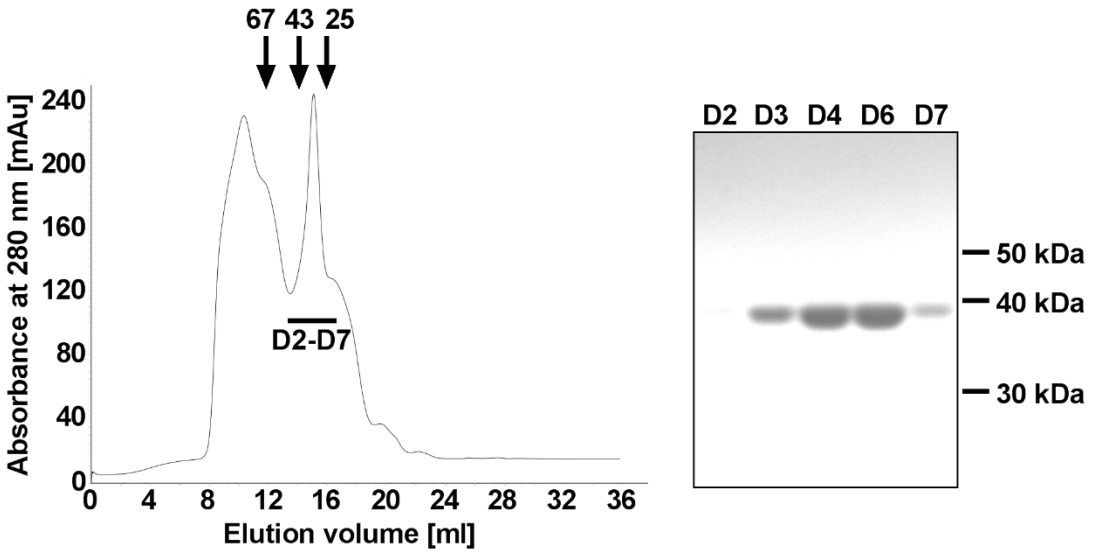


Figure 5. Size exclusion chromatography of the radixin FERM domain. *After cleavage of the GST tag, the monomeric radixin FERM domain was isolated by size exclusion chromatography (left) with a Superdex 200 10/30 GL column. Shown are the UV chromatograms for each protein. Standard protein weights in kDa are indicated at their elution volume. The radixin FERM domain has a molecular weight of 36 kDa. The fractions of the peak at the expected elution volume were analysed for purity by Coomassie staining (right).*

In a BIAcore 3000 instrument, the radixin FERM domain was injected over CD5 or CD6 SDSDY, pSDpSDpY or a FERM-binding ICAM-2 peptide. Radixin FERM bound to CD5 and CD6 pSDpSDpY with equilibrium dissociation constants of 1-2 µM for both sequences (Figure 6A and 6B). Binding to the unphosphorylated SDSDY CD5 and CD6 peptides resulted in a low response that did not reach saturation and therefore no equilibrium dissociation constants were determined. The raw traces reached equilibrium which suggested that the radixin FERM domain did not aggregate on the BIAcore chips but the response units were about 25 % of the expected response (Figure 6A and 6B right panels). A possible explanation for this lower response is that not all of the protein or peptide was active. Most of the radixin FERM domain aggregated during purification which is a sign of low protein stability and thus low protein activity may be the reason for the low response in SPR.

The affinities of radixin FERM for the ICAM-2 FERM-binding motif varied from 0.2 to 62 µM indicating that the true affinity of radixin FERM for ICAM-2 may be similar to the affinity for pSDpSDpY but this is difficult to conclude from the

varying K_D values (Figure 6C). The radixin-FERM affinity to ICAM-2 has been determined to be 16.4 nM but in this study the raw traces were not shown [145]. However, the same group published SPR data of radixin FERM binding to CD44 which showed signs of aggregation suggesting that they did not measure K_D values for monomeric FERM domains but for aggregates [159].

Measurements of direct binding of ezrin and moesin to pSDpSDpY was attempted but due to poor protein quality not further pursued (data not shown). All three ERM proteins were identified in pulldowns and the FERM domains of these three proteins possess about 85 % sequence homology suggesting that CD5 and CD6 pSDpSDpY can recruit all three ERM proteins.

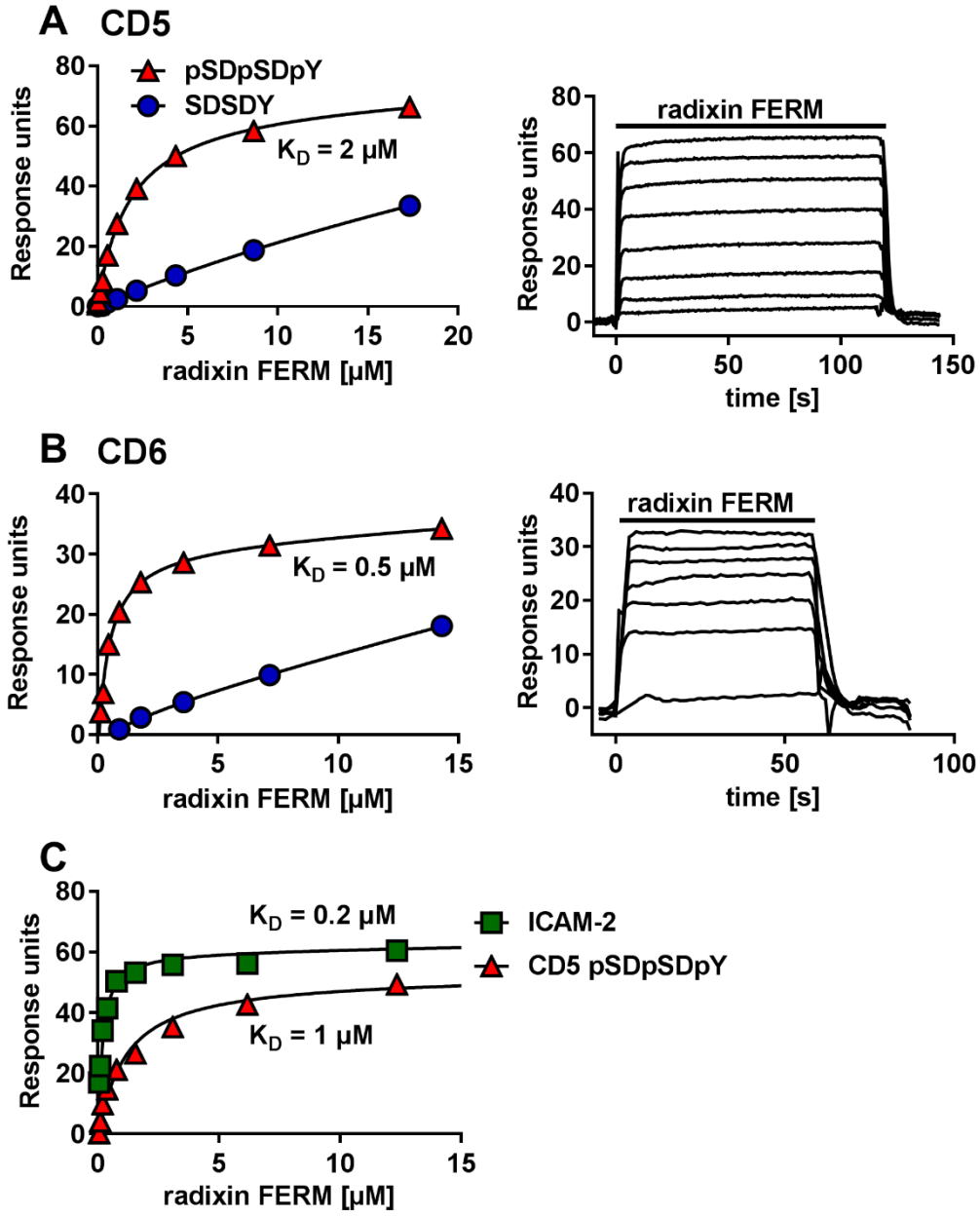


Figure 6. Equilibrium binding of radixin FERM to SDSDY and ICAM-2. *Radixin FERM was injected over immobilised CD5 (A) or CD6 (B) SDSDY or pSDpSDpY. The graphs on the left show equilibrium binding curves and the graphs on the right show raw traces of serial dilutions of the radixin FERM domain injected over the indicated peptides. Background signal of protein to an empty flow cell was subtracted for all results. The K_D for FERM binding to pSDpSDpY was in the range of 1-2 µM in three experiments. (C) Equilibrium binding of radixin*

FERM injected over CD5 pSDpSDpY and ICAM-2. The K_D for FERM binding to ICAM-2 was in the range of 0.2-62 µM in three experiments. All experiments were conducted three times at 25° C in 20 mM MES pH 6.8, 300 mM NaCl and 1 mM DTT and one example of each experiment is shown.

3.1.2 CD6 SDSDY has an activating effect on Jurkat 1G4 cells

3.1.2.1 CD6-EGFP transduced Jurkat 1G4 cells can be enriched with antibody-coated beads

To analyse the function of CD6 mutants in a T cell line, Jurkat 1G4 cells were transduced with human CD6-EGFP possessing different mutations. Transduction efficiency was >80 % for most constructs which is sufficient for experiments. However, some mutations of the SDSDY sequence of CD6 resulted in lower transduction efficiencies compared to the WT. To enrich for CD6 transduced cells, protein A/G beads were coated with the CD6 antibody T12.1 which has a low affinity for CD6 and therefore only cells expressing high levels of CD6 bind with high avidity to these beads. After several rounds of selection with beads with washing steps in between, cells were analysed for EGFP expression to determine the level of CD6 on the cell surface by flow cytometry. Figure 7A shows histograms showing EGFP expression in transduced cells before (bottom) and after (top) bead selection. The EGFP levels showed that after bead selection the CD6 expressing cells are highly enriched independently from the transduction efficiency. All SDSDY mutants and the WT had overlapping CD6 expression levels which allowed the comparison of cells with similar levels of CD6 (Figure 7B).

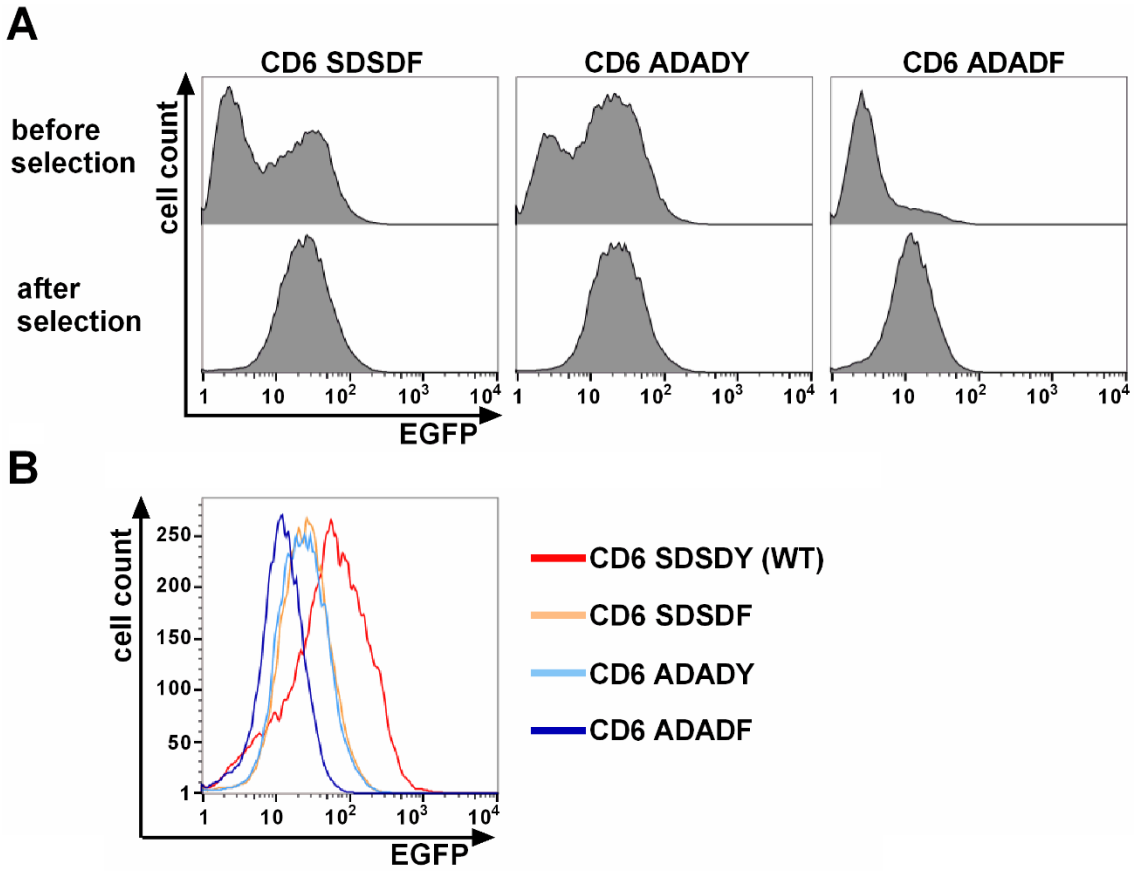


Figure 7. CD6-EGFP enrichment by bead selection. *WT and mutant CD6-EGFP expressing cells were enrichment by CD6 antibody-coated beads and assessed for EGFP signals before (top) and after (bottom) selection (A). WT and mutant CD6 constructs were compared for EGFP levels after bead selection (B).*

3.1.2.2 CD6 SDSDY mutant transduced Jurkat 1G4 cells express similar levels of TCR, CD3 and CD6

An important factor for comparing CD6 mutants is that the Jurkat 1G4 cells transduced with different CD6 constructs express the same level of receptors relevant for T cell activation. Figure 8A shows the expression levels of CD3, TCR as well as the surface expression of CD6 and total EGFP for WT and mutant

CD6. There was no difference for any of the examined receptors between the constructs, indicating that if these cells are used for functional experiments, an influence of different CD3, TCR or CD6 expression levels on Jurkat 1G4 cell activation can be excluded. Comparing EGFP and CD6 surface staining signals with dot plots showed a correlation of these two signals indicating that CD6-EGFP reached the cell surface (Figure 8B).

The phenotyping of the transduced Jurkat 1G4 cells suggested that all produced cell lines had similar capabilities to respond to CD3 and CD6 antibody stimulation due to the same expression levels of these receptors. Therefore, all measured T cell activation can be attributed to mutations in CD6.

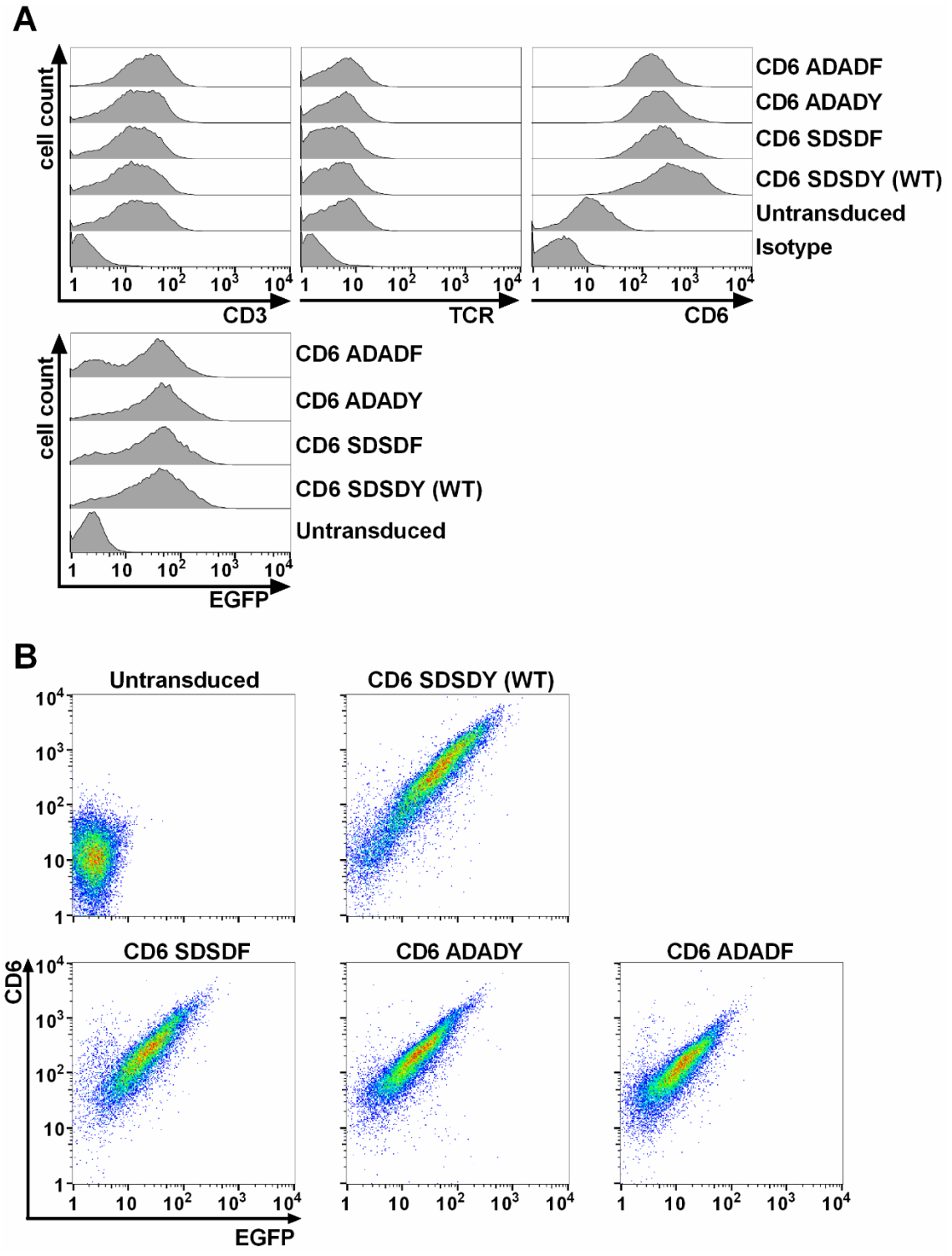


Figure 8. Similar expression levels of CD3, TCR and CD6-EGFP on Jurkat 1G4 cells transduced with WT, SDSDF, ADADY and ADADF CD6. *(A) WT or mutant CD6 was stained for CD3, TCR or CD6 and compared to an isotype control or analysed for CD6-EGFP expression shown as histograms. Transduced*

cells were gated on EGFP-positive cells. (B) Surface CD6 was stained and compared to the EGFP signal of transduced CD6. The isotype in (A) and the untransduced and WT samples in (A) and (B) are the same as in Figure 18 because all Jurkat 1G4 cell phenotyping was conducted together.

