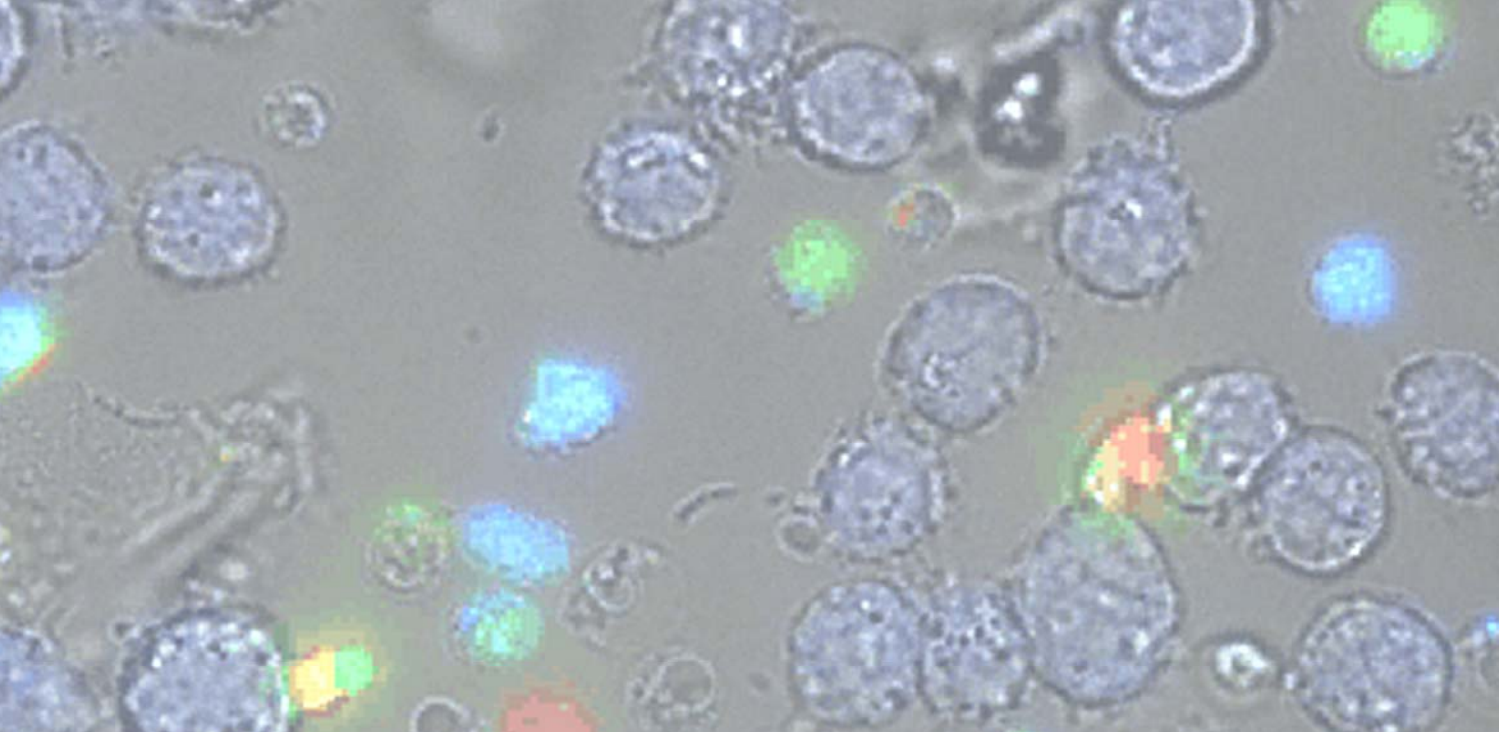
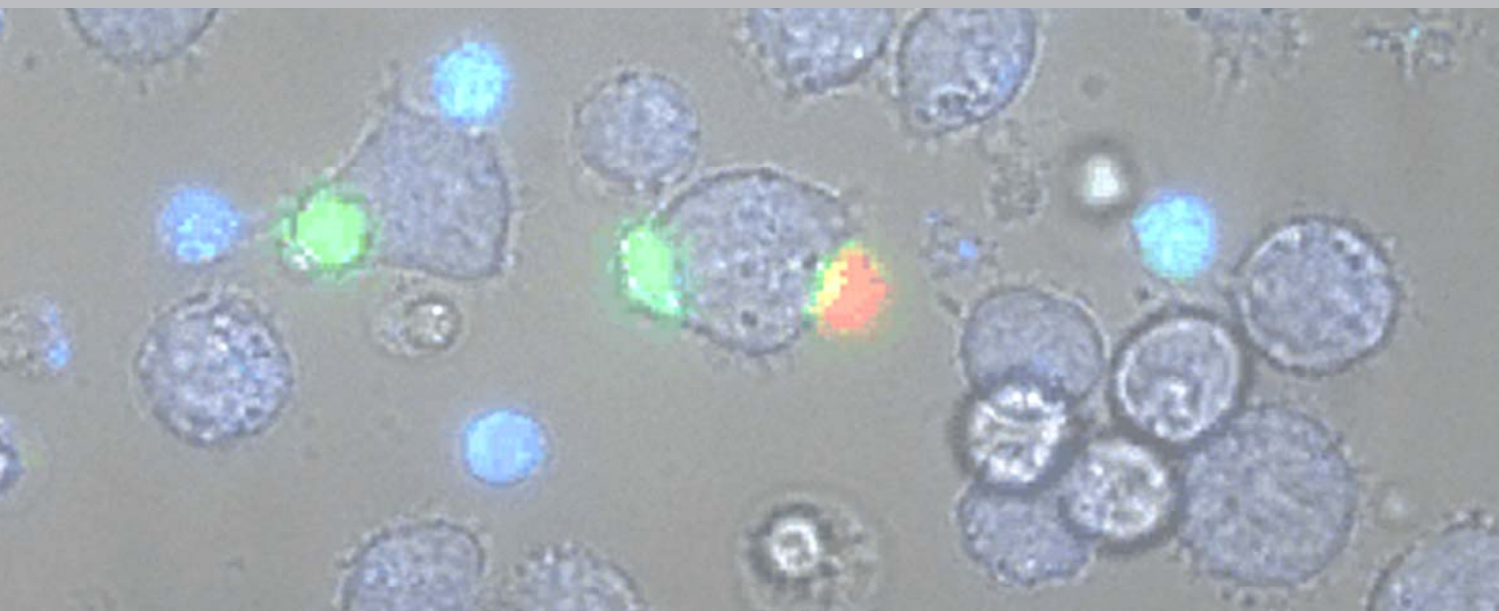




# THE SIGNALING ROLE OF THE ACCESSORY RECEPTORS CD2 AND CD6 IN T CELL ACTIVATION

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Dissertation to obtain the Degree of Doctor in Biomedical Sciences, speciality in  
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Ao Daniel e à Rita