3.1.2.3 The CD6 SDSDY sequence is involved in CD6 signalling

To avoid an impact of different CD6 expression levels on results, a single cell assay using flow cytometry was chosen. Cells were gated on similar EGFP levels which correlated with CD6 expression levels (Figure 9).

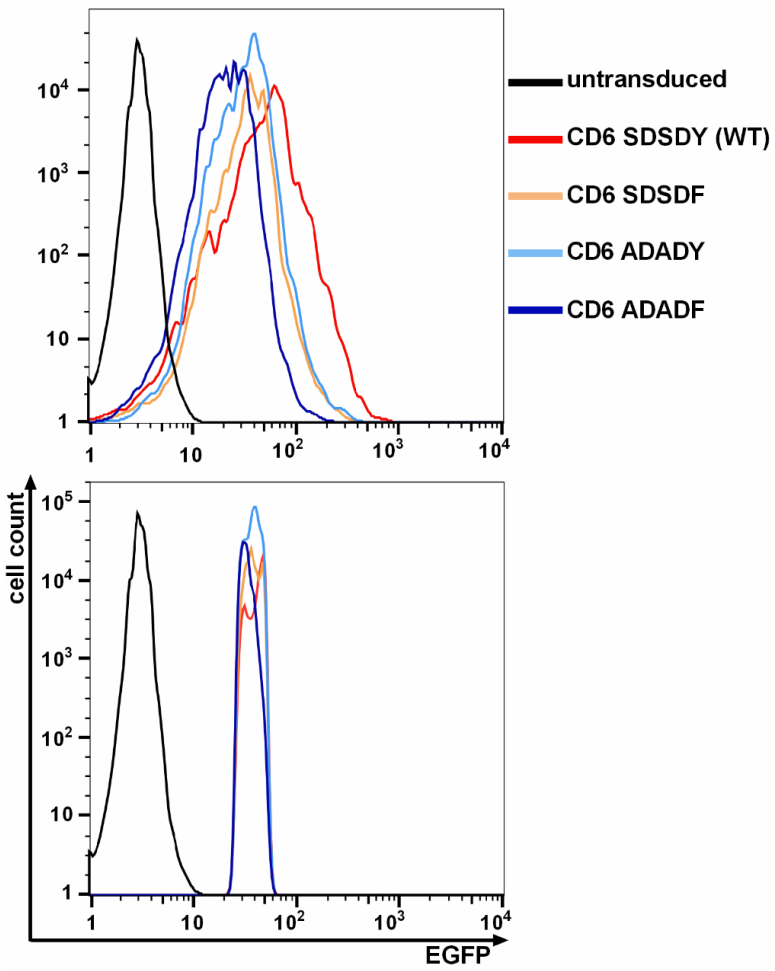


Figure 9. Gating strategy for single cell assay. *(Top) Different CD6 mutants are expressed at different levels shown for CD6 SDSDY (WT), SDSDF, ADADY*

and ADADF. (Bottom) A narrow gate was chosen to only analyse cells with similar EGFP levels for T cell activation.

Jurkat 1G4 cells were stimulated with different concentrations of plate-bound CD3 antibodies which mimic TCR signalling in the absence or presence of plate-bound CD6 antibodies which mimic CD6 ligation. CD69 was chosen as an early activation marker on the surface of T cells [160]. CD69 expression correlates with the strength of signalling through the TCR and costimulatory receptors. Higher T cell activation resulted in a shift towards higher CD69 expression which can be measured by assessing the mean fluorescence intensity (MFI) of CD69 expression (Figure 10).

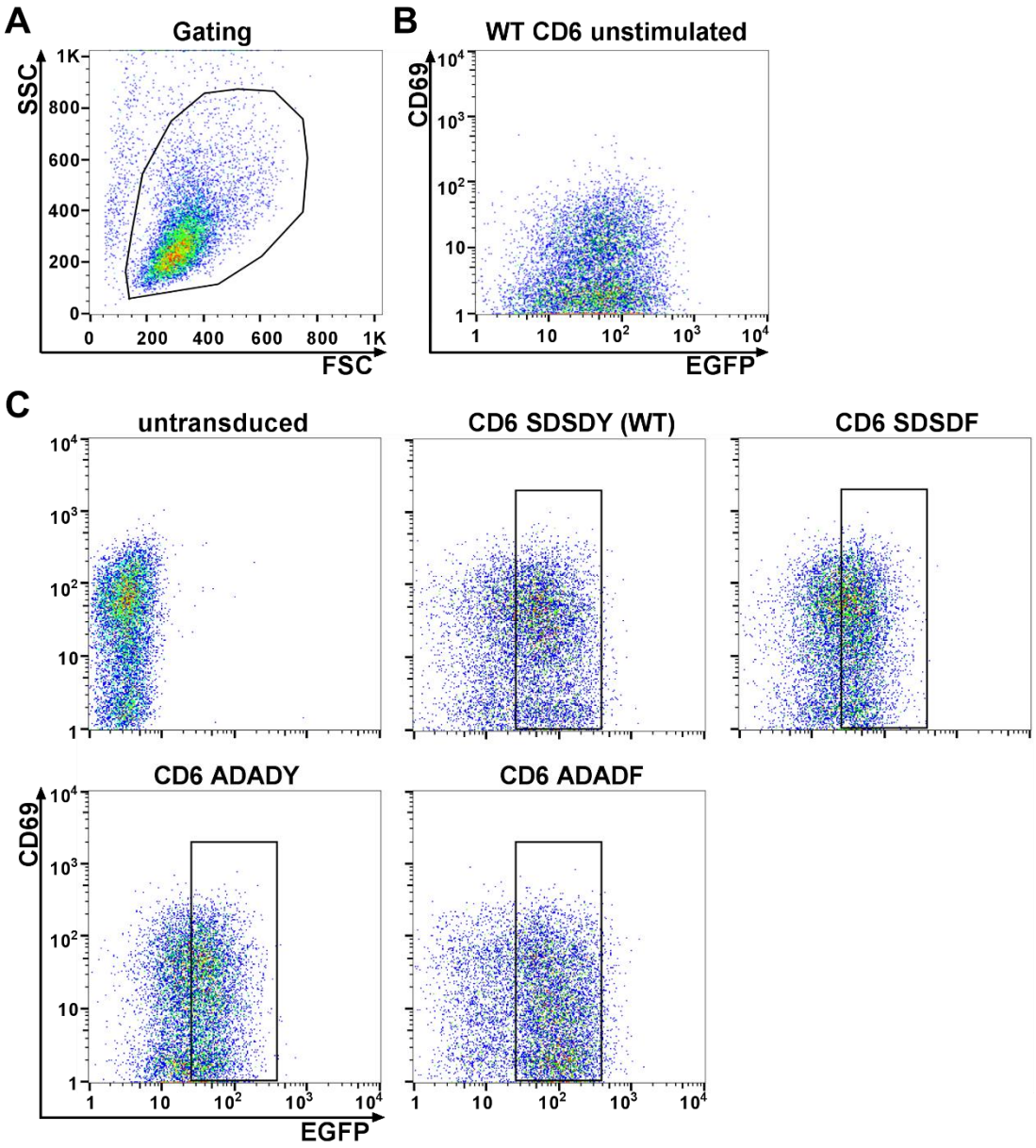


Figure 10. Example dot plots of activated Jurkat 1G4 cells. *(A) FSC-SSC plot showing the gating for cells that were assumed to be alive. (B) Shown is an example dot plot showing the CD69 signal versus the CD6-EGFP for unstimulated Jurkat 1G4 cells transduced with C6 SDSDY (WT). (C) 1G4 cells were stimulated with platebound 2 µg/ml CD3 antibody for 18 h and 2.5 µg/ml CD6 antibody T12.1 and stained for CD69 expression. Shown are example dot plots showing the CD69 signal versus the CD6-EGFP signal for untransduced*

Jurkat 1G4 cells and Jurkat 1G4 cells transduced with CD6 SDSDY (WT), SDSDF, ADADY or ADADF. CD69 expression was examined for all untransduced cells and for transduced cells in the shown gate.

Mutants of the CD6 SDSDY sequence were analysed in functional assays to assess the function of ERM protein binding to CD6 in T cell activation. The dose-dependent response to CD3 antibodies showed that these Jurkat 1G4 cells are able to upregulate the expression of CD69 in response to stimulation (Figure 11A). Jurkat 1G4 cells stimulated through CD3 but not CD6 had a significantly increased CD69 expression when WT CD6 was overexpressed but not when mutant CD6 was overexpressed. Stimulation with 2.5 µg/ml CD6 antibody T12.1 increased the CD69 expression levels further in a CD3 antibody-dependent manner (Figure 11B). WT CD6 transduced Jurkat 1G4 cells had significantly higher CD69 MFI than untransduced cells for any CD3 antibody concentration. Mutations of the tyrosine to phenylalanine did not result in a significant reduction of CD69 expression compared to WT CD6. Mutating the serine residues to alanine led to a significant reduction of CD69 expression for all three CD3 antibody concentrations. The same effect was achieved by mutating the serine residues and the tyrosine but this additional mutation did not reduce CD69 expression any further.

These results suggest an activating effect of overexpressing CD6 in Jurkat 1G4 T cells. The tyrosine in the CD6 SDSDY sequence did not influence CD6 function but the serine residues have an activating function in CD6 costimulation. However, mutating the SDSDY sequence of CD6 did not result in a complete

abrogation of CD6 costimulation indicating that other residues play a role in the costimulatory function as well.

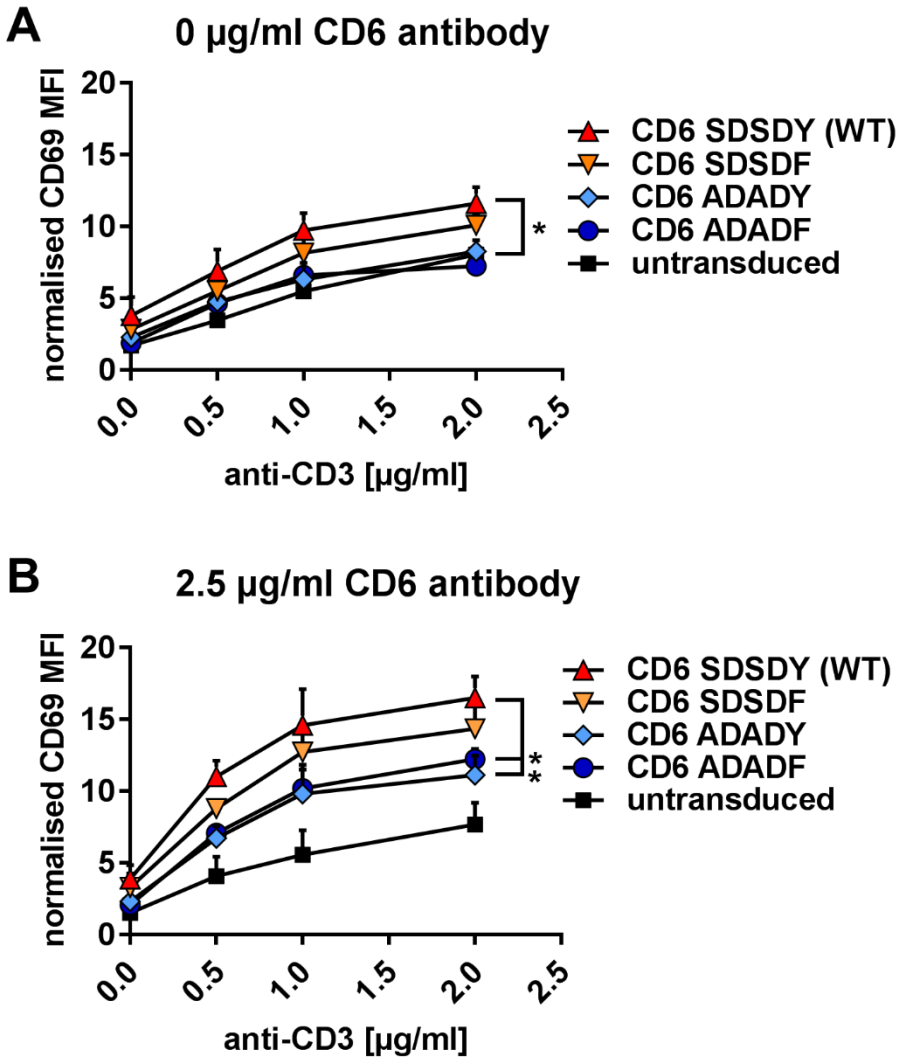


Figure 11. CD6 SDSDY has a costimulatory role in Jurkat 1G4 activation after CD3 and CD6 antibody stimulation. *(A) Jurkat 1G4 cells were stimulated with CD3 antibodies for 18 h and the CD69 MFI was examined for CD6 SDSDY (WT), SDSDF, ADADY, ADADF or untransduced cells. Statistics are shown for the comparison of WT CD6 transduced cells with untransduced cells. (B) Same*

*as in A but cells were also treated with 2.5 µg/ml CD6 antibody T12.1. CD69 MFI for WT CD6 was significantly higher ($p<0.01$) than for untransduced cells for any CD3 concentration. Statistics are shown for comparisons of mutant CD6 transduced cells with WT CD6 transduced cells. n = 3, error bars show the standard error of the mean, * $p<0.05$, student's t test for each CD3 antibody concentration above 0 µg/ml.*

3.2 The CD6 residues Y629 and Y662 recruit the GADS/SLP-76 complex and provide costimulation

3.2.1 CD6 Y629 and Y662 recruit the GADS/SLP-76 adapter complex

3.2.1.1 CD6 pY629 recruits SH2 domain-containing adapter proteins

The tyrosine at position 629 in human CD6 can be phosphorylated in T cells [133]. Pulldowns with a peptide containing the phosphorylated CD6 pY629 residue were conducted to find potential interaction partners with this residue. CD6 Y629 lies within a YXN motif which is predicted to recruit the SH2 domains of GADS, GRB2 and TSAd [161]. The pulldowns were conducted using lysates from Jurkat cells treated with 500 ng/ml ionomycin and 50 ng/ml PMA for 24 h which induced the expression of TSAd. Even though many SH2 domain-containing proteins were detected, only GADS,GRB2 and TSAd were identified in at least 2 out of 3 pulldowns and were not detected in a pulldown with CD5 Y1, an unrelated, unphosphorylated peptide derived from the first cytoplasmic tyrosine residue of human CD5 (Table 7).

Table 7. CD6 Y629 pulldown mass spectrometry results

Protein	Number of unique peptides*	% sequence coverage***
GADS	29, 43, 21**	60, 81, 72
GRB2	26, 18, 40	59, 66, 79
TSAd	3, 4, 0	11, 14, 0

*Peptides only found in the target protein
**each number represents one independent experiment
***protein sequence covered by the identified peptides

All three identified proteins are adapter proteins involved in TCR signalling [107,108,162]. They all have the same requirement for the phosphorylated

tyrosine and the asparagine of the YXN motif in CD6 and therefore it cannot be concluded from the screen which of these proteins is the main interaction partner [161]. It is also possible that these proteins do not directly bind CD6 but are part of a larger complex.

3.2.1.2 GADS, GRB2 and TSAd directly bind to CD6 pY629

SPR was chosen to test that the CD6 pY629 interactions detected in pulldown experiments were direct. Recombinant SH2 domains of GADS, GRB2 and TSAd were expressed in bacteria as his-tagged proteins. The SH2 domains were purified by nickel affinity and size exclusion chromatography. All three SH2 domains had monomer and dimer peaks in size exclusion chromatography and these peaks had very high purity shown by Coomassie staining (Figure 12). Monomeric peak fractions of GADS and GRB2 were used for SPR. The dimeric peak of TSAd was used because TSAd appeared to dimerise in solution.

Proteins in the C8 and D1 peaks of GADS and GRB2 gel filtration respectively eluted at the expected volume of the monomeric protein and contained the recombinant proteins (Figure 12A and 12B). The C5 peak for GADS and GRB2 contained the SH2 domain as well and might be a dimer. Dimerisation of SH2 domains has been shown before, mainly for the SH2 domain of Fyn [163]. Size exclusion chromatography of TSAd resulted in several peaks (Figure 12C) which probably contained aggregated protein because the TSAd SH2 domain has been shown to contain more hydrophobic residues than other SH2 domains which reduce solubility [164]. Nevertheless, the 5E3-6A5 peak

contained TSAd SH2 that eluted at the expected volume for a molecular weight of about 15 kDa when compared to standards.