Aos meus pais

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## Summary

It is widely accepted that the ultimate response to antigen-specific signals is also influenced by accessory receptors-ligand engagements that deliver costimulatory signals while strengthening the interaction between the cells. CD6, a type I cell surface glycoprotein, whose expression is largely restricted to peripheral T lymphocytes and medullary thymocytes, has been known to play a role in T cell activation. We have cloned by PCR the rat homologue of CD6 and biochemical analysis showed that CD6 interacts with CD5 at the surface of T lymphocytes and that the fraction of CD5 associated with CD6 was highly phosphorylated in kinase assays, in marked contrast with the low level of phosphorylation of CD5 associated with either TCR or CD2. Examination of protein kinases associating with CD6 showed the involvement of Lck, Fyn, and ZAP-70, and additionally the Tec family kinase Itk, which is absent from CD2, CD5 and TCR complexes. Together these results suggest that CD6 has an important role in the regulation of CD5 tyrosine phosphorylation, probably due to its unique feature of associating with kinases of different families. While cloning the rat homologue we discovered a novel isoform of the accessory molecule CD6 which lacks the CD166-binding domain, CD6 $\Delta$ d3. Subsequently, four novel human extracellular isoforms were identified and cloned, including isoforms lacking domain 1 or domain 3. Ensuing studies showed that the differential expression of the SRCR domain 3 resulted in a remarkable functional difference: whereas full-length CD6 targeted to the immunological synapse, CD6 $\Delta$ d3 was unable to localize at the T cell:APC interface during antigen presentation. This new finding constitutes a rare example of an alternatively splice-coded isoform with a distinct physiological function. Moreover, CD6 $\Delta$ d3 is markedly up-regulated upon activation of T lymphocytes, partially substituting full-length CD6, as evaluated by immunoblotting and by flow cytometry using antibodies recognizing SRCR domains 1 and 3 of human CD6. This isoform is considerably expressed in a significant number of thymocytes, constituting the sole species in a small percentage of cells, suggesting that CD6 switching between full-length and  $\Delta$ d3 isoforms may be involved in thymic selection as well. We have also focused our attention in the accessory receptor CD2 and have addressed the mechanisms that control the localization of rat CD2 at the plasma membrane, as well as its redistribution induced upon activation. We found no evidence that rat CD2 was subjected to lipid modifications that could favor the translocation to lipid rafts, preferred sites for signal transduction. On the other hand, using Jurkat cells expressing different CD2 and Lck mutants, we showed that the association of CD2 with the rafts fully correlates with its capacity to bind to Lck. As CD2 physically interacts with both Lck and Fyn, preferentially inside lipid rafts, and reflecting the increase of CD2 in lipid

rafts following activation, CD2 can mediate the interaction between the two kinases and the consequent boost in kinase activity in lipid rafts. These results are in agreement and contribute to the current view that the raft localization of cell surface molecules is mainly driven by protein-protein interactions.

## Sumário

A resposta específica resultante do reconhecimento do antígeno pelo receptor dos linfócitos T é influenciada por co-receptores que, quando associados aos respectivos ligandos, fornecem sinais co-estimulatórios e fortalecem a interacção entre os linfócitos T e as células apresentadoras de antígeno. O CD6 é uma proteína transmembranar que está envolvida em processos de activação de células T e cuja expressão está largamente restringida a linfócitos T periféricos e a timócitos da zona medular do timo. Nesta investigação, clonou-se a proteína homóloga de CD6 de rato e demonstrou-se que este co-receptor se associa ao CD5, à superfície de linfócitos T. Verificou-se também que a fracção de CD5 associada ao CD6 se apresentava fortemente fosforilada, contrastando assim com os baixos níveis de fosforilação do CD5 associado ao TCR e ao CD2. O CD6 tem a capacidade de se associar com diferentes cinases de tirosinas, tais como, a Lck, Fyn, ZAP-70 e Itk. Os resultados obtidos sugerem que o CD6 tem um papel importante na regulação da fosforilação de tirosinas do CD5 e que este facto se deve, provavelmente, à capacidade do CD6 de se associar com estas diferentes cinases. Nos trabalhos que se seguiram à clonagem da proteína CD6 de rato, verificou-se a existência de uma isoforma, resultante de “splincing” alternativo, que não possui o domínio de ligação ao ligando-CD166, que se denominou CD6 $\Delta$ d3. Posteriormente, identificaram-se e clonaram-se quatro novas isoformas extracelulares de CD6 humano, entre as quais isoformas sem domínio 1 ou domínio 3. A expressão diferencial do domínio 3 resulta numa função alternativa: enquanto a molécula de CD6 completa se encontra na sinapse imunológica, a isoforma CD6 $\Delta$ d3 não é recrutada para a zona de contacto entre as células T e as células apresentadoras de antígeno. Este resultado constitui um raro exemplo de uma molécula resultante de “splicing” alternativo com uma função fisiológica distinta. Verificou-se, usando técnicas de imunodeteção e de citometria de fluxo com anticorpos específicos para os domínios 1 e 3, que a expressão da isoforma CD6 $\Delta$ d3 está aumentada em linfócitos T activados. Mais ainda, esta isoforma é consideravelmente expressa em linfócitos, sendo a única presente num número significativo de células, sugerindo um papel na selecção tímica. Neste trabalho investigou-se outro co-receptor, o CD2. Foram determinados os mecanismos que controlam a localização do CD2 de rato na membrana celular, assim como a sua redistribuição após activação. Não foram encontradas evidências de que o CD2 de rato seja sujeito a modificações lipídicas que favoreçam a sua translocação para os “rafts”, locais preferenciais de transdução de sinal. Utilizando células T humanas Jurkat, que expressavam diferentes mutantes de CD2 e de Lck, foi possível demonstrar que a associação do CD2 com os “rafts” é determinada pela capacidade do CD2 de se associar com a Lck. O CD2 associa-se com a Lck e a Fyn,

preferencialmente nestes locais da membrana facilitando a interacção entre as duas cinases. Assim sendo, o aumento do CD2 nos "rafts" verificado após activação pode induzir a amplificação da actividade das cinases nos "rafts". Estes resultados estão em conformidade e fortalecem a visão corrente de que a localização de determinadas proteínas nos "rafts" é fundamentalmente determinada por interacções entre proteínas.

## Sommaire

Le concept que plusieurs molécules accessoires influencent la réponse des lymphocytes T déclenchée par la reconnaissance de l'antigène est largement accepté. CD6, une glycoprotéine de surface de type I dont la expression est spécifique des lymphocytes T et des thymocytes médullaires, joue un rôle méconnu dans la activation des lymphocytes T. Nous avons cloné CD6 de rat par PCR et ensuite montré que le CD6 interagit avec le CD5 à la surface des lymphocytes T. En plus, nous avons démontré que la fraction de CD5 en association avec le CD6 présente des forts niveaux de phosphorylation dans des essais kinase, contrairement à la fraction associé avec le TCR ou le CD2. L'analyse des différentes kinases associées avec le CD6 a démontré l'implication de Lck, Fyn, ZAP-70 et Itk, une kinase de la famille des Tec protéine kinases, absente des complexes CD2, CD5, et TCR. L'ensemble de ces résultats suggère que le CD6 a un rôle clé dans la régulation des niveaux de phosphorylation du CD5, possiblement en raison de son association avec des protéines kinases de différentes familles. Nous avons aussi découvert une nouvelle isoforme du CD6, CD6 $\Delta$ d3, dont le domaine de liaison au ligand CD166 est absent. Ensuite, nous avons identifié et cloné quatre nouvelles isoformes du domaine extracellulaire chez l'homme, notamment des isoformes sans les domaines 1 et 3. Des études qui ont suivi ont montré que l'expression différentielle du domaine SRCR 3 produit des effets fonctionnels importants : seule la forme entière du CD6 est localisé à la synapse immunologique lors du contact cellule T : APC en présence d'antigène. Ceci est un exemple rare de divergence fonctionnelle entre produits d'épissage alternatif du même transcrit. L'expression de CD6 $\Delta$ d3 augmente de forme significative après activation des lymphocytes T, substituant partiellement la forme entière de la molécule, comme le démontrent des expériences de western blot et cytométrie en flux. Cette isoforme est aussi présent dans un nombre significatif de thymocytes et c'est la seule isoforme détecté dans une fraction de thymocytes, ce que suggère l'existence d'un mécanisme de remplacement de la molécule de CD6 entière par la forme tronquée au cours de la sélection thymique. Nous avons aussi étudié les mécanismes impliqués dans le contrôle de la localisation de la molécule accessoire CD2 de rat dans la membrane cellulaire et au cours de l'activation des lymphocytes T. Nos résultats montrent que le CD2 n'est pas sujet à des modifications favorisant la translocation vers les rafts lipidiques. Au contraire, la localisation du CD2 dépende entièrement de sa capacité à lier Lck dans les rafts. Comme le CD2 interagit avec Lck et Fyn préférentiellement à l'intérieur des rafts lipidiques, le CD2 pourrait y jouer un rôle dans l'interaction entre les deux kinases et ainsi contribuer au renforcement de l'activité kinase après activation des lymphocytes T par l'antigène dans les rafts. Nos résultats contribuent au concept que des interactions du

type protéine-protéine jouent un rôle majeur dans le repositionnement des molécules de surface dans les rafts lipidiques.

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## Abbreviations

|                                  |                                                              |
|----------------------------------|--------------------------------------------------------------|
| TCR                              | T cell receptor                                              |
| MHC                              | Major histocompatibility complex                             |
| APC                              | Antigen presenting cell                                      |
| BCR                              | B cell receptor                                              |
| pMHC                             | peptide-MHC complex                                          |
| Ag                               | Antigen                                                      |
| Ig                               | Immunoglobulin                                               |
| ER                               | Endoplasmic reticulum                                        |
| ITAM                             | Immunoreceptor tyrosine based activation motif               |
| PLC $\gamma$ 1                   | Phospholipase C- $\gamma$                                    |
| IL-2                             | Interleukin-2                                                |
| IP3                              | Inositol-1,4,5-trisphosphate                                 |
| DAG                              | 1,2-diacylglycerol                                           |
| PKC                              | Protein kinase C                                             |
| [Ca <sup>2+</sup> ] <sub>i</sub> | Intracellular free calcium                                   |
| CRAC                             | Ca <sup>2+</sup> release-activated Ca <sup>2+</sup> channels |
| RyR                              | Ryanodine receptor                                           |
| cADPR                            | Cyclic adenosine diphosphoribose                             |
| NAADP <sup>+</sup>               | nicotinic acid adenine dinucleotide phosphate                |
| NFAT                             | Nuclear factor for activated T cells                         |
| SH2                              | Src-homology 2                                               |
| SH3                              | Src-homology 3                                               |
| LAT                              | Linker for activation of T cells                             |
| SLP-76                           | SH2-domain containing leukocyte protein of 76 KDa            |
| PKC $\theta$                     | Protein kinase C- $\theta$                                   |
| GEFs                             | Guanine nucleotide exchange factors                          |
| GAPs                             | GTPase activating proteins                                   |
| AP-1                             | Activator protein-1                                          |
| JNK                              | c-Jun N-terminal kinase                                      |
| GRP                              | Guanyl nucleotide-releasing protein                          |
| MAPKs                            | Mitogen-activated protein kinases                            |
| PI3K                             | Phosphatidylinositol 3-kinase                                |
| CD2BP2                           | CD2-binding protein                                          |
| ZAP-70                           | Zeta-chain-associated protein 70                             |
| PTK                              | Protein tyrosine kinases                                     |

|       |                                                                        |
|-------|------------------------------------------------------------------------|
| PTP   | Protein tyrosine phosphatases                                          |
| PAG   | Phosphoprotein associated with glycosphingolipid-enriched microdomains |
| Csk   | C-terminal Src kinase                                                  |
| Cbp   | Csk binding protein                                                    |
| PH    | Pleckstrin homology                                                    |
| IS    | Immunological synapse                                                  |
| IgSF  | Immunoglobulin superfamily                                             |
| SRCR  | Scavenger receptor cysteine rich                                       |
| cSMAC | Central supramolecular activation clusters                             |
| pSMAC | Peripheral supramolecular activation clusters                          |
| dSMAC | Distal supramolecular activation clusters                              |
| MCs   | Microclusters                                                          |
| TIRF  | Total internal reflection fluorescence                                 |

## Publications

In this thesis are included the following articles which are relevant for the present work:

1. Castro, MAA, Tavares, PA, Almeida, MS, **Nunes, RJ**, Wright, MD, Mason, D, Moreira, A and Carmo, AM (2002). CD2 physically associates with CD5 in rat T Lymphocytes with the involvement of both extracellular and intracellular domains. *Eur. J. Immunol.* **32**: 1509-1518.
2. Castro, MAA, **Nunes, RJ**, Tavares, PA, Oliveira, MI, Simões, C, Parnes, JR, Moreira, A and Carmo, AM (2003). OX52 is the rat homologue of CD6: evidence for an effector function in the regulation of CD5 phosphorylation. *J. Leukoc. Biol.* **73(1)**:183-90.
3. **Nunes, RJ**, Castro, MAA, Carmo, AM (2006). Protein crosstalk in lipid rafts. Review. *Adv. Exp. Med. Biol.* **584**:127-36.