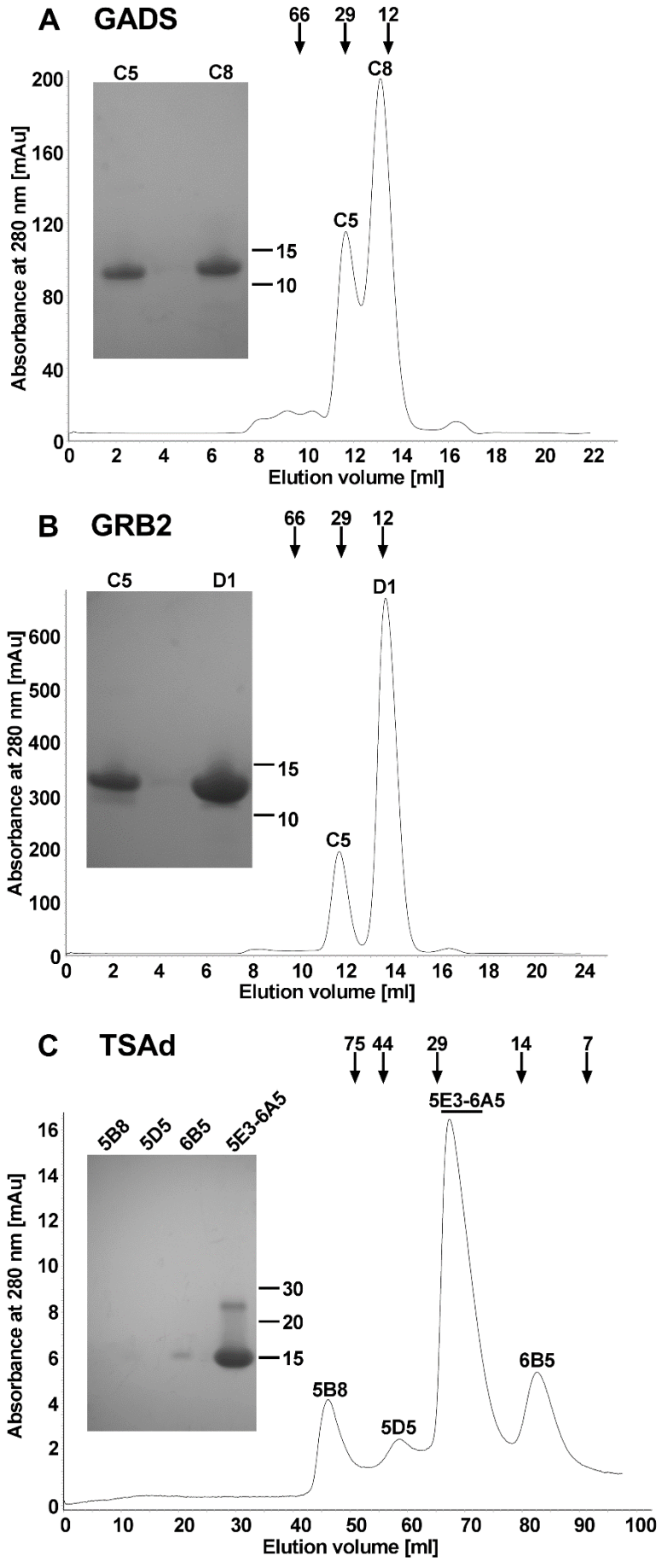

A GADS
66 29 12
C5 C8
C8
C5
15
10
Absorbance at 280 nm [mAu]
Elution volume [ml]
B GRB2
66 29 12
C5 D1
D1
C5
15
10
Absorbance at 280 nm [mAu]
Elution volume [ml]
C TSAd
75 44 29 14 7
5B8 5D5 6B5 5E3-6A5
5E3-6A5
6B5
5B8
5D5
30
20
15
Absorbance at 280 nm [mAu]
Elution volume [ml]

Figure 12. Size exclusion chromatography of SH2 domains of GADS (A), GRB2 (B) and TSAd (C). *Proteins were purified using a nickel column and then loaded onto a size exclusion chromatography column (Superdex 75 10/30 GL column for GADS SH2 and GRB2 SH2 and the Superdex 75 pg HiLoad 16/600 column for TSAd SH2). Standard protein weights in kDa are indicated at their elution volume. The GADS, GRB2 and TSAd SH2 domains have molecular weights of 12 kDa, 12 kDa and 13 kDa, respectively. Shown are the UV chromatograms for each protein and the Coomassie staining of peak fractions.*

In a BIAcore 3000 instrument, the SH2 domains were flowed over phosphorylated and unphosphorylated CD6 Y629 peptides and as positive control a phosphorylated CD28 pYMN peptide. The CD28 pYMN peptide contained a YXN motif and was shown to recruit GADS and GRB2 [146]. The experiments were conducted in cytoplasmic buffer at 37° C. Three experiments per protein were conducted using different protein preparations and BIAcore CM5 chips each time.

GADS SH2 bound to CD6 pY629 with an affinity range of 1-3 µM and to CD28 pYMN with a range of 2-3 µM which agrees with equilibrium dissociation constants measured by other groups [146] (Figure 13A). GRB2 SH2 bound to CD6 pY629 and CD28 pYMN with 2-4 µM and 3 µM, respectively, also similar to measurements in the literature (Figure 13B) [146]. TSAd SH2 bound with a lower affinity of 26-58 µM to CD6 pY629 and 74 µM to CD28 pYMN (single measurement) (Figure 13C). However, considering that TSAd SH2 tended to aggregate, the lower affinity may be due to lower protein activity. Binding to the

unphosphorylated CD6 Y629 residue was not observed for any of the SH2 domains.

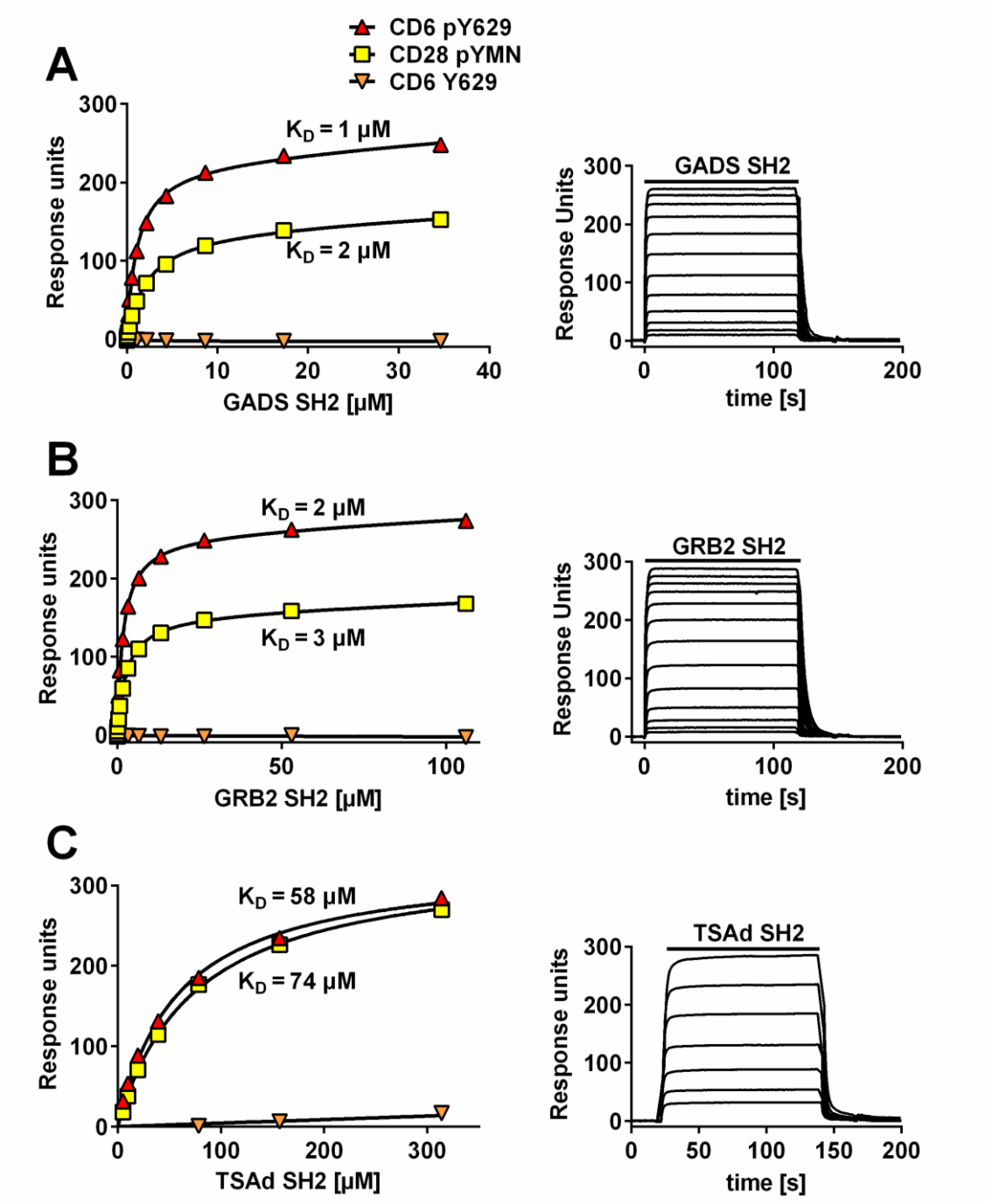


Figure 13. Equilibrium binding of GADS (A), GRB2 (B) and TSAd (C) SH2 to CD6 pY629. *The graphs on the left show equilibrium binding curves of proteins injected over CD6 Y629 and CD6 pY629 and a phosphorylated CD28 pYMN peptide. The graphs on the right show raw traces of serial dilutions of the SH2*

domains injected over CD6 pY629. Background signal of protein to an empty flow cell was subtracted for all results. Similar amounts of peptide were immobilised in each flow cell. GADS SH2 bound to CD6 pY629 with an affinity range of 1-3 µM and to CD28 pYMN with a range of 2-3 µM. GRB2 SH2 bound to CD6 pY629 and CD28 pYMN with 2-4 µM and 3 µM, respectively. TSAd SH2 bound with an affinity of 26-58 µM to CD6 pY629 and 74 µM to CD28 pYMN (single measurement). *All experiments were conducted at 37°* C in cytoplasmic buffer. *The results shown here are examples of one out of three experiments except for TSAd SH2 binding to CD28 pYMN which was only conducted once at 37° C.*

These SPR experiments show that the SH2 domains of GADS, GRB2 and TSAd directly bind to CD6 pY629 in a phosphorylation-dependent manner, confirming the pulldown data. However, these experiments do not show if any of these direct binding partners is the dominant binding partner of CD6 Y629 in cells or if all three proteins bind to CD6 equally.

3.2.1.3 GADS is the main interaction partner of CD6 Y629

Peptide pulldowns with tandem mass spectrometry analysis and SPR studies revealed that the phosphorylated CD6 Y629 residue can bind GADS, GRB2 and TSAd but does not provide information about the main interaction partner of CD6 Y629 in cells. Co-immunoprecipitation of CD6 from Jurkat 1G4 cells transduced with a CD6-EGFP fusion construct was performed to find the main interaction partners of the CD6 C-terminal tyrosine residues. Samples were stained for the presence of EGFP as evidence for the CD6-EGFP fusion protein,

the CD6 Y629 binding partners GADS, GRB2 and TSAd and the Y662 binding partner SLP-76 [79].

Immunoprecipitation of CD6 strongly enriched CD6. (Figure 14A). The two bands represent different degrees of glycosylation with the majority of CD6 being fully glycosylated [165]. Bands for all interaction partners can be seen in the whole cell lysate indicating that the proteins were present in the sample and can be detected by the antibody. SLP-76 was detected in the co-immunoprecipitation sample, showing that proteins can be co-precipitated (Figure 14A and 14B). Of the three potential CD6 Y629 interaction partners, only GADS showed a signal. This indicated that GADS is the main interaction partner of CD6 Y629 in Jurkat 1G4 cells.

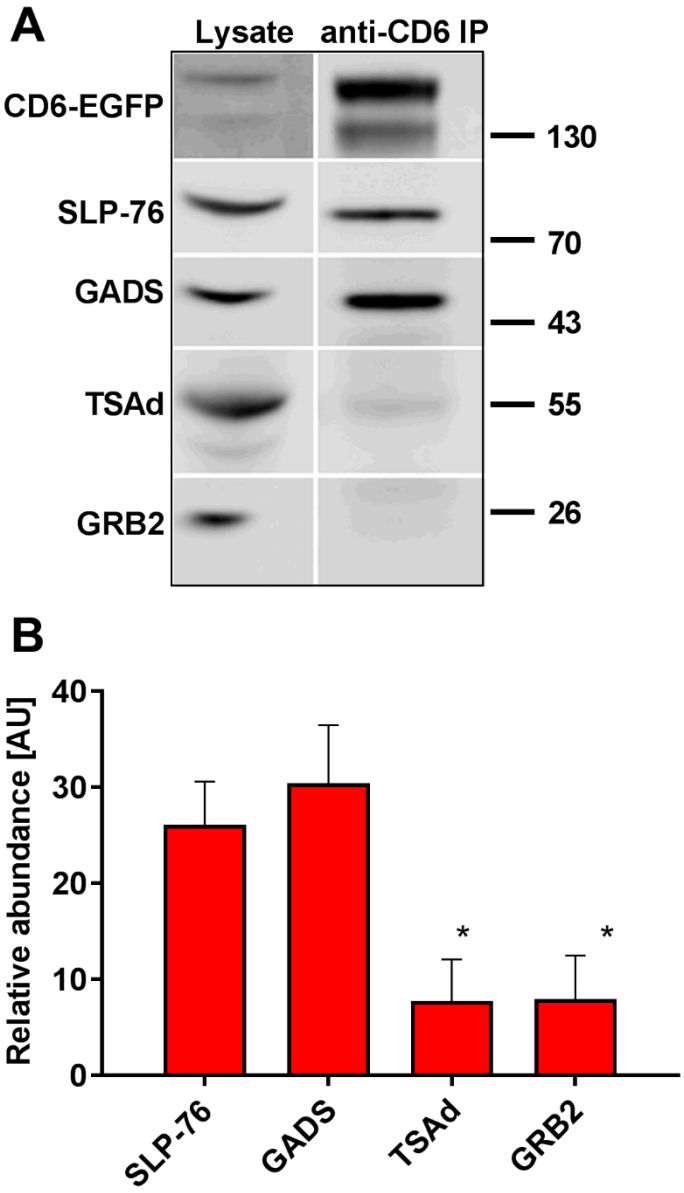


Figure 14. CD6 co-immunoprecipitation in Jurkat 1G4 cells reveal GADS as main binding partner of CD6 Y629. *(A) Lysate of 8x10^5 Jurkat 1G4 cells treated with the phosphatase inhibitor* Na_3VO_4 *or immunoprecipitation samples of 3x10^6 cells were loaded on gels and after electrophoretic separation they were blotted on nitrocellulose membranes. The membranes were probed with the indicated antibodies and visualised using LI-COR western blotting. (B) Western Blot bands of three independent CD6 co-immunoprecipitations were quantified*

*using the software ImageJ. Each co-immunoprecipitation value is divided by the intensity of the band in the lysate to compensate for differences in cell numbers used. Samples were analysed using the unpaired Student t test comparing values of mutants to the WT. n = 3, error bars show the standard error of the mean, * p<0.05.*

3.2.1.4 CD6 Y629 and Y662 cooperate in recruiting signalling molecules

To investigate the contribution of the individual tyrosine residues to binding, WT CD6 was compared to the CD6 Y629F mutant, the CD6 Y662F mutant and the double mutant CD6 Y629F Y662F. Co-immunoprecipitation samples were examined for the presence of CD6-EGFP and the CD6 binding partners SLP-76, GADS, GRB2 and TSAd.

Figure 15A shows an example of a CD6 co-immunoprecipitation experiment analysed with LI-COR western blots for EGFP and CD6 interaction partners and the densitometry of 3 independent co-immunoprecipitation experiments is shown in Figure 15B. Blotting for EGFP shows similar levels of CD6-EGFP in the lysates and a similar enrichment for the different CD6 constructs in the immunoprecipitation. SLP-76 and GADS were co-immunoprecipitated with WT CD6 but the co-immunoprecipitation of SLP-76 and GADS was strongly reduced with CD6 Y629F and CD6 Y662F mutants. Mutating CD6 Y629 and Y662 further reduced the signal and almost no SLP-76 and GADS was detected in the co-immunoprecipitation sample. The signals for TSAd and GRB2 were weak and not dependent on the CD6 Y629 or Y662

residues which could mean that they weakly bind to other residues or were a contamination in the immunoprecipitation.

This suggests that binding of GADS and SLP-76 to CD6 was dependent on both tyrosine residues. CD6 Y629 and Y662 alone can only recruit a small amount of GADS and SLP-76 and both residues are required for efficient recruitment of GADS and SLP-76.

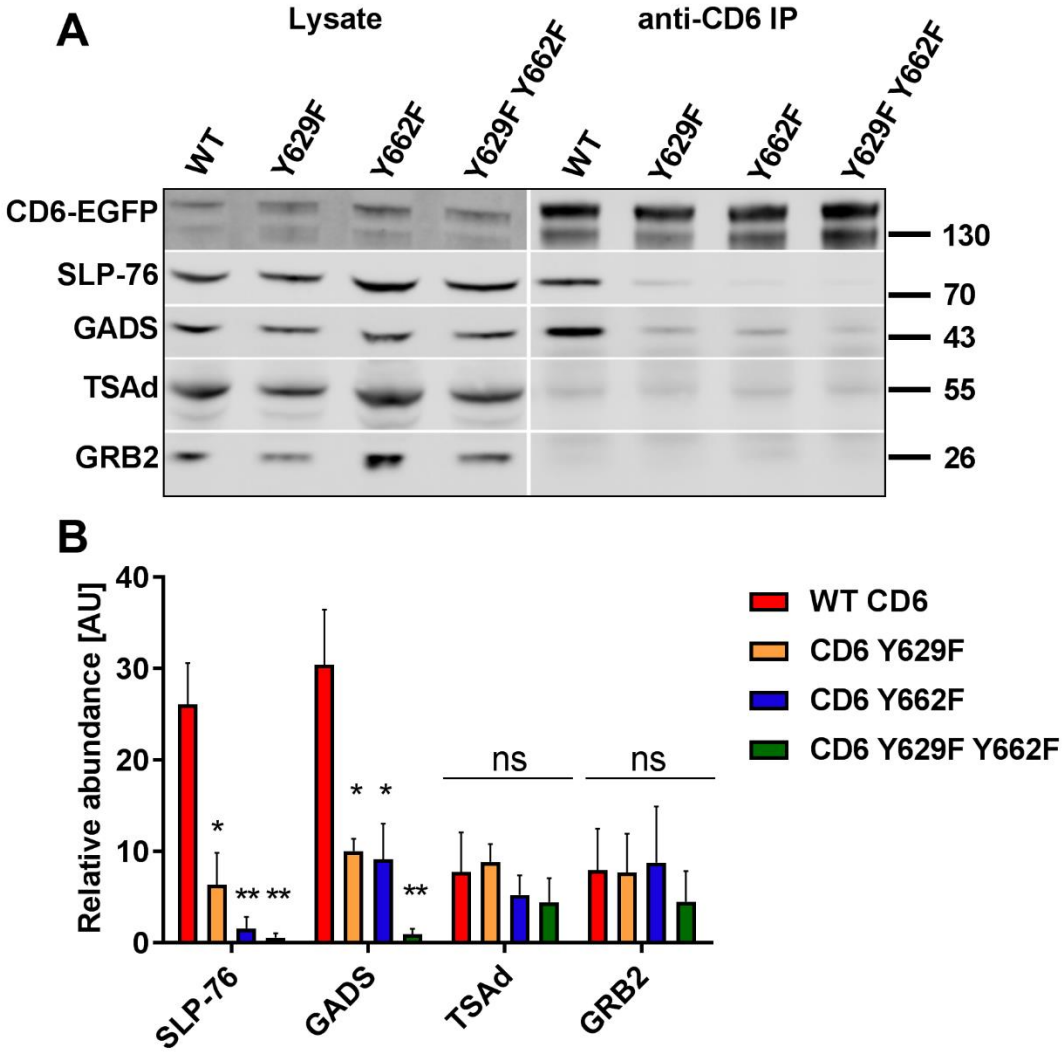


Figure 15. Co-immunoprecipitation of CD6 mutants shows a cooperation of CD6 Y629 and Y662. *(A) Lysate of 8x10^5 Jurkat 1G4 cells treated with the phosphatase inhibitor* Na_3VO_4 *or immunoprecipitation samples of 3x10^6 cells were loaded on gels and after electrophoretic separation they were blotted on nitrocellulose membranes. The membranes were probed with the indicated antibodies and visualised using LI-COR western blotting. (B) Western Blot bands of three independent CD6 co-immunoprecipitations were quantified using the software ImageJ. Each co-immunoprecipitation value is divided by the intensity of the band in the lysate to compensate for differences in cell numbers used and is normalised to the intensity of EGFP in the co-immunoprecipitation divided by the intensity of EGFP in the lysate to compensate for different expression levels of the constructs. Samples were analysed using the unpaired Student t test comparing values of mutants to the WT. n = 3, error bars show the standard error of the mean, * p<0.05, ** p<0.01, ns = not significant.*

It is possible that the GADS and SLP-76 SH2 domains can bind to both tyrosine residues equally and thus mutating a single tyrosine affects binding of both proteins.