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4. Castro MAA, Oliveira MI, **Nunes RJ**, Fabre S, Peixoto A, Brown MH, Parnes JR, Bismuth G, Moreira A, Rocha B, Carmo AM (2007). Extracellular isoforms of CD6 generated by alternative splicing regulate targeting of CD6 to the immunological synapse. Resubmitted to *J. Immunol.*
5. **Nunes RJ**, Castro MAA, Pereira CF, Bismuth G, Carmo AM (2007). Protein interactions between CD2 and Lck are required for the lipid raft distribution of CD2. Submitted to *J. Immunol.* and returned for modifications.
6. Oliveira MI, Fabre S, Castro, **Nunes RJ**, Bismuth G, Carmo AM (2006). CD6 attenuates calcium signals on the onset of T cell activation. On hold.
7. Castro MAA, **Nunes RJ**, Bamberger M, Maia AR, Oliveira MI, Carmo AM (2006). Lck-dependent and independent mechanisms in the activation-induced translocation of T lymphocyte accessory molecules to lipid rafts. On hold.

## **I. Introduction**

The vertebrate immune system recognizes infectious agents and then uses innate and adaptive responses to eradicate them. The adaptive immune response, which plays a critical role in this process, relies on diverse antigen-specific B and T cell receptors (BCRs and TCRs). Together these receptors can discriminate essentially the total world of potential foreign antigens.

T lymphocytes play a key role in controlling the adaptive immune response by producing cytokines and in some cases acting as cytotoxic cells to kill infected host cells. Activation of T lymphocytes involves the recognition of microorganism-derived peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs) by the TCR. The tight regulation of this recognition process is thus critical to prevent inappropriate and potentially harmful responses to self-antigens. Productive stimulation or positive TCR engagement leads to a succession of events that include activation of protein kinases and phosphorylation of many substrates, including the TCR, and the formation of protein complexes containing adaptors and enzymes. These “proximal” events culminate in cellular responses such as proliferation, differentiation and secretion of cytokines and growth factors.

The ultimate response to antigen-specific signals is also influenced by accessory receptor-ligand engagements that deliver costimulatory signals while strengthening the interaction between the cells. How can such complexity and specificity be achieved through the stimulation of a single class of receptors? How are the decisions made? The answer must depend on the different biochemical activation pathways. Although the TCR signal transduction pathways have been extensively studied there are still critical questions that remain unanswered and conflicting data that should be reconciled.

In this chapter a brief description of current knowledge and recent advances towards the understanding of basic events that regulate T lymphocyte activation is presented. It is now well documented that initiation and propagation of the TCR-generated signal is critically dependent on the transient assembly and spatial reorganization of specific components used by T cells. These include accessory cell surface receptors and associated enzymes that will be described here in more detail.

## 1. Signal transduction in T lymphocytes

### 1.1. TCR/CD3 complex

The T cell receptor is a multicomponent structure that comprises two functional subunits. The antigen-binding subunit, that recognizes peptide-MHC complex (pMHC), is a highly polymorphic heterodimer of clonotypic  $\alpha$  and  $\beta$  chains [1, 2], or  $\gamma$  and  $\delta$  in 2-5% of T lymphocytes [3]. These membrane-spanning chains show great similarity to the domain structure of the immunoglobulins (Igs) displaying one variable and one constant extracellular domain [4]. The variable region domains of  $\alpha$  and  $\beta$  or  $\gamma$  and  $\delta$  come together to form the antigen binding cleft and the mechanisms for generating the vast TCR diversity and antigen specificity involve rearrangements of the multiple germline variable (V), diversity (D) and joining (J) gene segments. The TCR  $\beta$  and  $\delta$  chain are assembled from V, D and J segments while TCR  $\alpha$  and  $\gamma$  chain are assembled from V and J segments (reviewed in [5]). The affinity of  $\alpha\beta$  TCRs binding to pMHC is relatively low ( $K_d \sim 1-100 \mu\text{M}$ ) and this binding occurs through complementarity-determining regions present in their variable domains [6].

TCR heterodimers are non-covalently associated with the non-polymorphic transmembrane proteins CD3 $\gamma$ ,  $\delta$  and  $\epsilon$  chains and a  $\xi$  homodimer [7, 8], which form the signal transducing subunit. A coordinated expression of the ligand-recognizing and signal-transducing subunits is required to achieve full expression of the TCR/CD3 complex on the cell surface. However, despite precedent efforts, the stoichiometry of the TCR/CD3 complex is still unclear. The complex is formed in the endoplasmic reticulum (ER) and is assembled after a series of pairwise interactions involving the formation of dimers of CD3 $\epsilon$  with either CD3 $\gamma$  or CD3 $\delta$ . These dimers assemble with TCR  $\alpha$  and TCR  $\beta$  chains, and finally, the  $\xi$  homodimer is added to allow export of the full complex from the ER [9-12]. As a result, a typical TCR $\alpha\beta$  is associated with at least six signaling subunits, including CD3 $\epsilon\gamma$  and CD3 $\epsilon\delta$  heterodimers and CD3 $\xi\xi$  homodimer.

Despite the extensive work performed in the attempt of unravelling TCR/CD3 complex assembly, the enormous amount of conflicting data on this subject reflects the experimental difficulties of examining such a complex receptor structure in the available cellular systems. There have been two major models of different stoichiometry and valency proposed for the TCR/CD3 complex assembly. A model in which a single TCR heterodimer associates with all signaling components [11, 13, 14], in contrast to the model in which two TCR $\alpha\beta$  heterodimers form two hemicomplexes containing either CD3 $\epsilon\gamma$  or CD3 $\epsilon\delta$  dimers which become associated via the  $\xi$  homodimer [15, 16]. More recent data

from Call *et al.*, using direct biochemical approaches instead of transgenic mice, support the model where the TCR/CD3 complex is monovalent, with a stoichiometry of TCR $\alpha\beta$ -CD3 $\epsilon\gamma$ -CD3 $\epsilon\delta$ - $\xi\xi$  [13].

The TCR  $\alpha$  and  $\beta$  chains have very small cytoplasmic tails and are unable to communicate signals produced by antigen recognition. Therefore, the CD3 and  $\xi$  components of the receptor are accountable for transmitting the signal into the cell. They do so via a conserved amino acid motif present in their cytoplasmic tails, consisting of the sequence YXX(L/I)X<sub>6-8</sub>YXX(L/I) and known as immunoreceptor tyrosine based activation motif (ITAM) [17-19]. Given the current structural model of the TCR complex a total of 10 ITAMs are present in a single receptor complex: each  $\xi$  chain contains three ITAMs in tandem while each of the CD3 chains has only one of these modules [20].