Another possible explanation is that there is cooperation between CD6 Y629 and Y662. The C-terminal GADS SH3 domain binds to the SLP-76 proline-rich region with an affinity of 0.1 µM at 25° C and in cells these proteins are often seen as a complex [108,109]. The dependence of GADS and SLP-76 on both residues for binding to CD6 could be explained by bivalent binding of the GADS/SLP-76 complex to CD6 pY629 and CD6 pY662. Mutating individual

tyrosine residues significantly reduces the affinity of the GADS/SLP-76 complex for CD6 and mutating both residues abolishes binding of the GADS/SLP-76 complex.

3.2.1.5 GADS SH2 and SLP-76 SH2 specifically bind to CD6 pY629 and pY662

To understand how GADS and SLP-76 are recruited to the CD6 C terminus, it is essential to determine the specificity of the GADS and SLP-76 SH2 domains to CD6 Y629 and Y662, respectively. The recombinant GADS SH2 and SLP-76 SH2 domains were expressed in bacteria as his tagged proteins and purified according to their nickel affinity and size. On a streptavidin-coated CM5 chip, biotinylated CD6 peptides containing the Y629 and the Y662 residues with either tyrosine or both residues phosphorylated were immobilised. In a BIAcore 3000, equilibrium binding constants of the SH2 domains were measured at 15° C and 37° C. 37° C was chosen to measure specificity at a physiological temperature and 15° C was chosen to determine if cross-specificity is revealed at lower temperatures.

Table 8. K_D for GADS SH2 and SLP-76 SH2 for pY629 and pY662

	CD6 peptide	GADS SH2	SLP-76 SH2
15° C	**pY629 Y662**	0.15-0.62*	-
	Y629 pY662	-	0.31-1.87
	pY629 pY662	0.14-2.05	0.26-1.82
37° C	**pY629 Y662**	0.60-0.88	-
	Y629 pY662	-	0.89-3.09
	pY629 pY662	0.77-1.58	0.62-2.82

*K_D given in µM

The GADS SH2 domain bound to the CD6 pY629 Y662 and the CD6 pY629 pY662 peptide with similar affinities but it did not bind to the CD6 Y629 pY662 peptide at 15° C and 37° C (Table 8 and Figure 16A and 16B). The affinities measured for GADS SH2 were higher than the affinities measured in Figure 13 which is likely to be due to the longer peptides used in this experiment.

The SLP-76 SH2 domain bound to the CD6 Y629 pY662 and the CD6 pY629 pY662 peptide with similar affinities but it did not bind to the CD6 pY629 Y662 peptide at 15° C and 37° C (Table 8 and Figure 17A and 17B). Differences in the maximal response units between the CD6 Y629 pY662 and CD6 pY629 pY662 peptides can be explained by different amounts of peptide immobilised on the CM5 chip. The equilibrium dissociation constants measured for the SLP-76 SH2 domain for CD6 pY662 are similar to published measurements [79].

These results prove that the GADS SH2 domain specifically binds to CD6 pY629 and that the SLP-76 SH2 domain specifically binds to CD6 pY662 at physiological temperatures and also at 15° C.

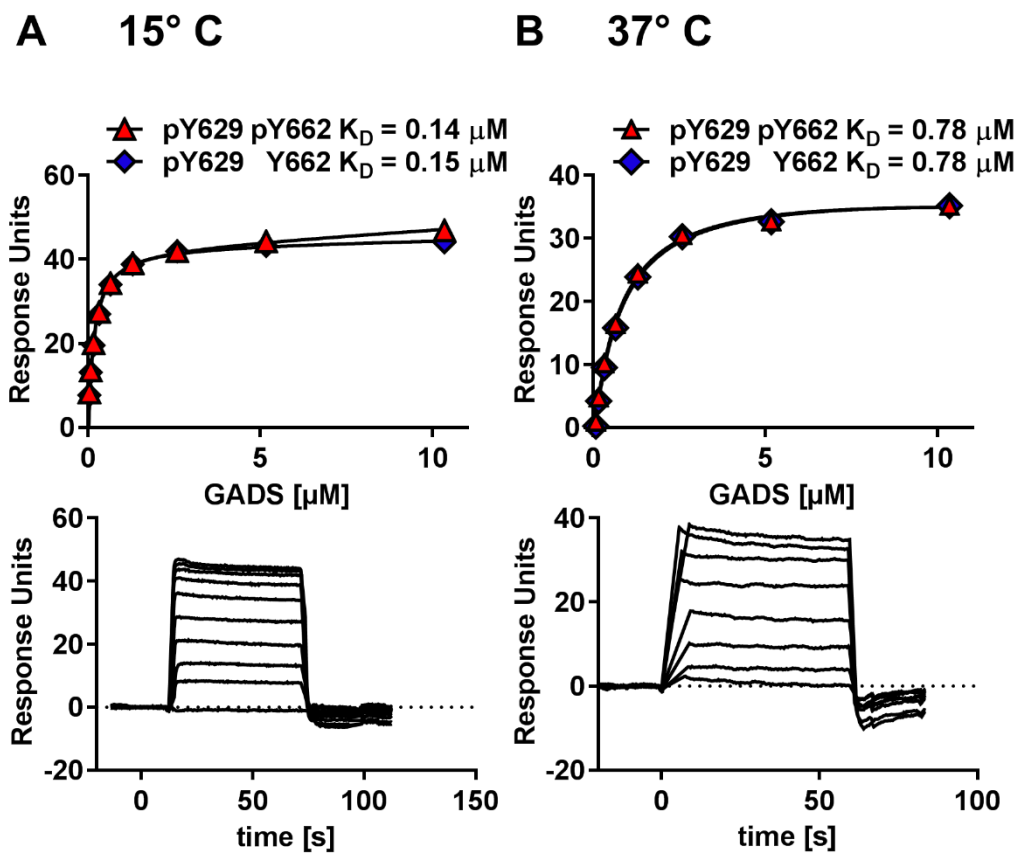


Figure 16. GADS specifically binds to CD6 pY629. *Serial dilutions of GADS SH2 were injected over CD6 peptides with phosphorylation of Y629, Y662 or both residues. Equilibrium binding measurements (top) were conducted at 15° C (A) and 37° C (B) in cytoplasmic buffer and raw traces are shown for binding to the CD6 pY629 Y662 single phosphorylation peptide (bottom). Background signal of protein to an empty flow cell was subtracted for all results. Binding to CD6 Y629 pY662 did not produce any signal and therefore is not shown. Results from one out of three independent experiments are shown.*

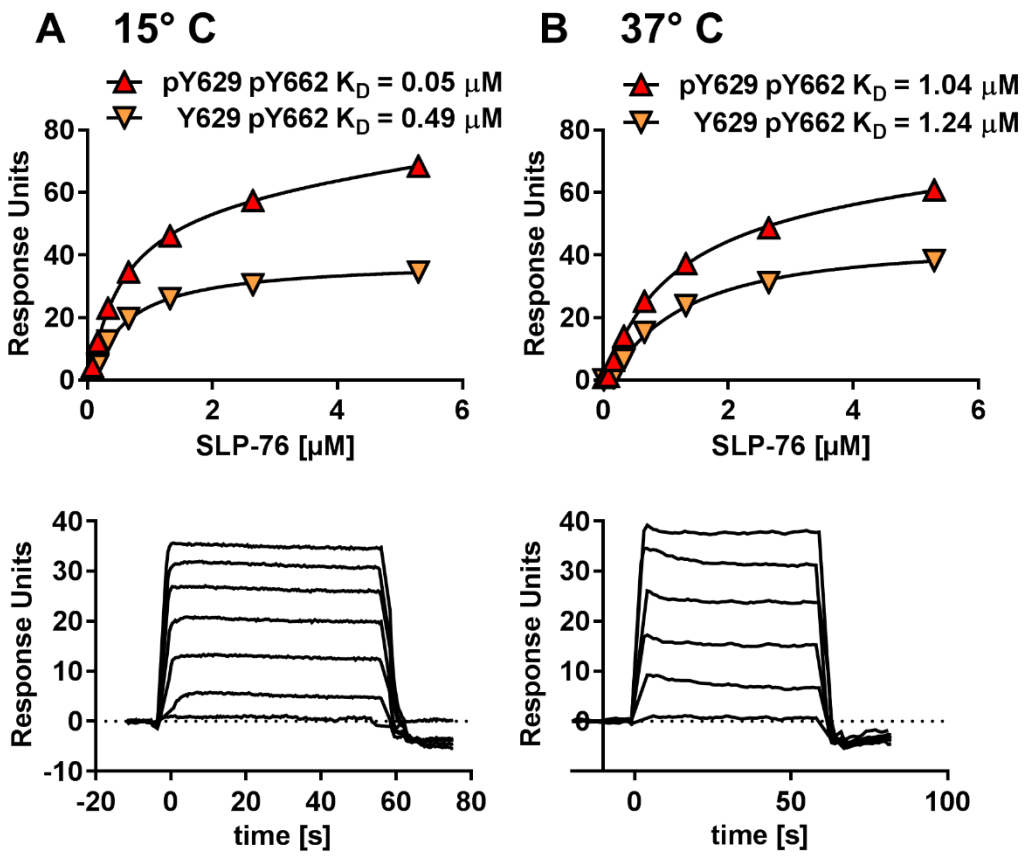


Figure 17. SLP-76 specifically binds to CD6 pY662. *Serial dilutions of SLP-76 SH2 were injected over CD6 peptides with phosphorylation of Y629, Y662 or both residues. Equilibrium binding measurements (top) were conducted at 15° C (A) and 37° C (B) in cytoplasmic buffer and raw traces are shown for binding to the CD6 Y629 pY662 single phosphorylation peptide (bottom). Background signal of protein to an empty flow cell was subtracted for all results. Binding to CD6 pY629 Y662 did not produce any signal and therefore is not shown. Results from one out of three independent experiments are shown.*

3.2.2 Recruitment of the GADS/SLP-76 complex has a costimulatory effect on Jurkat 1G4 cells

3.2.2.1 CD6 Y629F and Y662F transduced Jurkat cells express similar levels of TCR, CD3 and CD6

Jurkat 1G4 cells transduced with different Y629 and Y662 mutant CD6-EGFP constructs were analysed for TCR, CD3 and CD6 surface expression and EGFP signal. There was no difference for any of the examined receptors between the constructs, indicating that if these cells are used for functional experiments, an influence of different CD3, TCR or CD6 expression levels on Jurkat 1G4 cell activation can be excluded (Figure 18A). Comparing EGFP and CD6 surface staining signals with dot plots showed a correlation of these two signals indicating that most of the CD6-EGFP reached the cell surface (Figure 18B).

The phenotyping of the transduced Jurkat 1G4 cells suggested that all produced cell lines had similar capabilities to respond to CD3 and CD6 antibody stimulation due to the same expression levels of these receptors. Therefore, all measured T cell activation can be attributed to mutations in CD6.

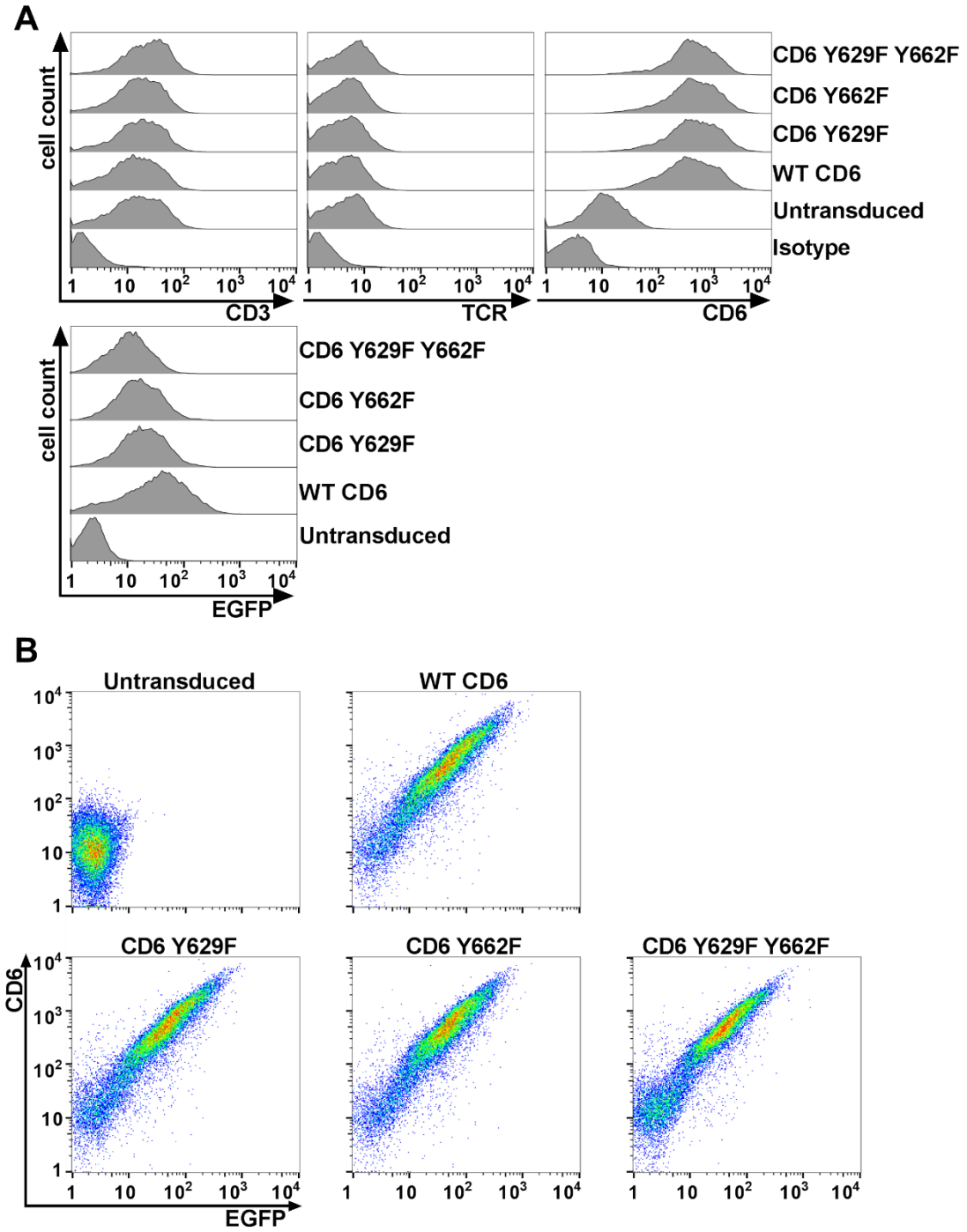


Figure 18. Similar expression levels of CD3, TCR and CD6-EGFP on Jurkat 1G4 cells transduced with WT CD6, CD6 Y629F, CD6 Y662F and CD6 Y629F Y662F. *(A) WT or mutant CD6 was stained for CD3, TCR or CD6 and compared to an isotype control or analysed for CD6-EGFP expression shown as histograms. Transduced cells were gated on EGFP-positive cells. (B) Surface*

CD6 was stained and compared to the EGFP signal of transduced CD6. The isotype in (A) and the untransduced and WT samples in (A) and (B) are the same as in Figure 8 because all Jurkat 1G4 cell phenotyping was conducted together.

3.2.2.2 CD6 Y629 and Y662 are required for CD6 costimulation

Cells were transduced with WT, Y629F, Y662F and Y629F Y662F CD6-EGFP to prevent binding of GADS, SLP-76 and a complex of both proteins, respectively. Jurkat 1G4 cells were stimulated and analysed as described in section 3.1.2.3. Stimulated Jurkat 1G4 cells upregulated CD69 in a mutant CD6-dependent manner (Figure 19). Transducing cells with WT CD6 but not the CD6 Y629F, CD6 Y662F or CD6 Y629F Y662F mutants resulted in a significant increase of CD69 expression in response to CD3 antibodies in the absence of CD6 antibodies (Figure 20A). Because costimulation required CD3 antibodies, it is possible that the activating function of CD6 in the absence of ligand engagement represents a function of CD6 as a signalling molecule downstream of the TCR instead of a function as a receptor. This has been suggested by Malissen and colleagues who have proposed that CD6 can function as a scaffold protein which recruits several signalling molecules important for TCR signalling in the absence of the ligand [106].