Two non-exclusive models incorporating previous results were put forward by Pitcher *et al.* to argue the contribution of the ten ITAMs to T-cell signaling [21]. The ITAM multiplicity model describes a functional redundancy for distinct ITAMs. In fact, several reports support this view: a CD8/ $\xi$  chimera was able to induce early and late signaling events that were undistinguishable from those elicited by the intact TCR, in the absence of CD3 chains [18]. Later on the same authors have also established that trimerization of the  $\xi$  ITAMs yielded more intense intracellular signals than single ITAMs, implying that  $\xi$  ITAMs functioned in a cumulative manner and induce a similar pattern of tyrosine phosphorylation [17]. Moreover, chimeric receptors comprising the cytoplasmic domains of TCR $\xi$  versus CD3 $\epsilon$  were equally capable of restoring thymopoeisis [22] implying functional redundancy between the TCR $\xi$  and CD3 $\epsilon$  ITAMs.

The differential signaling model, which proposes distinct functions for the CD3 $\gamma$ ,  $\delta$  and  $\epsilon$  and TCR $\xi$  modules is also supported by other studies which report that activation of T cells through either TCR $\xi$  or CD3 $\epsilon$  results in a distinct pattern of tyrosine phosphorylation and in specific interactions with a number of intracellular proteins [23-26]. The authors suggested that T-cell development, signal transduction and effector functions incorporate both ITAM multiplicity and differential signaling, the relative contributions may depend on the avidity of the TCR for its particular cognate antigen, the developmental or differentiation state of a T cell, and/or the environmental context in which a T cell is activated.

A number of mechanisms can be envisaged for how information is transferred upon TCR triggering. Several models were proposed even though they converge to the idea that ligand engagement results in a perturbation of the extracellular region of the TCR/CD3 complex through either a rigid body or intermolecular conformational change. Also, multimerization of TCR/CD3 complex into dimers or higher order complexes is

suggested as a common model for TCR triggering [27]. Although different models invoking aggregation, conformational changes and segregation of the TCR/CD3 complex are proposed they are not mutually exclusive and triggering may involve a combination of all mechanisms.

Full activation through the T cell receptor results in the phosphorylation of multiple ITAMs that become high-affinity binding sites for intracellular proteins that couple antigen recognition to the subsequent cascade of downstream intracellular signals. These events, essential requirements for the cellular effector functions of T cells, particularly, the signaling pathways and the signaling machinery will be briefly described in this chapter.

## 1.2. Signaling pathways

### 1.2.1. $\text{Ca}^{2+}$ /PLC $\gamma$ signaling pathway in T cells

Activation of T cells triggers the recruitment of tyrosine kinases that initiate a signaling cascade that transmits the stimulus across the membrane giving rise to the phosphorylation and activation of phospholipase C- $\gamma$  (PLC $\gamma$ ) [28, 29]. Hydrolysis of inositol phospholipids, mainly phosphatidylinositol-4,5-bisphosphate due to the activity of PLC $\gamma$ , at the inner leaflet of the plasma membrane [30-32] generates the second messengers inositol-1,4,5-trisphosphate (IP $_3$ ), which causes entry of  $\text{Ca}^{2+}$  to cytosol from the ER and the extracellular space; and 1,2-diacylglycerol (DAG), which activates a protein kinase C (PKC) dependent pathway [33].

#### 1.2.1.1. Calcium signaling: triggering, release and influx

The rise of intracellular free calcium ( $[\text{Ca}^{2+}]_i$ ) is recognized as a crucial triggering signal for T cell activation and the nature of this signal is thought to be strongly influenced by factors such as T cell type and maturation state as well as APC and antigen characteristics. Because some of these factors are known to influence the outcome of TCR triggering towards activation, anergy or death, it is reasonable to imagine that together with the large number of participants in  $\text{Ca}^{2+}$  signal generation they add complexity for signal modulation [34].

The T cell  $\text{Ca}^{2+}$  response begins with the binding of IP $_3$  to its receptors in the ER and plasma membranes causing the opening of  $\text{Ca}^{2+}$  channels which leads to the rising of

$[Ca^{2+}]_i$  [35, 36]. Multiple  $IP_3$  molecules bind to the tetrameric receptor illustrating a highly cooperative mode of binding of the ligand. The outcome is that the release generates a rapid transient peak in  $[Ca^{2+}]_i$  with half of the stored  $Ca^{2+}$  being released within 200ms after addition of saturating ( $1\mu M$ )  $IP_3$  [37]. Although the role of  $IP_3$  in the initial  $Ca^{2+}$  release is evident, it is still not clear how  $Ca^{2+}$  stores are maintained depleted for long periods, sustaining activation of  $Ca^{2+}$  influx. Indeed, Putney showed that if  $Ca^{2+}$  stores were kept in a depleted state,  $Ca^{2+}$  influx was maintained even though average  $IP_3$  levels have returned to near basal levels [38]. One possible explanation introduced by Guse and colleagues, was that cyclic adenosine diphosphoribose (cADPR) takes over  $IP_3$  in keeping the stores empty by activating the ryanodine receptor (RyR), another ER  $Ca^{2+}$  release channel. While  $IP_3$  acts in the initial phase of  $Ca^{2+}$  signaling, cADPR is involved in both initial and subsequent sustained phases [39]. An alternative possibility is that local  $IP_3$  generation remains sufficiently high to keep the  $IP_3$  receptor channel open. It is also possible that once depleted by  $IP_3$ , the  $Ca^{2+}$  stores cannot refill, due to a massive  $Ca^{2+}$  leak, as was demonstrated during adhesion-induced T cell priming [40].

Moreover, a role for nicotinic acid adenine dinucleotide phosphate (NAADP<sup>+</sup>) as a  $Ca^{2+}$  mobilizing compound in T cells, has been described [41]. The experiments established that NAADP<sup>+</sup> acts as a first messenger to provide a  $Ca^{2+}$  trigger which then activates both  $IP_3$ - and cADPR -mediated  $Ca^{2+}$ -signaling, in a  $Ca^{2+}$ -induced  $Ca^{2+}$ -release mechanism. More recently, Langhorst and colleagues, using combined microinjection and single cell calcium imaging, show data that demonstrate that in T cells RyRs are quantitatively the major intracellular  $Ca^{2+}$  release channels involved in  $Ca^{2+}$  mobilization by NAADP<sup>+</sup>, and that such  $Ca^{2+}$  release events are largely amplified by  $Ca^{2+}$  entry [42].

In resting T lymphocytes, due to the thin cytoplasmic compartment, the amount of  $Ca^{2+}$  found in intracellular stores is usually extremely low. Since the amplitude of  $Ca^{2+}$  release is small, sustaining the  $Ca^{2+}$  response is mainly achieved by  $Ca^{2+}$  influx, through store-operated  $Ca^{2+}$  channels, named CRAC ( $Ca^{2+}$  release-activated  $Ca^{2+}$  channels). These are highly  $Ca^{2+}$ -selective and essential for generating the prolonged  $[Ca^{2+}]_i$  elevation required for the expression of T-cell activation genes. Although considerable efforts have been focusing on the elucidation of the molecular mechanism of store-operated  $Ca^{2+}$  entry, important questions remain and the models proposed are not fully satisfactory (see [43] for a review).

Lymphocyte activation does not lead to a unique outcome. There are multiple processes that originate from an intracellular  $Ca^{2+}$  increase, being enzymatic activation, cytoskeleton reorganization and exocytosis the nearly immediate, and gene activation the more delayed. A central but elusive aim is to know how different patterns of  $Ca^{2+}$  signaling can lead to such diverse and selective effects, namely the activation of different

transcription factors. The nuclear factor for activated T cells (NFAT), a transcription factor that regulates the expression of several T-cell proliferation associated genes including IL-2 (interleukin-2), is established as the main target in sensing  $[Ca^{2+}]_i$  oscillations.  $Ca^{2+}$  activates the phosphatase calcineurin, which in turn dephosphorylates and activates NFAT [44].

Studies performed in Jurkat T cell line and on rat basophilic leukaemia cells showed the relevance of  $Ca^{2+}$  oscillations on gene activation [45, 46]. The results suggested that for low levels of stimulation, oscillations in  $[Ca^{2+}]_i$  are more efficient in activating NFAT. Hence,  $[Ca^{2+}]_i$  oscillations may function to maximize the sensitivity of low antigen concentration. Moreover, the frequency of the oscillations appears to direct cells into diverse patterns of activation.

#### 1.2.1.2. Coupling TCR to PLC $\gamma$ 1 activity

Mobilization of  $Ca^{2+}$  via activation of PLC $\gamma$ 1 is crucial for the activation of lymphocytes by antigens (Ag) and consequently the understanding of the mechanism of PLC $\gamma$ 1 activation following TCR engagement of an extreme importance. T cells predominantly express the PLC $\gamma$ 1 isoform which is ubiquitously expressed, whilst PLC $\gamma$ 2 is limited to cells of hematopoietic lineage [47]. All PLC enzymes contain a split catalytic domain comprised of two conserved regions, termed X and Y. In PLC $\gamma$ , these regions are separated by a regulatory domain which includes a tandem of Src-homology 2 (SH2) domains followed by a single Src-homology 3 (SH3) domain. PLC $\gamma$ 1 contains also at least 5 potential sites for tyrosine phosphorylation [48]. All these features provide PLC $\gamma$ 1 with the ability to interact with many different intracellular proteins.

Triggering of the TCR leads to the recruitment of PLC $\gamma$ 1 towards its substrate at the membrane to the TCR-proximal signaling complex, which is followed by PLC $\gamma$ 1 phosphorylation and activation [49, 50]. Given that the TCR itself has no intrinsic kinase activity, a number of proteins that are involved in the formation of the TCR signaling complex have also been shown to be implicated in the regulation of PLC $\gamma$ 1. The hallmark of PLC $\gamma$ 1 recruitment is its association with the adaptor protein LAT (linker for activation of T cells), through the interaction of PLC $\gamma$ 1 N-terminal SH2 domain with phosphotyrosine 132 of LAT [51]. SLP-76 (SH2-domain containing leukocyte protein of 76 KDa), another adaptor protein is constitutively associated with Gads and after TCR engagement Gads binds to tyrosine-phosphorylated LAT through its SH2 domain [52]. The presence of a

trimolecular SLP-76-Gads-LAT complex generates a platform for the recruitment of multiple signaling molecules, such as PLC $\gamma$ 1, Grb2, Nck, Vav, c-Cbl and Itk [48].

Recently, a model summarizing the current knowledge of PLC $\gamma$ 1 recruitment and activation was put forward. Braiman and colleagues [48], propose that upon TCR triggering, PLC $\gamma$ 1 is recruited to the SLP-76-Gads-LAT complex but, this binding through the N-terminal SH2 domain of PLC $\gamma$ 1 is not sufficient for PLC $\gamma$ 1-LAT association to be kept. The SH3 or the C-terminal SH2 domain of PLC $\gamma$ 1, with the participation of Vav, c-Cbl and SLP-76 are required to stabilize PLC $\gamma$ 1 recruitment, as shown in figure 1. Furthermore, they report that all three PLC $\gamma$ 1 SH domains are necessary for an efficient phosphorylation and activation of PLC $\gamma$ 1 in T cells. These findings are in contradiction with the previous current model, which suggested that the N-terminal SH2 domain of PLC $\gamma$ 1 was both necessary and sufficient for its recruitment and phosphorylation. The molecules and their signaling modules described above will be dealt with in more detail in the following sections.

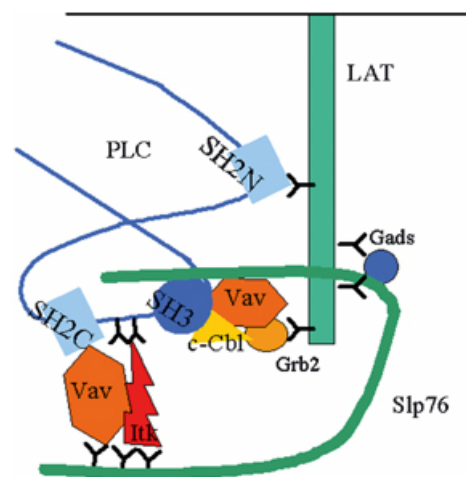


**Figure 1.** Schematic model of PLC $\gamma$ 1 recruitment and activation. Taken from [48].

### 1.2.2. Protein Kinase C- $\theta$ (PKC $\theta$ ) functions

The importance of PKC $\theta$  in T-cell biology stems from the fact that pharmacological agents, such as phorbol esters synergize with calcium ionophores that respectively activate PKC $\theta$  and elevate calcium levels, are able to mimic many aspects of antigen receptor triggering. Another relevant aspect is that upon TCR activation the DAG produced, resulting from PLC $\gamma$ 1 activity, is a PKC-activating second messenger [53]. T

cells express several PKC isotypes, but PKC $\theta$  found in T cells and in muscle cells, has an expression profile that is unique and distinct from all other PKC [54].