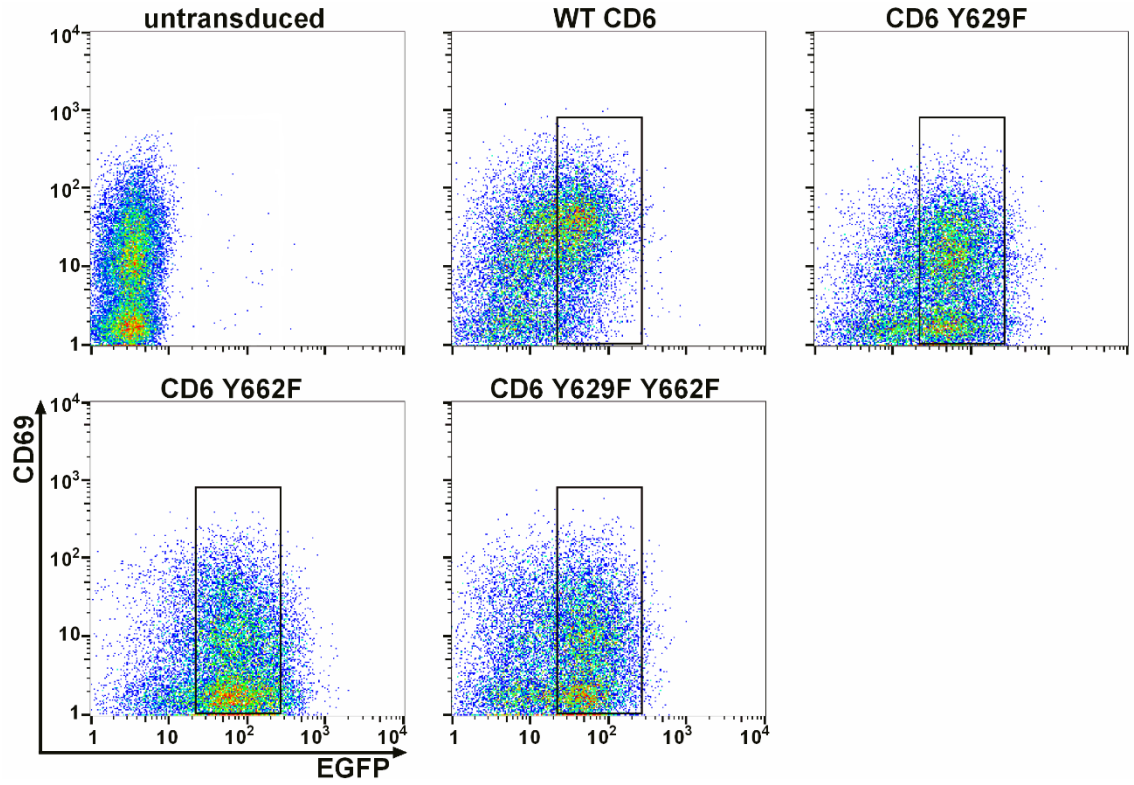


Figure 19. Example dot plots of activated Jurkat 1G4 cells. *Jurkat 1G4 cells were stimulated with 2 µg/ml CD3 antibody for 18 h and 2.5 µg/ml CD6 antibody T12.1 and stained for CD69 expression. Shown are example dot plots showing the CD69 signal versus the CD6-EGFP signal for untransduced Jurkat 1G4 cells and Jurkat 1G4 cells transduced with WT CD6, CD6 Y629F, CD6 Y662F and CD6 Y629F Y662F. CD69 expression was examined for all untransduced cells and for transduced cells in the shown gate. FSC-SSC gating for Jurkat 1G4 cells is shown in Figure 10.*

This response was further upregulated when cells were also stimulated with CD6 antibodies (Figure 20B). When CD6 was crosslinked by antibodies, mutating the GADS binding site CD6 Y629 resulted in a reduction of CD69 expression. Mutating the SLP-76 binding site CD6 Y662 led to lower T cell

activation compared to the CD6 Y629F mutation. This response was further reduced when CD6 Y629 and Y662 are mutated. In fact, the CD6 Y629F Y662F construct did not provide any CD6-mediated costimulation.

Remarkably, mutating CD6 Y629 and Y662 significantly inhibited the response of Jurkat 1G4 cells stimulated with CD3 antibodies but not CD6 antibodies indicating that CD6 can mediate inhibitory signalling which is counteracted by signalling through CD6 Y629 and Y662 (Figure 20A).

From this experiment it can be concluded that the GADS/SLP-76 recruitment to CD6 was crucial for costimulation by CD6. The single mutants showed that recruitment of SLP-76 to CD6 had a stronger effect on T cell activation than the recruitment of GADS. This is in line with the evidence that GADS knockout has a milder phenotype than the SLP-76 knockout [166]. The main role of GADS appears to be the recruitment of SLP-76 to the scaffold protein LAT because GADS itself has no known additional downstream signalling pathways [110]. When SLP-76 binds to CD6, GADS may just strengthen the interaction to ensure stable recruitment of SLP-76 to CD6. SLP-76 can signal through VAV-1 and PLCγ1 which activate several downstream pathways, explaining the lower T cell activation when SLP-76 does not bind to CD6 [110].

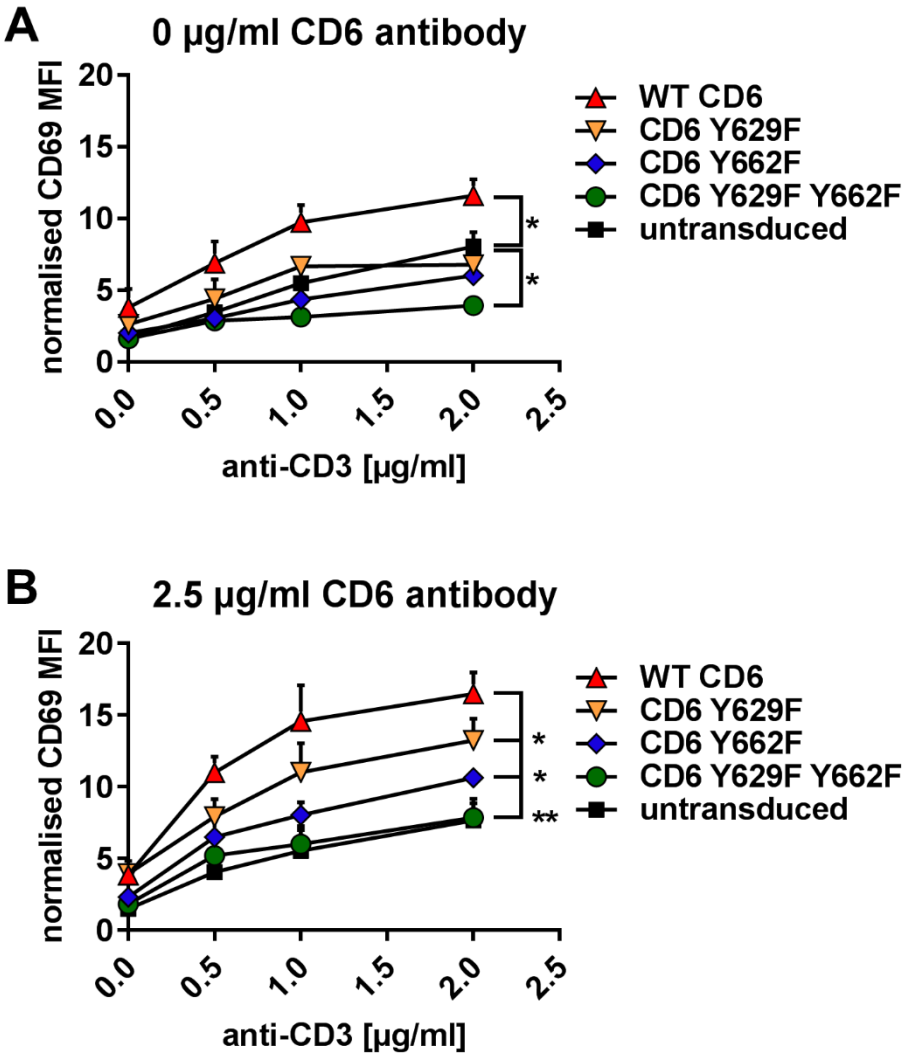


Figure 20. Role of CD6 Y629 and Y662 in Jurkat 1G4 activation after CD3 and CD6 antibody stimulation. *(A) Jurkat 1G4 cells were stimulated with CD3 antibodies for 18 h and the CD69 MFI was examined for WT CD6, CD6 Y629F, CD6 Y662F or CD6 Y629F Y662F transduced cells or untransduced cells. Statistics are shown for comparisons of WT or mutant CD6 transduced cells with untransduced cells (B) Same as in A but cells were also treated with 2.5 µg/ml CD6 antibody T12.1. CD69 MFI for WT CD6 was significantly higher ($p<0.01$) than for untransduced cells for any CD3 concentration. Statistics are shown for comparisons of mutant CD6 transduced cells with WT CD6 transduced cells.*

*n = 3, error bars show the standard error of the mean, * p<0.05, ** p<0.01 student's t test for each CD3 antibody concentration above 0 µg/ml.*

3.2.3 Recruitment of the GADS/SLP-76 complex has a costimulatory effect on primary T cells

3.2.3.1 The rat domain 1 human CD6 chimera can be used in functional experiments

A main challenge of investigating the function of CD6 mutants in primary T cells is the high expression of endogenous CD6 compared to the CD6 expression by Jurkat cells. Experiments that use ligand engagement or antibody crosslinking would also activate endogenous CD6 which masks the signal of the transduced CD6. Knockdown or knockout of endogenous CD6 is a possible solution but the most efficient methods reducing endogenous CD6 would also target transduced CD6 because they target the coding region of CD6.

Specific antibody crosslinking of transduced CD6 would allow experiments without the influence of endogenous CD6. The chosen method to specifically crosslink transduced CD6 was the exchange of human SRCR domain 1 with rat domain 1 and crosslinking with the rat domain 1-specific antibody OX52 [152]. Human CD6 possesses a HindIII restriction site in SRCR domain 2 which was used for the exchange resulting in SRCR domain 1 and a part of domain 2 being rat and the rest of the CD6 construct being human (termed rd1 hCD6).

To test whether the rat domain 1 antibody OX52 can crosslink this chimeric CD6 protein, the rd1 hCD6 extracellular region was fused to the CD3 ζ cytoplasmic region which provides strong signalling for T cell activation due to its three ITAMs [167]. The 2B4 Saito T cell hybridoma line was chosen to test the

chimeric CD6 construct [151]. OX52 detected rd1 hCD6 but did not bind to human CD6 domain 1 (Figure 21).

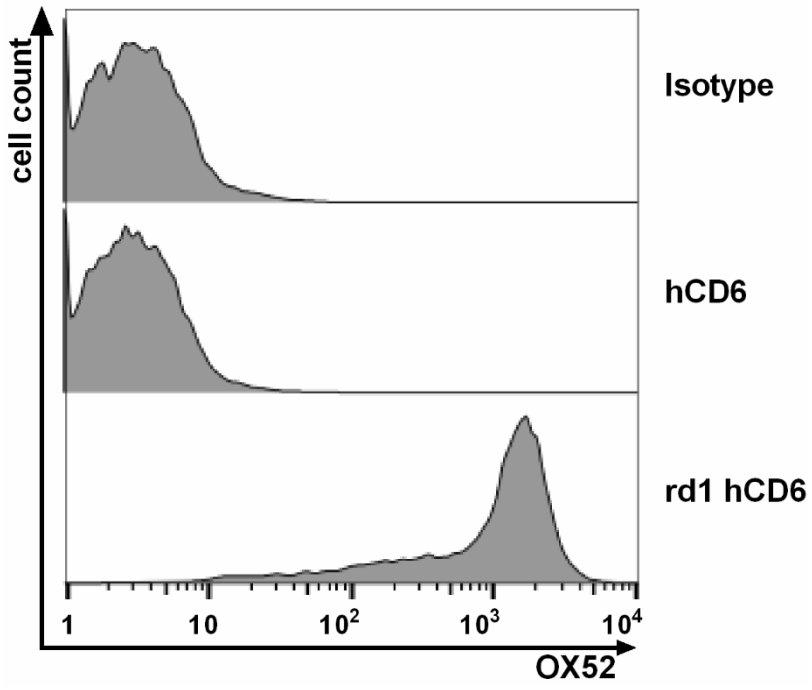


Figure 21. Specificity of OX52 for rat CD6 domain 1. *2B4 Saito hybridoma cells expressing human CD6 or rd1 hCD6 ζ chimera were stained with OX52 and compared to 2B4 Saito cells stained with a mouse IgG2a isotype.*

To find out whether OX52 can crosslink rd1 hCD6 to trigger signalling, 2B4 Saito cells were incubated with plate-bound mouse IgG2a isotype or OX52 for 18 h and IL-2 production was measured by ELISA. OX52 did not trigger IL-2 production by 2B4 Saito cells expressing human CD6 (Figure 22). 2B4 Saito cells expressing rd1 hCD6 produced IL-2 in response to OX52 in an antibody concentration-dependent manner.

These results suggest that using rd1 hCD6 together with OX52 is a suitable system to test the function of CD6 mutants in primary T cells because this recombinant protein can be specifically crosslinked by OX52.

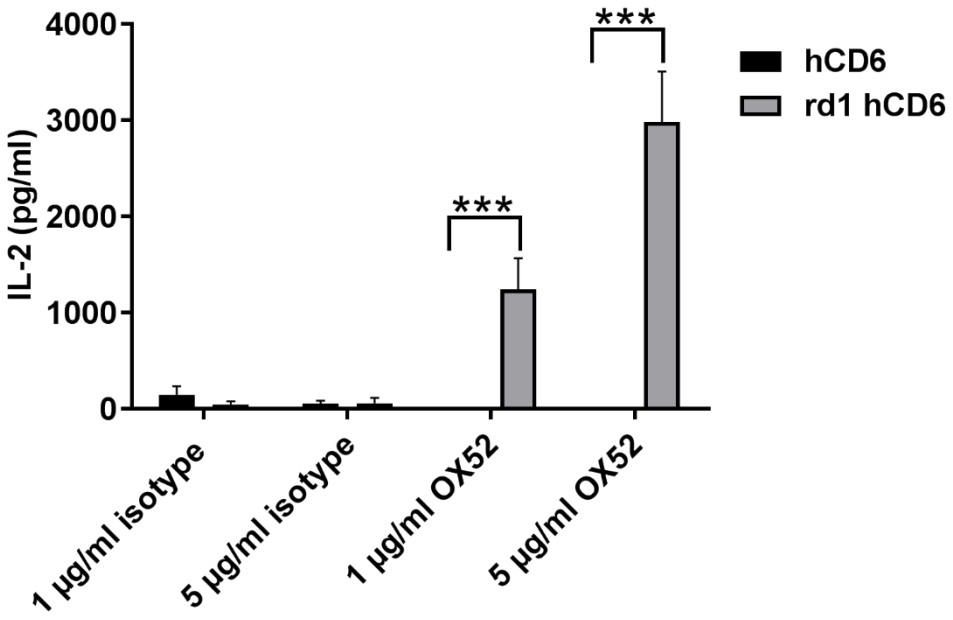


Figure 22. IL-2 production induced by crosslinking with OX52. *2B4 Saito cells transduced with human CD6 or rd1 hCD6 ζ chimera were stimulation with plate-bound 1 µg/ml or 5 µg/ml mouse IgG2a isotype or OX52 for 18 h. Mouse IL-2 production was measured by ELISA. n=3, error bars show the standard error of the mean, *** p<0.001 student's t test comparing hybridoma cells expressing human CD6 or rd1 hCD6.*

3.2.3.2 CD6 mutants are expressed on primary T cells

Jurkat cells are a good model to examine T cell signalling because most of the TCR signalling machinery is present in Jurkat cells [168]. However,

changes in signalling molecule expression levels may lead to different functional outcomes [169], highlighting the necessity to study T cell activation in primary T cells. Therefore, CD4+ T cells were isolated from individuals, activated with beads coated with CD3 and CD28 antibodies and transduced with rd1 hCD6 WT or rd1 hCD6 Y629F Y662F. Figure 23 shows CD3 and CD6 expression levels of donor 1 but all 3 donors used in functional experiments had similar transduction efficiencies and expression levels. Both constructs were transduced into CD4+ T cells with an efficiency of 40-55 % and with similar levels of expression (Figure 23A). The OX124 antibody binds to human CD6 domain 3 and therefore binds to endogenous CD6 and transduced rd1 hCD6. This means OX124 can be used to estimate the ratio of transduced CD6 and endogenous CD6. Comparing the OX124 signal indicates that transduced rd1 hCD6 was about 40-50 times more abundant than endogenous CD6 (Figure 23A). Transduction of these constructs did not alter the levels of CD3 expression. The constructs were expressed on the CD4+ T cell surface and did not accumulate inside (Figure 23B). Due to similar rd1 hCD6 expression levels, these CD4+ T cells can be used for functional experiments to examine the effect of CD6 mutations on primary T cell activation.

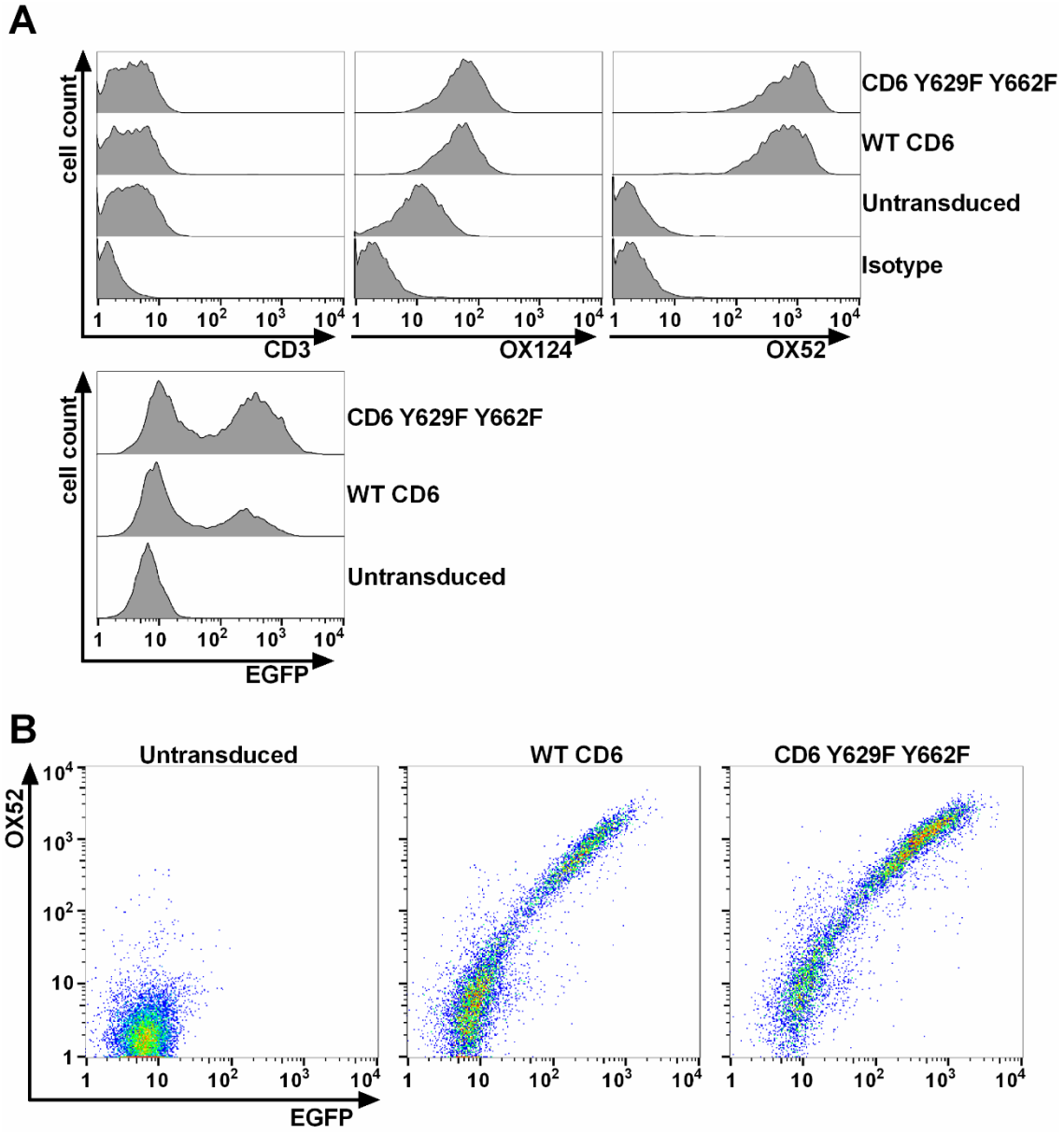


Figure 23. Similar expression levels of CD3, total CD6 and rd1 hCD6-EGFP on untransduced primary CD4+ T cells or cells transduced with WT rd1 hCD6 or rd1 hCD6 Y629F Y662F. *(A) Untransduced or WT or mutant rd1 hCD6 transduced cells were stained for CD3, total CD6 with OX124 and rd1 hCD6 alone with OX52 and compared to an isotype control or analysed for rd1 hCD6-EGFP expression shown as histograms. Transduced cells were gated on EGFP-positive cells. (B) Surface rd1 hCD6 was stained with OX52 and compared to the EGFP signal of transduced rd1 hCD6.*

3.2.3.3 CD69 is a suitable readout for primary T cell activation

CD4+ T cells were stimulated as described in section 3.1.2.3 but instead of crosslinking human CD6, rat CD6 domain 1 was crosslinked using the CD6 antibody OX52. Unstimulated primary T cells did not express CD69 but expression was induced after activation and expression levels were similar independent of genetic modifications (Figure 24). CD69 positive cells can be identified as a separate population and therefore, assessing the percentage of CD69 expressing cells rather than the expression level was chosen as readout for primary T cells.