PKC $\theta$  is a crucial serine/threonine kinase which has been implicated in the regulation of important transcription factors involved in T cell activation and the production of T cell survival factors such as IL-2. The transcription factor activator protein-1 (AP-1) is composed of two protein components: Jun and Fos. Jun proteins are in the cytoplasm of resting cells and translocate to the nucleus after being phosphorylated by c-Jun N-terminal kinase (JNK) where they bind Fos to form AP-1 complex. It was shown that PKC $\theta$  is able to activate AP-1, which has an important role in the induction of genes such as the IL-2 gene. More specifically, it was reported that PKC $\theta$  is capable of activating JNK leading to AP-1 activation, but the physiological significance of this activation is unclear given that PKC $\theta$ -deficient mice show normal TCR/CD28-induced JNK activation. Another transcription factor activated by PKC $\theta$  is NFAT; this conclusion came from the observation that in one of the two knockout mice for PKC $\theta$  shows a deficient mobilization of NFAT. The final pathway in which PKC $\theta$  is reported to be involved is that of NF- $\kappa$ B activation. Both PKC $\theta$ -deficient mouse strains show a deficient stimulation of both AP-1 and NF- $\kappa$ B leading to a severe defect in TCR/CD28-induced IL-2 production and proliferation [53, 55]. PKC $\theta$  is therefore an essential regulator of several pathways critical to T cell activation ultimately affecting IL-2 production.

### 1.2.3. Ras regulation in T cells

The general interest in Ras stems from the fact that it was early on recognized as an oncogene, regulating pathways that lead to cellular proliferation and survival. Lymphocytes are among the cells in which the function of Ras has been extensively studied [56]. In fact, the guanine nucleotide binding protein, Ras, was shown to accumulate in its active GTP-bound form after TCR engagement [57]. Ras has also been shown to be required for thymocyte development, T cell proliferation and IL-2 production [58]. Like for all GTPases, the binding cycle of Ras is controlled by two main classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) that promote the formation of active GTP-bound Ras complexes, and GTPase activating proteins (GAPs) which stimulate GTPase inactivation [59]. The Ras GEF Sos, the mammalian homologue of the *Drosophila* "Son of Sevenless" protein, associates constitutively with the SH3 domain of an adaptor molecule Grb2 [60]. In activated T cells, tyrosine-phosphorylated LAT interacts with SH2 domain of Grb2, thereby forming complexes that regulate the

membrane localization and catalytic activity of Sos. Phosphorylated LAT also acts as the scaffold of other signaling pathways: it can bind the SH2 domain of PLC $\gamma$ 1, possibly coupling the two pathways [51, 59]. The recognition of another Ras GEF in lymphocytes, Ras guanyl nucleotide-releasing protein (GRP), which has a DAG/phorbol-ester binding domain, supports a model where Ras is activated via DAG recruitment of Ras GRP. Several lines of evidence suggest that this model is not completely satisfactory and it may be that depending on the cell type, either DAG/GRP or Grb2/Sos pathways are more relevant [59].

Once Ras is activated it couples to several effector signaling pathways, the best characterized being Raf-1/MEK/Erk1,2 pathway. Raf-1, a serine/threonine kinase, is recruited to the plasma membrane where it is activated and subsequently activates MEK (MAPK/Erk kinase) that triggers the mitogen-activated protein kinases (MAPKs) Erk1 and Erk2 [56]. However, the Raf-1/MEK/Erk pathway is not the only Ras effector signaling pathway. Activated Ras also couples to signaling pathways controlled by the Rac/Rho GTPases [59]. Moreover, several Ras effectors, proteins whose function is regulated by the binding of GTP-Ras, have been described. This is the case of phosphatidylinositol 3-kinase (PI3K), which activates Akt thus promoting cell survival [56].

In peripheral T cells, the ultimate Ras function is to activate transcription factors that are involved in cytokine gene induction, including AP-1 and NFAT. It is now evident that the plasma membrane is not the only platform for Ras/MAPK signaling. This signaling pathway has also been viewed on endosomes, the ER, the Golgi apparatus and mitochondria but it appears that the Golgi apparatus is the primary platform for signaling. Studies with a spatiotemporal fluorescent probe revealed that the pathway of activation on the Golgi involves PLC $\gamma$  and Ras GRP1 and thereby differs from the Grb2/Sos-mediated pathway for Ras activation on the plasma membrane. Thus, compartmentalized signaling can be accomplished by using distinct upstream pathways [56].

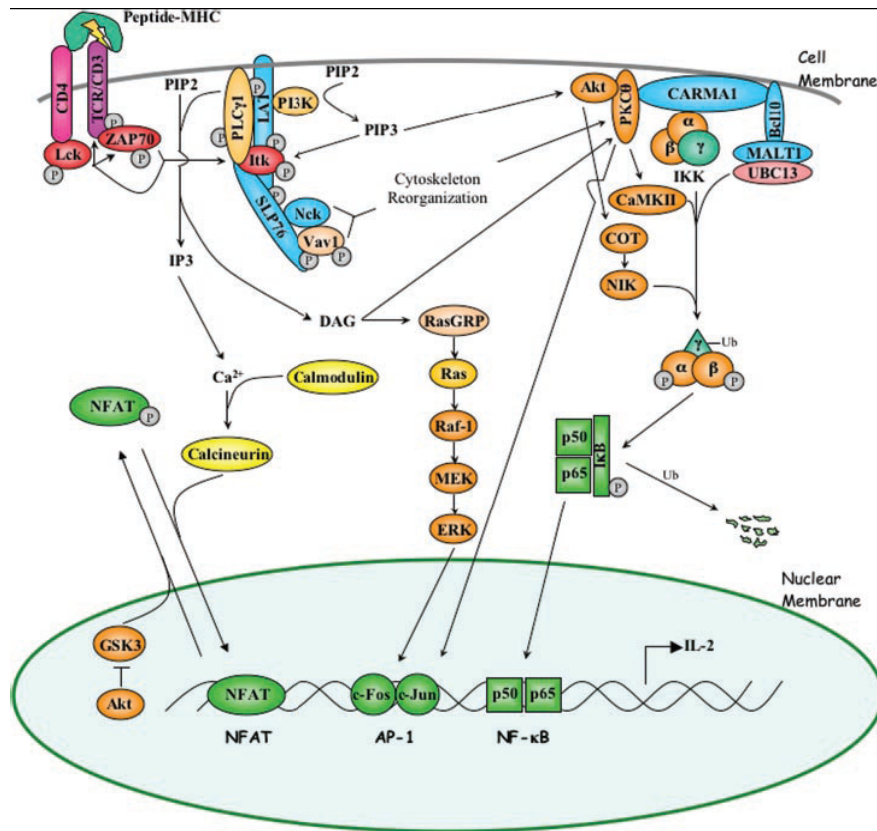


**Figure 2.** T cell receptor signaling events leading to activation of transcription factors. This figure presents an overview of some of the key signaling events triggered by the binding of peptide- MHC to the T cell receptor (TCR/ CD3). Taken from [61].

### 1.3. Non-receptor molecules and modules involved in signal transduction in T cells

Proximal events that immediately follow TCR triggering include the activation of protein kinases and phosphorylation of many substrates, including the TCR/CD3 complex itself, and the assembly of signaling pathways and networks, that involve numerous enzymes and adaptor proteins. It is now indisputably accepted the idea that specific protein-protein interactions control numerous cellular processes. Other than temporal and spatial factors, the specificity of pairwise binding events and the subsequent protein networks established, largely determine the specificity of signal transduction.