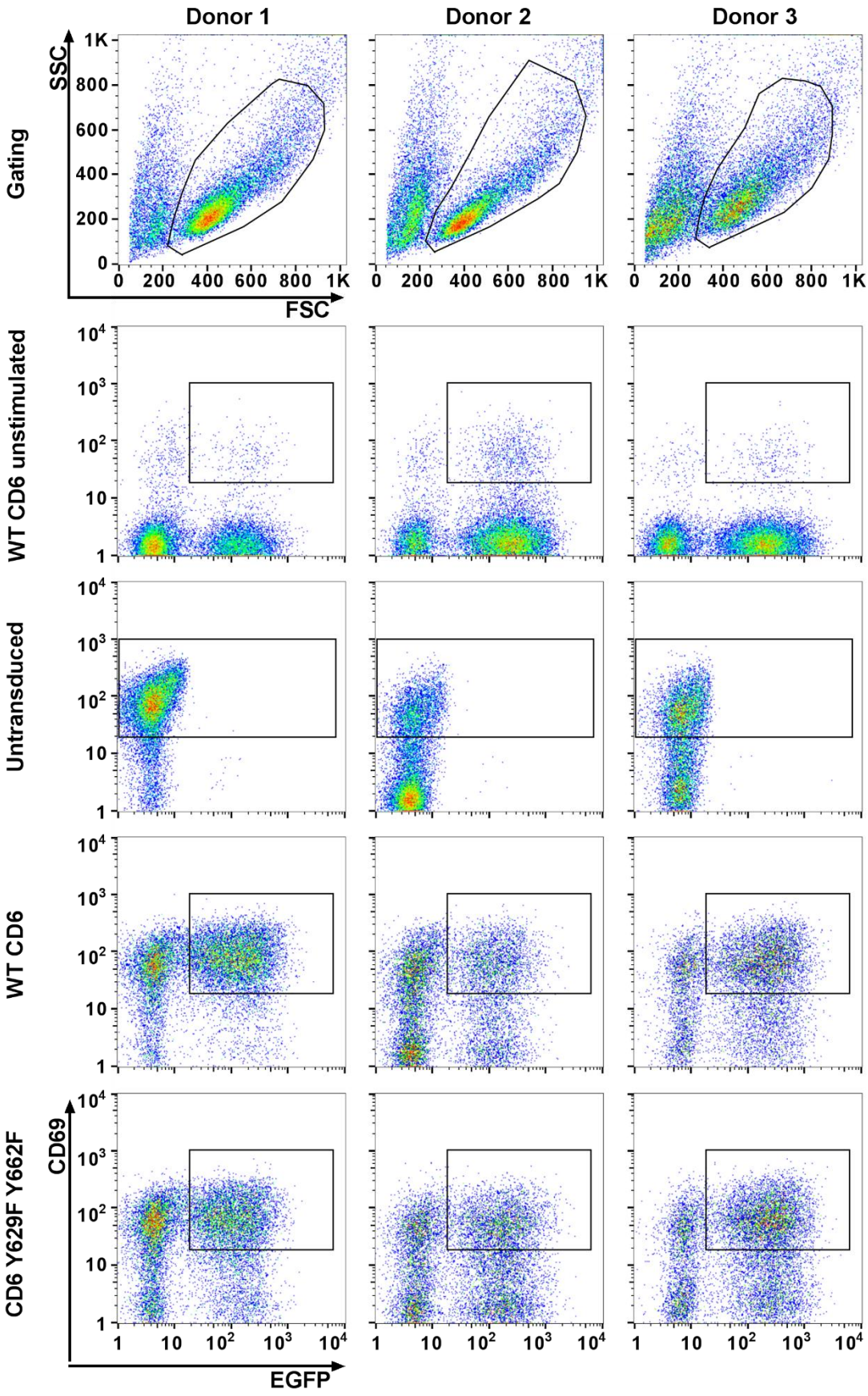

Donor 1
Donor 2
Donor 3
Gating
SSC
FSC
WT CD6 unstimulated
Untransduced
WT CD6
CD6 Y629F Y662F
CD69
EGFP

Figure 24. Example EGFP-CD69 dot plots of activated primary T cells. *The FSC-SSC gating strategy is shown in the top row. Example dot plots for untransduced cells or WT rd1 hCD6 or rd1 hCD6 Y629F Y662F transduced cells are shown for all three donors. Cells were stimulated with 5 µg/ml OX52 and 2 µg/ml CD3 antibody, unstimulated WT CD6 transduced cells are shown as well. The gate for untransduced cells shows that the percentage of all CD69 positive cells were considered to be activated T cells. Transduced cells were gated on EGFP expression and the percentage of CD69 positive cells was assessed within this gate.*

3.2.3.4 Y629 and Y662 mutant CD6 is inhibitory in primary T cells

CD4+ T cells were stimulated as described in section 3.2.3.3 with CD3 antibodies but not CD6 antibodies. Under these conditions, only donor 2 showed a significant increase in the percentage of CD69 positive cells when transduced with WT rd1 hCD6 (Figure 25). Donor 2 had overall lower numbers of cells responding to stimulation compared to the other two donors, indicative of a weaker activation. This suggests that the weaker activation was required to reveal the activating effect of WT rd1 hCD6 in the absence of CD6 crosslinking.

T cells transduced with rd1 hCD6 Y629F Y662F showed a significant reduction of T cell activation for donor 2 at high CD3 antibody concentrations and for donor 3 at several CD3 antibody concentrations (Figure 25).

These results suggest that CD6 can induce inhibitory signalling in the absence of ligand engagement but this inhibitory function is masked by activating signalling. Under certain circumstances, inhibitory signalling may dominate in WT

CD6. The inhibitory effect of CD6 seen in primary cells confirms data obtained with Jurkat 1G4 cells (section 3.2.2.2).

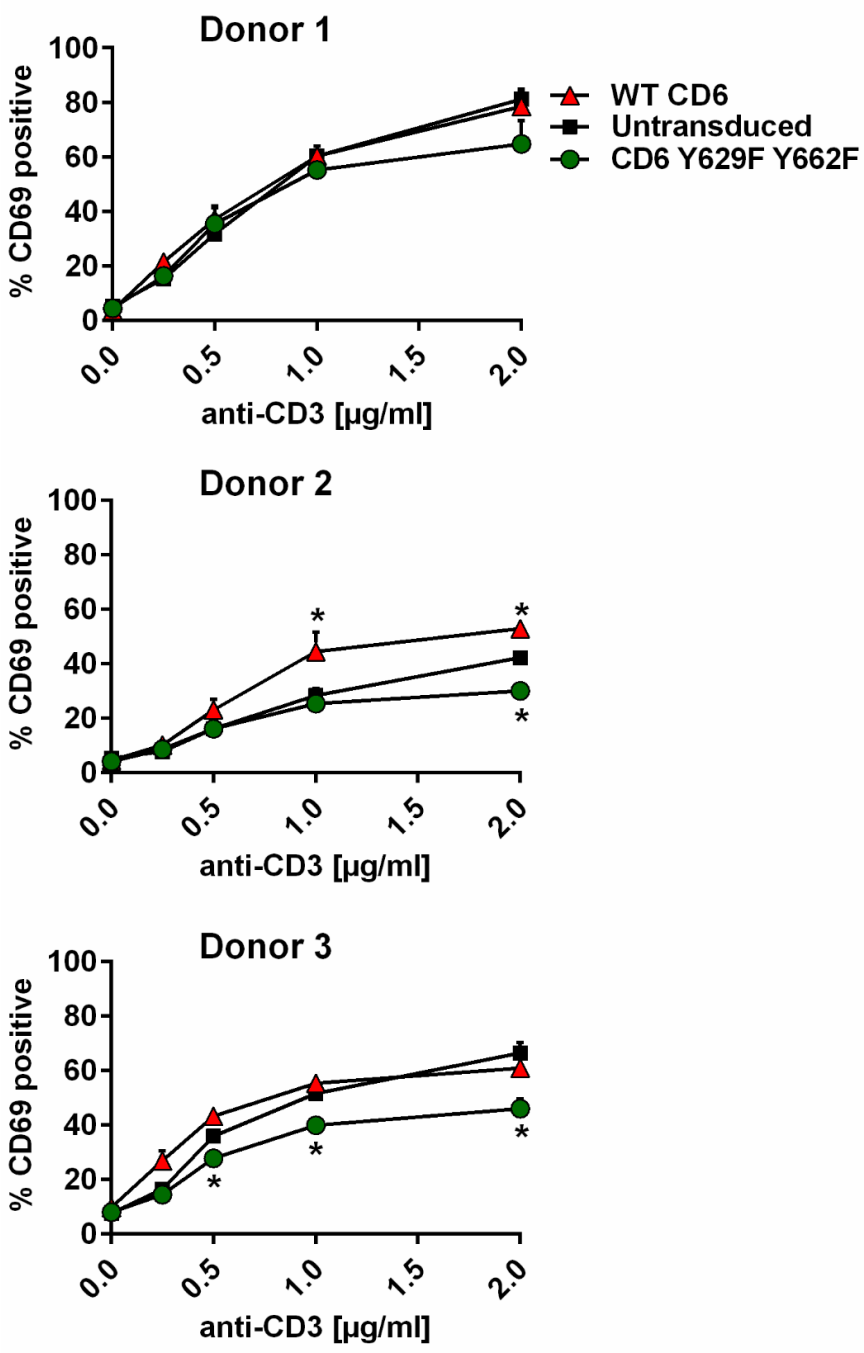


Figure 25. Mutating Y629 and Y662 in CD6 revealed an inhibitory function of CD6 in primary T cells. *T cells were stimulated with plate-bound CD3 antibodies for 18 h and assessed for the percentage of CD69 positive cells. Statistics are shown for comparisons of WT or mutant rd1 hCD6 transduced cells*

*with untransduced cells at the indicated CD3 antibody concentration using the student's t test. n = 3, error bars show the standard error of the mean, * p<0.05.*

3.2.3.5 CD6 costimulation depends on the residues Y629 and Y662 in primary T cells

CD4+ T cells were stimulated as described in section 3.2.3.3 with CD3 antibodies and 5 µg/ml OX52 to crosslink transduced rd1 hCD6. WT rd1 hCD6 transduced T cells of all three donors had a significantly increased percentage of CD69 positive cells, confirming the activating effect of crosslinking CD6 together with crosslinking CD3 seen in Jurkat 1G4 cells (Figure 26). The costimulatory function of WT CD6 was seen with different CD3 antibody concentrations but not in the absence of CD3 antibodies, suggesting that CD6 ligation alone cannot activate T cells (Figure 26).

Mutating the rd1 hCD6 tyrosine residues Y629 and Y662 significantly reduced the T cell response compared to WT rd1 hCD6 transduced T cells (Figure 26). In fact, there was no significant difference between untransduced cells and rd1 hCD6 Y629F Y662F transduced cells. The difference between WT rd1 hCD6 and Y629F Y662F rd1 hCD6 or untransduced cells was increased at 0.25 µg/ml CD3 antibody for donor 1 and 3 compared to higher CD3 antibody concentrations, suggesting that at weaker TCR stimulation, CD6 has a stronger costimulatory effect. These results indicate that the CD6 Y629 and Y662 residues are essential for CD6-mediated costimulation.

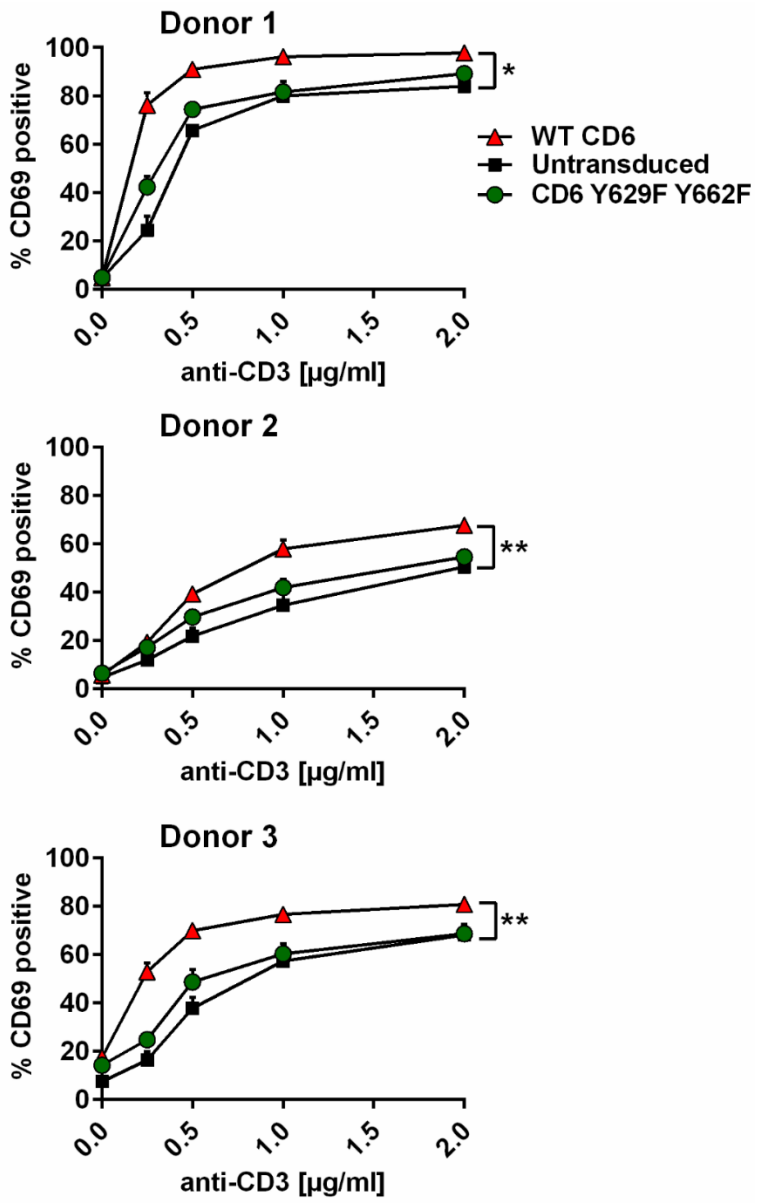


Figure 26. The activating effect of CD6 in primary T cells assessed by CD69 expression is mediated by the CD6 Y629 and Y662 residues. *T cells were stimulated with plate-bound CD3 antibodies and 5 µg/ml OX52 for 18 h and assessed for the percentage of CD69 positive cells. Statistics are shown for comparisons of WT rd1 hCD6 transduced cells with untransduced cells and rd1 hCD6 Y629F Y662F transduced cells at the CD3 antibody concentrations 0.5, 1 and 2 µg/ml using the student's t test. n = 3, error bars show the standard error of the mean, * p<0.05, ** p<0.01 for all indicated CD3 antibody concentrations.*

3.2.3.6 The CD6 Y629 and Y662 residues affect IL-2 production in primary T cells

CD69 is a common marker for T cell activation but CD6 stimulation may also have an effect on T cell activation that is more downstream of CD69 surface expression. The production of IL-2 was chosen as an additional marker of T cell activation because IL-2 production is induced during T cell activation and IL-2 production has direct functional consequences on the immune system [170]. In addition, IL-2 production has been shown to be affected by CD6 signalling [79]. The concentration of secreted IL-2 would be affected by stimulation of untransduced cells which may mask the effects seen on transduced T cells. To specifically measure IL-2 in transduced cells, secretion was blocked by Brefeldin A and the level of intracellular IL-2 in single cells was determined by flow cytometry.

Even after stimulation, many cells remained IL-2 negative but a higher percentage of cells became IL-2 positive compared to unstimulated cells (Figure 27). Therefore, assessing the percentage of IL-2 positive cells, similarly to CD69, was chosen as functional readout.

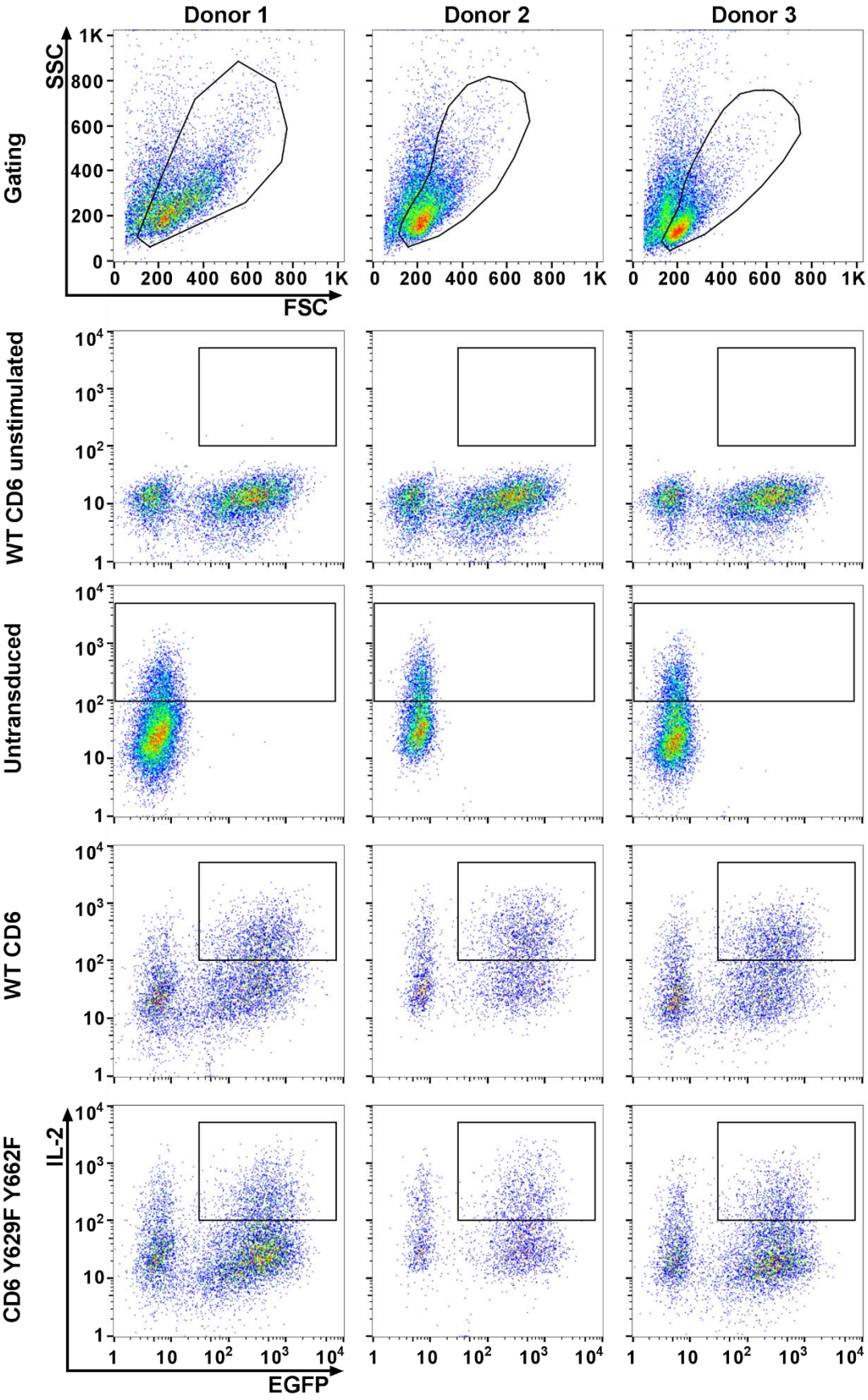

Donor 1
Donor 2
Donor 3
Gating
SSC
FSC
WT CD6 unstimulated
Untransduced
WT CD6
CD6 Y629F Y662F
IL-2
EGFP

Figure 27. Example EGFP-IL-2 dot plots of activated primary T cells. *The FSC-SSC gating strategy is shown in the top row. Example dot plots for untransduced cells or WT rd1 hCD6 or rd1 hCD6 Y629F Y662F transduced cells are shown for all three donors. Cells were stimulated with 5 µg/ml OX52 and 2 µg/ml CD3 antibody, unstimulated WT CD6 transduced cells are shown as well. The gate for untransduced cells shows that the percentage of all IL-2 positive cells were considered to be activated T cells. Transduced cells were gated on EGFP expression and the percentage of CD69 positive cells was assessed within this gate.*

Untransduced, WT rd1 hCD6 transduced and rd1 hCD6 Y629F Y662F transduced T cells were stimulated with 2 µg/ml CD3 antibody and/or 5 µg/ml OX52 and the percentage of IL-2 positive cells was determined for all three donors (Figure 28).

When T cells were stimulated with CD3 antibodies but not CD6 antibodies, WT rd1 hCD6 transduced T cells had a significantly increased percentage of IL-2 positive cells. T cells expressing rd1 hCD6 Y629F Y662F did not show a significantly increased level of T cell activation and in donor 2, there is evidence that rd1 hCD6 Y629F Y662F can inhibit the percentage of IL-2 positive cells (Figure 28).

This difference in behaviour of WT rd1 hCD6 and rd1 hCD6 Y629F Y662F for CD69 and IL-2 may be due to different kinetics of these readouts and certain time points and stimulations may reveal the activating effect and some may reveal the inhibitory effect.

Crosslinking of rd1 hCD6 with OX52 without CD3 stimulation resulted in no T cell activation for donor 2 and 3 but there was a significant increase in T cell activation with WT rd1 hCD6 transduced T cells for donor 1 (Figure 28). The donor 1 intracellular IL-2 (but not CD69) experiments were conducted 18-20 days after T cell transduction compared to 9-11 days for donor 2 and 3 and this may have altered T cell behaviour.

Stimulation of T cells with CD3 antibodies and OX52 significantly increased the response of WT rd1 hCD6 transduced T cells for all three donors (Figure 28). T cells transduced with rd1 hCD6 Y629F Y662F did not show a significantly increased percentage of IL-2 positive cells.

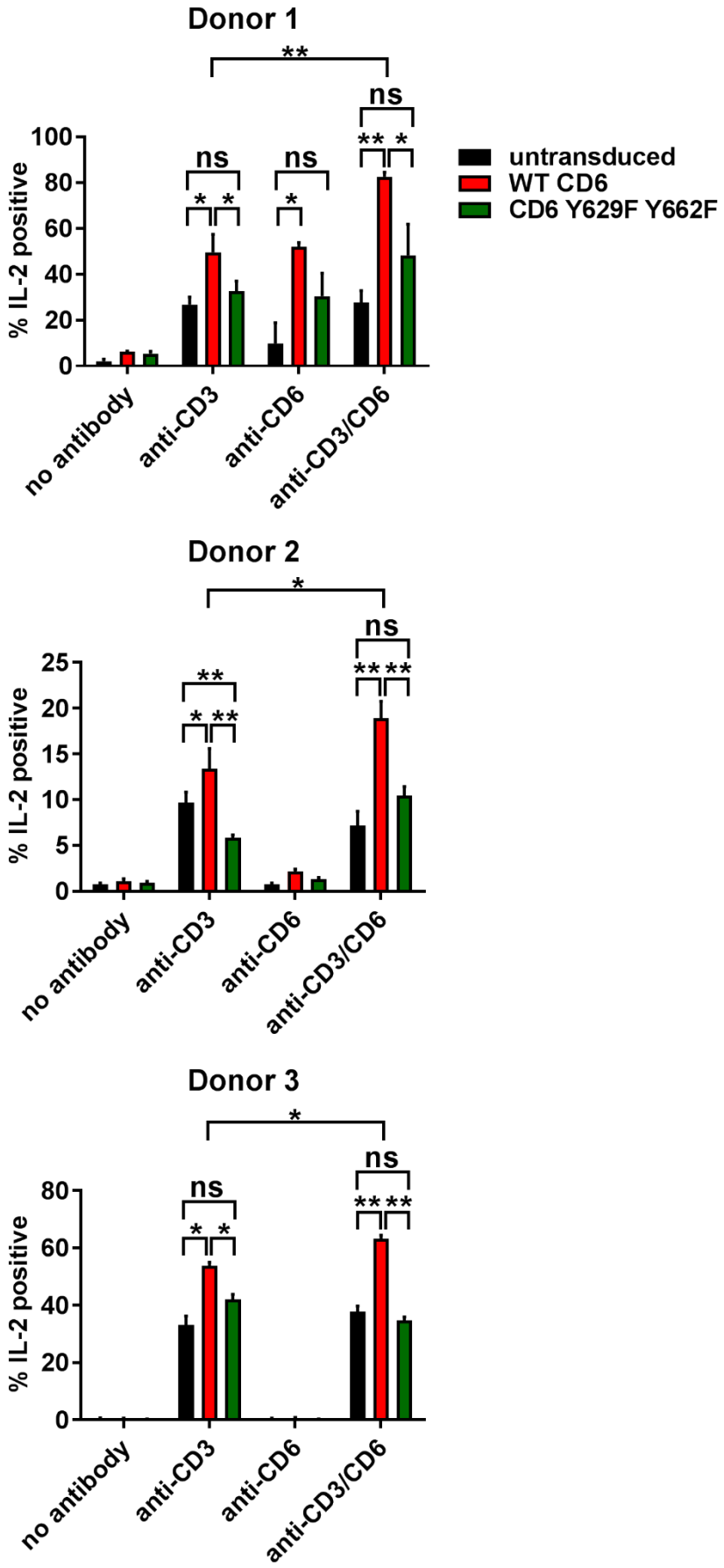

Donor 1
**
ns
ns
ns
** *
* *
*
100
80
60
40
20
0
% IL-2 positive
untransduced
WT CD6
CD6 Y629F Y662F
no antibody
anti-CD3
anti-CD6
anti-CD3/CD6
Donor 2
*
ns
**
** **
* **
25
20
15
10
5
0
% IL-2 positive
no antibody
anti-CD3
anti-CD6
anti-CD3/CD6
Donor 3
*
ns
ns
** **
* *
80
60
40
20
0
% IL-2 positive
no antibody
anti-CD3
anti-CD6
anti-CD3/CD6

Figure 28. The activating effect of CD6 on IL-2 production in primary T cells is mediated by the CD6 Y629 and Y662 residues. *T cells were stimulated with 2 µg/ml CD3 antibody and 5 µg/ml OX52 for 18 h and assessed for the percentage of IL-2 positive cells. n = 3, error bars show the standard error of the mean, * p<0.05, ** p<0.01, ns = not significant, student's t test.*

Thus, results obtained with intracellular IL-2 as readout for T cell activation confirm the CD69 data for primary T cells and Jurkat 1G4 cells. This means that it is likely that the effects of WT CD6 and CD6 Y629F Y662F are not specific to a particular readout but are real influences on T cell activation.

4. Discussion

4.1 Summary and CD6 costimulation model

CD6 is a costimulatory receptor on T cells that enhances T cell activation when it is ligated by its ligand CD166 and it can inhibit T cell activation in the absence of ligand engagement (Figure 29A) [28]. The molecular mechanisms of costimulation are poorly understood and the only confirmed interaction is the adapter molecule SLP-76 that binds to the tyrosine residue Y662 [79]. However, the human CD6 cytoplasmic region has a length of 244 amino acids and some serine and tyrosine residues have been shown to be phosphorylated, suggesting that CD6 can recruit other proteins as well [133,135]. The aim of my project was to identify and characterise novel cytoplasmic interactions with these phosphorylated residues.

I have shown that the FERM domain of ERM proteins binds to the serine- and tyrosine phosphorylated SDSDY sequence of CD5 and CD6. Mutating this sequence in CD6 led to reduced costimulation by CD6 in Jurkat cells. A potential explanation is that the cytoskeletal interaction via ERM proteins is required for CD6 migration towards the TCR where it then influences TCR signalling (Figure 29B). This would mean that only CD6 in proximity of the TCR influences TCR signalling, reducing the overall contribution of CD6 on T cell activation.

I have also shown that phosphorylated CD6 Y629 can recruit the SH2 domain-containing adapter molecules GADS, GRB2 and TSAd but GADS is the main binding partner in T cells. GADS and SLP-76 cooperatively bind to the CD6 C terminus and mutating their binding sites abrogates CD6-mediated costimulation (Figure 29C). This suggests that CD6 costimulation depends on the recruitment of GADS and SLP-76.

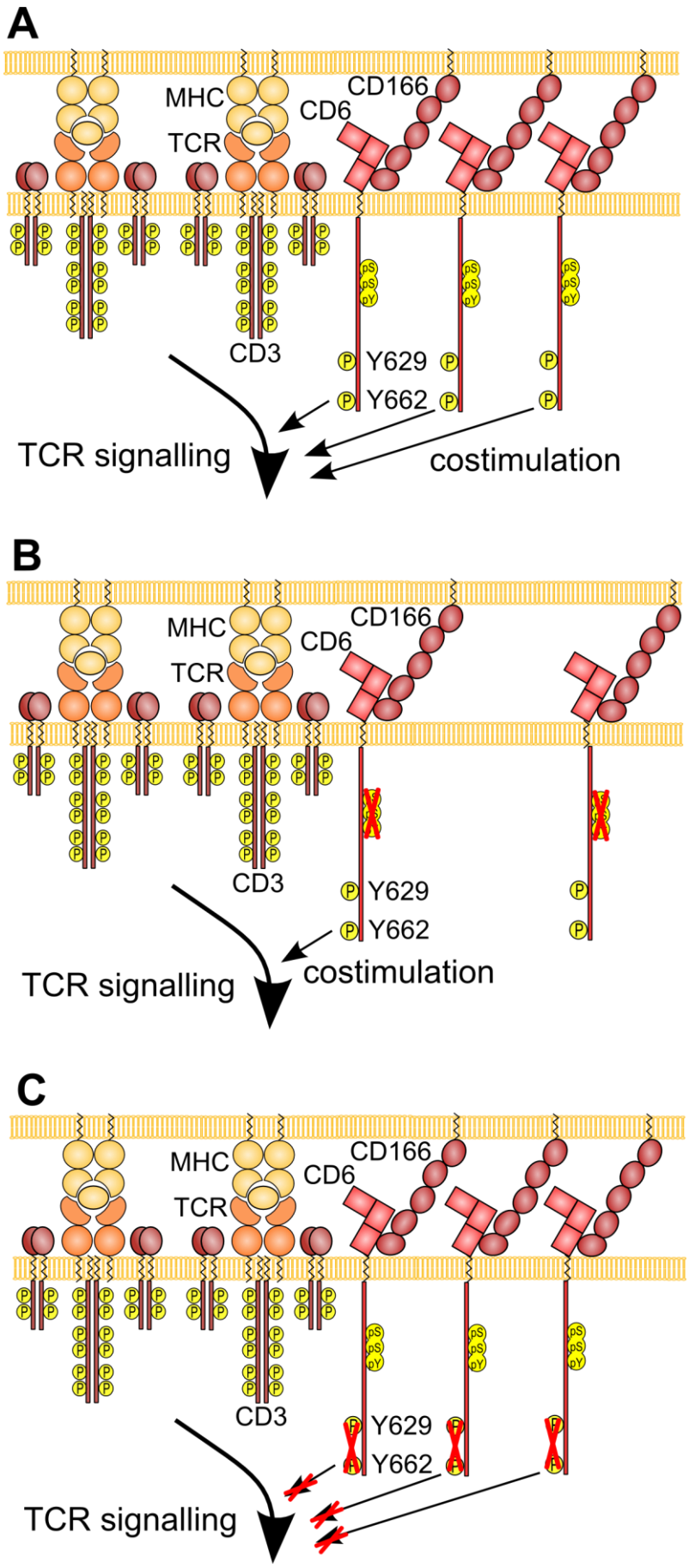

A
MHC
TCR
CD6
CD166
CD3
Y629
Y662
TCR signalling
costimulation
B
MHC
TCR
CD6
CD166
CD3
Y629
Y662
TCR signalling
costimulation
C
MHC
TCR
CD6
CD166
CD3
Y629
Y662
TCR signalling

Figure 29. Model of CD6 SDSDY and C terminus function in T cell costimulation. *When a T cell is engaged by an APC, the TCR binds to peptide:MHC which results in TCR signalling. (A) WT CD6 is engaged by CD166 leading to colocalisation of CD6 with the TCR and costimulation by CD6 through the cytoplasmic regions (B) CD6 with the mutated SDSDY sequence cannot bind to the cytoskeleton via ERM proteins. Thus, CD6 migration towards the TCR is reduced and only CD6 in proximity of the TCR is able to provide costimulation. (C) CD6 lacking the C terminal Y629 and Y662 residues may colocalise with the TCR but it does not provide any costimulation because it cannot recruit the GADS/SLP-76 complex required for CD6-mediated costimulation.*

In conclusion, TCR signalling induces CD6 tyrosine phosphorylation and subsequent recruitment of the GADS/SLP-76 complex to CD6. CD6 ligand engagement is required for CD6-mediated costimulatory function. A potential mechanism is increased serine- and tyrosine phosphorylation when CD6 is engaged by CD166. This results in a stronger connection to the actin cytoskeleton and increased recruitment of the GADS/SLP-76 complex and therefore stronger signalling in the proximity of the TCR.

4.2 pSDpSDpY is an unusual binding site for FERM domains

ERM proteins have a large number of interaction partners which demonstrates their important function in the organisation of plasma membrane receptors and the underlying cytoskeletal rearrangements [156]. Most of these interactions are mediated by the FERM domain with only a few binding partners binding to non-conserved residues outside of the FERM domain [156,171]. The

FERM domain of ERM proteins can bind to several different consensus sequences that all have positive charges in common due to the many negative charges of the FERM domain binding pocket [145,158,172,173].

I have identified an interaction of the ERM protein FERM domain with the CD5 and CD6 SDSDY sequence in a serine- and tyrosine phosphorylation dependent manner. The CD5 and CD6 SDSDY sequences which contain two aspartic acid residues and several phosphorylations are highly unusual binding sites for FERM domains due to their negative charges. The aspartic acid residues in the FERM domain binding site of CD5 and CD6 may be sufficient for a low affinity interaction with FERM domains which is then strengthened by adding further negative charges by phosphorylation.

To my knowledge, no other ERM binding partners that bind the FERM domain have been shown to depend on phosphorylation or negative charges. Only the FERM domain of the protein 4.1G has been shown to bind the receptor FcγRI in a serine phosphorylation-dependent manner but the interaction has not been analysed in detail [174]. Protein 4.1G belongs to the same class of FERM domain-containing proteins and therefore the binding mode might be similar to ERM proteins [175].

This makes the SDSDY-FERM domain interaction highly unusual by not just having reversed charges compared to other receptor-FERM domain interactions but also because CD5 and CD6 need to be phosphorylated for an interaction.

ERM protein activation is tightly regulated by its own C terminus that folds into the N-terminal FERM domain when the protein is inactive [176]. Release of the C terminus and subsequent linking of receptors to the actin cytoskeleton

require two steps for activation [177]. The initial step requires binding of negatively charged phospholipids such as PIP_2 and IP_3 to a positively charged pocket of the FERM domain between the ubiquitin-fold-like subdomain and the phosphotyrosine-binding-like subdomain. This may lead to conformational changes that reduce the binding affinity of the C terminus to the negatively charged FERM domain binding pocket [176,178]. This allows a C-terminal threonine residue to be phosphorylated adding a negative charge that further reduces binding of the C terminus to the FERM domain [179].

This unusual FERM domain binding site raises two questions: Does the binding of ERM proteins to CD5 and CD6 establish a link to the actin cytoskeleton similarly to other ERM interaction partners [156]? Does the negatively charged SDSDY sequence function similarly to negatively charged phospholipids and activate ERM proteins [176]? This would also mean that the FERM binding pocket could bind to other receptors and allow receptor clustering in an ERM protein- and cytoskeleton-dependent manner.

4.3 The SDSDY-FERM domain interaction may regulate receptor localisation

Receptors on the lymphocyte surface can be actively recruited to sites of cell-cell contact and CD5 and CD6 may migrate in an actin-dependent manner similar to other receptors (Figure 30) [180,181].

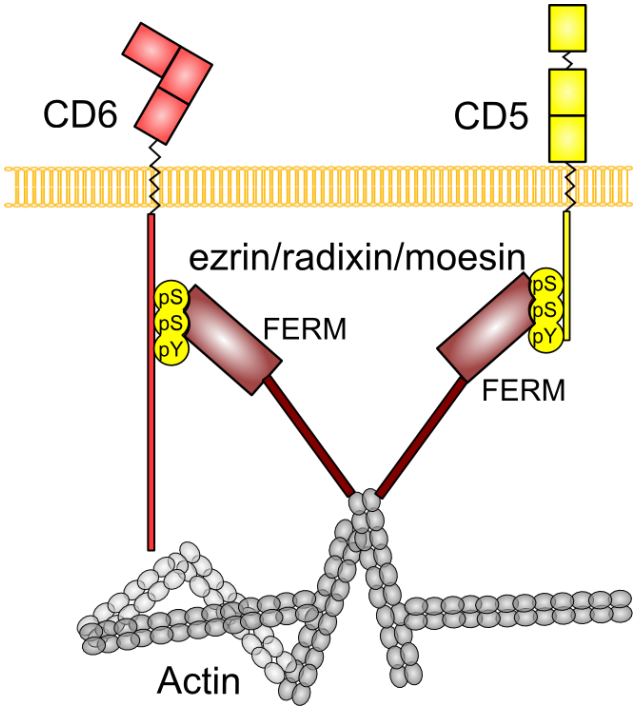


Figure 30. CD5 and CD6 are connected to the cytoskeleton in an ERM protein-dependent manner. *ERM proteins are recruited to the phosphorylated SDSDY sequence of CD5 and CD6 via their FERM domain. The C terminus of ERM proteins contains an actin binding site which may connect CD5 and CD6 to the actin cytoskeleton in a phosphorylation-dependent manner.*

In fact, ERM proteins are required for T cell activation and mouse T cells with an ezrin knockout and an additional moesin knockdown have significantly reduced IL-2 production and transcription factor activation [182]. This might be due to altered signalling microcluster formation and thus altered signalling strength which has been shown in B cells with ERM protein knockdown or constitutively active ERM proteins [181,183]. In B cells, knockdown of ezrin resulted in reduced B cell receptor cluster formation and reduced B cell signalling [181]. Constitutively active ezrin led to larger B cell receptor clusters but also

reduced B cell signalling, suggesting that dynamic regulation of ERM protein activation is required for proper B activation [181].

An important question is, when are CD5 and CD6 connected to the cytoskeleton. Phosphorylation of SDSDY appears to be dependent on TCR signalling and occurs soon after TCR triggering [122]. However, there is evidence that the serine residues of CD5 and CD6 SDSDY are constitutively phosphorylated which indicates an ERM protein-SDSDY interaction in resting T cells which may further increase during activation [130,135].

Mutating SDSDY may reduce the actin polymerisation-dependent migration speed of CD5 and CD6 or may reduce colocalisation with receptors such as the TCR. This would add another layer of regulation to CD5 and CD6 function because compared to other ERM binding partners, CD5 and CD6-ERM binding is inducible by phosphorylation.

The implications of the unusual SDSDY-ERM protein interaction can be studied using a microscopy approach by assessing the localisation of WT CD5 and CD6 and SDSDY mutants on the surface of T cells. The CD6-EGFP fusion constructs used for functional experiments can be used to track receptor movement and localisation with tagged ERM proteins and other receptors such as CD3.

4.4 CD6 costimulation depends on the recruitment of the GADS/SLP-76 complex

My experiments provide strong evidence that the GADS/SLP-76 complex binds to CD6 Y629 and Y662 in cells (Figure 31). An activating function for the recruitment of SLP-76 to CD6 Y662 has been shown before but this paper has

not shown whether other signalling contributes to the activating signal provided by CD6 [79]. In this study, transducing 2B4 hybridoma cells with CD6 led to an increase in IL-2 production when cells were engaged by APCs, suggesting a ligand-dependent effect of the Y662 residue.

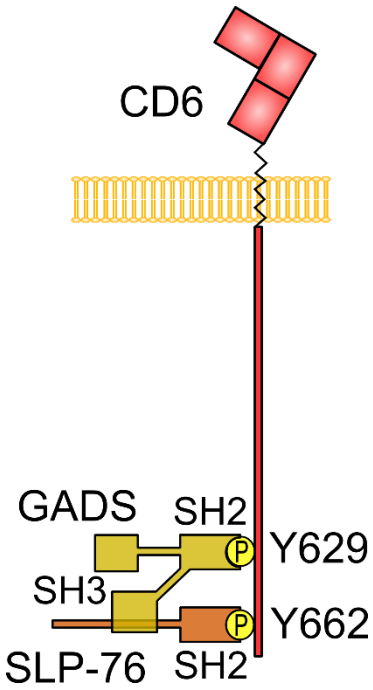


Figure 31. The GADS/SLP-76 complex binds bivalently to CD6 Y629 and Y662. *The GADS and SLP-76 SH2 domains bind to the phosphorylated tyrosine residues Y629 and Y662 of CD6, respectively. The C-terminal SH3 domain of GADS binds to a proline-rich region of SLP-76 and there is evidence that the GADS/SLP-76 complex binds bivalently to the CD6 C terminus.*

My work showed that overexpression of CD6 in Jurkat cells or primary T cells leads to a stronger T cell activation when CD6 signalling is induced by antibody crosslinking. Mutating CD6 Y629 and Y662 resulted in CD69 expression levels similar to cells that were not transduced with CD6. This could mean that

these two residues are sufficient for CD6 costimulation because no signalling contribution to TCR signalling is detected. An alternative explanation is that other activating signalling occurs but this is counteracted by inhibitory signalling.

Interestingly, phosphorylation of CD6 Y629 and Y662 depends on TCR signalling but my experiments show that the costimulatory role of these two residues required CD6 ligation [134]. A possible explanation would be that the function of CD6 Y629 and Y662 requires other factors. CD6 possesses partially phosphorylated serine residues but the effect of CD6 ligation on serine phosphorylation is unknown [134,135]. Crosslinking of CD5 increased serine phosphorylation together with TCR signalling, suggesting that CD6 ligation may increase serine phosphorylation in cooperation with TCR signalling as well and therefore induce or strengthen the connection to the cytoskeleton via ERM proteins [135,184,185].

Thus, CD6 costimulation may be dependent on the recruitment of the GADS/SLP-76 complex and cytoskeleton-mediated migration to the TCR where SLP-76 can act on TCR signalling.

4.5 Inhibitory signalling may depend on other cytoplasmic interactions

There is evidence that deletions of parts of the CD6 cytoplasmic region can have activating or inhibitory functions shown by Ca^{2+} influx in Jurkat cells [133]. Carmo and colleagues have proposed that CD6 has an inhibitory function on early TCR signalling shown by a reduced Ca^{2+} influx and on T cell proliferation [78]. In their experiments they do not find an inhibitory function of CD6 when it is engaged by CD166, providing evidence that inhibitory signalling downstream of CD6 only occurs or dominates in the absence of ligand engagement. There is

some evidence that CD6 ligand engagement can be inhibitory. Starling et al. used a mixed leukocyte reaction mouse model to show that CD6 antibodies that block ligand engagement can increase proliferation of splenic immune cells [186]. However, they did not define which immune cells showed enhanced proliferation and whether the antibodies really blocked ligand engagement [186].

I have shown that overexpressing WT CD6 in primary human CD4+ T cells does not affect T cell activation in the absence of CD6 crosslinking antibodies. This discrepancy between my work and work done by Carmo's lab may be due to the experimental system and functional readout used [78]. TCR engagement with peptide:MHC instead of CD3 crosslinking antibodies may reveal an inhibitory effect of WT CD6 on T cell activation. Hassan et al. have shown that WT CD6 can have an inhibitory function in T cells when these T cells are incubated with APCs expressing CD166 but only at low APC:T cell ratios [79]. This effect is not dependent on the strength of TCR signalling but it is dependent on the level of CD6 ligand engagement.

The results of crosslinking CD6 with antibodies may also vary with which domain and epitope of CD6 is targeted and with different affinities. Mutating the CD6 Y629 and Y662 residues reduced T cell activation without crosslinking CD6 antibodies. This suggests that baseline signalling in the absence of ligand engagement or in response to CD166 expressed by other T cells occurs. These mutations show that CD6 Y629 and Y662 provide a low level of activating signalling. However, the activation of WT CD6 transduced cells is not increased compared to untransduced cells. This indicates that inhibitory signalling occurs at the same time as activating signalling and counteracts the activating signalling.

It is likely that CD6 can also recruit inhibitory proteins, possibly to other motifs such as proline-rich regions or phosphorylated residues. The phosphorylation-dependent inhibition is indicated by the increased inhibition of CD6 Y629F Y662F when cells were stimulated with CD3 antibodies. An example of phosphorylation-dependent recruitment of inhibitory molecules is the recruitment of SHP-1 and SHP-2 to PD-1 [14]. However, according to Tinti et al. who conducted a screen for binding motifs of SH2 domains and predicted GADS, GRB2 and TSAd binding to CD6 Y629 and SLP-76 binding to CD6 Y662, no other SH2 domain containing proteins are likely to bind to any of the tyrosine residues in the cytoplasmic region of CD6 in a physiological setting [161]. It is still possible that SH2 domain-mediated interactions with CD6 occur that were not predicted or that have a low affinity. Evidence for other functional tyrosine residues is that mutating the tyrosine at position 489 resulted in increased overall tyrosine phosphorylation of CD6 [79]. Alternatively, inhibitory proteins that do not contain an SH2 domain may be recruited. An example for this would be the recruitment of the E3 ubiquitin ligase c-cbl to CD5 which only possesses an SH2-like domain [127].

Another explanation for the activation and inhibition mediated by CD6 could be a role of CD6 in the function of the serine/threonine kinase HPK1. Phosphorylation by HPK1 can induce the degradation of substrates as shown for SLP-76 and is therefore inhibitory [137]. HPK1 binds to the SLP-76 SH2 domain and may compete with CD6 for SLP-76 binding [120]. It can be conceived that binding of HPK1 to SLP-76 is inhibitory and that CD6 displaces HPK1 from the SLP-76 SH2 domain when CD6 Y662 is phosphorylated. Baseline phosphorylation of Y662 may be sufficient to displace some HPK1 and ligand

engagement and TCR signalling which lead to CD6 Y662 phosphorylation would increase displacement of HPK1. When CD6 Y629 and Y662 are mutated, a high amount of HPK1 is bound to SLP-76 and exerts its inhibitory function, explaining the mutant CD6 phenotype. WT CD6, especially when ligated, reduces the function of HPK1 explaining the activating function of CD6.

To test this hypothesis, it is important to examine whether CD6 and HPK1 compete. This can be done by immunoprecipitating SLP-76 in the presence of WT CD6 and CD6 Y629F Y662F and blotting for HPK1. Another possibility is to measure the affinities for the GADS/SLP-76 complex and of HPK1 for the CD6 pY629 pY662 peptide. If the GADS/SLP-76 has a higher affinity for CD6 it is likely that it would displace HPK1. To investigate a role of CD6 on HPK1 function, HPK1 knockout cells could be transduced with WT or mutant CD6 and examined for the influence of CD6 on T cell activation in the absence of HPK1. If the contribution of CD6 to TCR signalling is reduced, CD6-mediated regulation of HPK1 is a potential mechanism of CD6 function.

The adapter protein ADAP also binds to the SLP-76 SH2 domain and is therefore likely to compete with HPK1 and CD6 for SLP-76 binding because all three proteins share the YDD sequence as a binding site for the SLP-76 SH2 domain [120]. ADAP is important for integrin activation and actin polymerisation in response to TCR activation [118]. It would be important to show whether CD6 displaces ADAP from SLP-76 and whether this affects cytoskeletal rearrangements and adhesion. However, as my data show that CD6 is likely to be connected to the cytoskeleton and it contributes to adhesion by binding to its ligand CD166 [38], it is possible that CD6 can compensate for reduced ADAP recruitment to SLP-76.

An important question is if inhibitory signalling by CD6 can be triggered by antibody crosslinking. This has direct implications on immunotherapy. Inhibition of autoimmune responses could be achieved by blocking the activating function or triggering the inhibitory function of CD6. In addition, if an activating function of CD6 is desired, for example in cancer immunotherapy, triggering of inhibitory signalling needs to be avoided.

4.6 CD6 may function as an alternative scaffold protein to LAT

A knockout of the scaffold protein LAT in mice did not result in a complete block of T cell development, hinting at the existence of alternative scaffold molecules for TCR signalling [105]. SLP-76 is recruited to LAT via GADS [187]. Remarkably, phosphorylation of SLP-76 in response to crosslinking CD3 and CD4 still occurs in the absence of LAT in mice and Jurkat cells [188,189].

Malissen and colleagues suggested that CD6 may function as a scaffold protein similarly to LAT [106]. Knocking out both LAT and CD6 in mice would reveal whether CD6 can function as an alternative to LAT and completely block T cell development.

Mutation of LAT Y136 in mice leads to a pathogenic lymphoproliferative disorder [188]. This tyrosine residue is involved in the activation of the phospholipase PLCγ1, which is essential in T cell activation [190]. It is not understood how the lack of PLCγ1 recruitment leads to more proliferation but an initial lymphopenia was observed with uncontrolled expansion of T cells in the periphery [188,191]. Full PLCγ1 activation also requires the recruitment of PLCγ1 to SLP-76 [115]. My data and previous work in the lab show that CD6 is involved in the recruitment of SLP-76 but the reduced T cell activation in the absence of

LAT suggests that this does not fully activate PLCγ1 [79,106]. Alternatively, CD6 may be sufficient for PLCγ1 activation but other pathways downstream of LAT, such as the MAPK pathway, may not be activated by CD6 [107]. This raises the question why binding of the GADS/SLP-76 complex to CD6 provides an activating signal to T cells if recruitment of SLP-76 to LAT is an essential step for T cell activation. One possibility would be the displacement of HPK1 from SLP-76 as described in section 4.5.

Alternatively, CD6 may cooperate with LAT in recruiting SLP-76. Confocal and TIRF microscopy have shown that LAT only transiently colocalised with the TCR before ubiquitination of LAT which led to protein internalisation and subsequent degradation [192]. SLP-76 can be internalised and degraded together with LAT. However, not all SLP-76 is degraded suggesting a mechanism of keeping SLP-76 at the membrane or recycling SLP-76 through endosomes without degradation [193].

The engagement of CD6 by CD166 enhances T cell activation [28]. This engagement strengthens the contact of APCs and T cells leading to longer interaction times between these cells [38]. This study showed that recruitment of CD6 and CD166 to the contact interface happens after about 6 min suggesting that the interactions between these molecules enhance long-term interactions rather than stabilising the initial immunological synapse formation. These CD6-CD166 interactions may increase adhesion but it could also keep CD6 localised to TCR microclusters and it could keep SLP-76 in proximity to activating signals. Alternatively, CD6 may enhance TCR signalling by recruiting additional SLP-76 to TCR signalling complexes.

Microscopy in live cells can be used to determine when CD6 and SLP-76 colocalise and how long these interactions last. This would reveal when CD6 provides costimulation to enhance TCR signalling. This may also show whether CD6 resides in the same microclusters as LAT and therefore competes with LAT for SLP-76 binding or whether an additional pool of SLP-76 binds to CD6.

Analysing the signalling pathways downstream of CD6 may also shed light on its niche in TCR signalling. A knockout of LAT does not attenuate ERK phosphorylation when T cells are stimulated with a crosslinking CD6 antibody [106]. Therefore, CD6 affects ERK activation either by an unknown link to the MAPK signalling cascade or by inducing a Ca^{2+} influx which has been shown to activate ERK by several mechanisms [194]. The Ca^{2+} influx may be triggered by the recruitment of PLCγ1 to SLP-76 bound to CD6 [116]. Analysing transcription factor activation downstream of CD6 would show whether CD6 only influences certain signalling pathways or whether it influences all TCR signalling pathways.

4.7 Using CD6 in chimeric antigen receptor T cells

Using the immune system to combat cancer shows great promise in clinical trials and tumour immunotherapy has therefore been called “breakthrough of the year 2013” by Science [195]. One of the most promising tumour immunotherapy approaches is using T cells expressing a chimeric antigen receptor (CAR) [196]. CARs contain an extracellular single chain variable fragment from an antibody that bind to antigens on the surface of tumour cells and a cytoplasmic region providing activating signals to the T cell [197]. The design of the cytoplasmic region of CARs is critical for CAR T cell function because signalling downstream of the CAR needs to be strong enough for T cell

activation but should not trigger a cytokine storm. First generation CARs used the CD3 ζ chain as cytoplasmic region but because signalling through CD3 ζ alone does not activate T cells, the addition of other cytoplasmic regions were investigated [196,198]. The addition of the CD28 or 4-1BB cytoplasmic region (second generation CARs) to the CD3 ζ chain increases T cell activation, *in vitro* tumour cell lysis and survival in a leukaemia mouse model [199,200]. Third generation CARs that use CD3 ζ and both CD28 and 4-1BB or cytoplasmic regions from other receptors have also been used but it remains to be shown whether third generation CARs are more efficient than second generation CARs [196].

The specific role for CD6 in T cell activation may make the CD6 cytoplasmic region an attractive component for CAR design [28]. An important question is whether the addition of further cytoplasmic regions to CARs affect the expression level. The 244 amino acids in the cytoplasmic region of human CD6 might reduce the CAR expression level and it may be advantageous to only use parts of the CD6 cytoplasmic region. Another factor that needs to be considered is whether signalling sequences require a certain distance from the membrane. The conserved distance from the membrane of the SDSDY sequence in CD5 and CD6 suggests that this distance is important for the connection to the cytoskeleton. In addition, due to its activating and inhibitory function in T cells, the use of inhibitory regions of CD6 should be avoided for CARs.

Because the region containing CD6 Y629 and Y662 is the dominant costimulatory region of CD6, it may be sufficient to just add this sequence to the CAR. Considering that the length of a third generation CAR containing CD3 ζ, CD28 and 4-1BB has a length of 193 residues and CD6 Y629 is located at

position 205 of the human CD6 cytoplasmic tail it can be conceived that the CD6 C terminus containing Y629 and Y662 would be functional if added to the C terminus of a CAR.

Using CD6 in CARs is particularly interesting because unlike receptors it can be a scaffold for signalling complexes [106]. Instead of simulating early signalling events with CARs, providing the scaffold for larger signalling complexes that result in the activation of several signalling pathways may result in more sustained T cell activation.

5 References

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