### 1.3.1. Protein interaction domains: Src homology domains

Generally, regulatory proteins are composed of domains, in a cassette-like fashion, that have enzymatic activity or mediate molecular interactions. The latter, the modular interacting domains, are conserved regions specialized in mediating interactions between proteins or between proteins and other molecules such as lipids or nucleic acids. By doing so, they can target proteins to a specific subcellular location, confer a means for recognition of protein posttranslational modifications, centre the establishment of multiprotein complexes and control the conformation, activity and substrate specificity of enzymes, hence directing specific cellular responses to external signals [62]. Among all protein interaction modules, the Src homology domains are one of the best characterized members. These domains were first recognized as regions of sequence similarity between the non-catalytic domains of the Src family of tyrosine kinases and the divergent signaling proteins Crk and PLC $\gamma$ 1 [63, 64]. Since these modules were first noted in proteins of the Src family they were designated Src-homology 2 (SH2) and Src-homology 3 (SH3) domains [65].

SH2 domains are modules of about one hundred amino acids that in addition to their common ability to bind to phosphotyrosine recognize between 3–6 residues C-terminal to the phosphorylated tyrosines in a fashion that differs from one SH2 domain to another. The SH2 domain comprises a central anti-parallel  $\beta$ -sheet flanked by two  $\alpha$ -helices which form a positively charged pocket on one side of the  $\beta$ -sheet for binding of the phosphotyrosine moiety of the ligand and an extended surface on the other for binding residues C-terminal to the phosphotyrosine [66]. Indeed, specificity of SH2 domains seems to be determined by the recognition of these C-terminal residues. For instance, it was shown by Songyang and colleagues [67] that the SH2 domains of the PI3K bind preferentially to phosphorylated YMXM motif, while the Src SH2 domain bound to an optimal phosphopeptide with a YEEI motif. The same author also demonstrated that Grb2 SH2 domain binds phosphorylated YXN sequences [68]. The specificity can also be increased if proteins have two SH2 domains in tandem, such is the case of ZAP-70 (zeta-chain-associated protein 70), SHIP2 and PLC $\gamma$ 1 [66].

After TCR engagement, tyrosine phosphorylation of receptor tyrosine kinases (RTK) acts as a switch to recruit SH2-containing targets. These targets can function by simply binding to the receptor, and therefore, juxtaposing them next to membrane associated substrates. Illustrating this re-location is PLC $\gamma$ 1, PI3K and Ras GAP, that after binding through their SH2 domains to the cytoplasmic tail of RTK come closer to the membrane, where their substrates are located [69]. In addition, SH2 domains can function to regulate enzymes activity through intramolecular interactions. In the case of

autoinhibited Src-family kinases, the SH2 domain binds to a C-terminal phosphotyrosine site, positioning the SH3 domain to recognize the linker sequence between the SH2 and kinase domains [70-72]. One more function of the SH2-containing protein once bound to the receptor is simply to become a substrate for phosphorylation, resulting either in a conformational change or in the creation of docking sites for additional SH2 proteins.

SH3 domains are a group of small interaction modules of about sixty amino acids that are ubiquitous in eukaryotes. These domains were initially shown to always bind to proline-rich sequences and the PXXP was identified as the core conserved binding motif [73]. The SH3 domains have a relatively flat hydrophobic binding surface that contains three shallow pockets of which two are occupied by the two hydrophobic prolines in the PXXP motif whereas the third is negatively charged and interacts with a basic residue either NH<sub>2</sub>- or COOH-terminal of the PXXP core [74]. A number of SH3 domains have also been shown to recognize a (R/K)XX(R/K) motif, where positively charged residues appear to play a dominant role. These include SH3 domains from PLC $\gamma$ 1, Grb2 and Nck [75].

These domains can regulate a variety of biological activities as diverse as signal transduction, protein and vesicle trafficking, cytoskeletal organization, cell polarization and organelle biogenesis [76, 77]. Their function is in several circumstances overlapping to those of the SH2 domains. Although the affinity of SH3 domains for their ligands is relatively low and selectivity is generally marginal, SH3 domains show specific biologically significant interactions. One of the best examples of a specific SH3-mediated intermolecular interaction, which is also evidence of their function as protein re-locators, is the role of Grb2 in Ras activation. Grb2 has a SH2 domain flanked by two SH3 domains that bind to the proline-rich tail of Sos. Sos is recruited by Grb2 to the tyrosine phosphorylated sites at the membrane, where Ras (the substrate) is located. For that reason, Grb2 couples tyrosine phosphorylation to the activation of Ras [78]. An illustration of the importance of intramolecular SH3-mediated interactions comes from the regulation of the Src family of kinases (mentioned above). In this case SH2 and SH3 domains together with phosphorylation of at least two regulatory sites are involved in complex regulatory interactions, such as a delicate conformational switch.

Since the interaction between an SH3 and a proline-rich sequence is relatively weak, and that these domains are able to interact with several different ligands, it has been suggested that affinity and selectivity could result from the collective contribution of sub-optimal interactions [75]. Providing an alternative for increasing specificity are factors such as cellular context, co-operative events and subcellular localization. The importance of the latter is depicted by the CD2BP2 (CD2-binding protein). In T cells, Fyn SH3 domain binds to the same proline-rich motifs in CD2 cytoplasmic tail as does the GYF domain of

CD2BP2. However, these potential competitors are segregated since CD2BP2 associates with CD2 outside lipid rafts (membrane microdomains discussed latter) while Fyn is present only in lipid rafts to which CD2 is translocated upon its crosslinking [79]. Affinity and specificity can also be achieved by the recognition of residues outside the conventional PXXP motif and, as for SH2 domains, by the tandem organization of these domains. Although posing a challenge for the explanation of signaling specificity, the weak and promiscuous interactions of SH3 domains can be valuable in circumstances where the formation and dissolution of signaling complexes have to take place rapidly to favour the dynamic flux of protein networks [75].

### 1.3.2. The organization of membrane proximal TCR signaling: implicated protein tyrosine kinases and phosphatases

The molecular aspects of signal transduction and cellular activation have been the centre of attention of many immunologists during the last decade. On the other hand, interconnecting a given biochemical mechanism with a physiological phenomenon is an essential but in many cases still elusive objective. Important questions regarding how molecules function within the immune cells have and will continue to be addressed with the use of improved research technologies. Among these questions are the functions of several protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) in the early T cell signaling events.

Although much of the past work as focused on the PTK-mediated signaling, it is becoming obvious that many PTP play equally important roles in T cells and that the subtle equilibrium required for an appropriate effector function results from the fine-tuned regulation of tyrosine kinases and phosphatases. This balance between kinases and phosphatases can also be perceived from the human genome that encodes the same number of PTK and their counterbalanced, PTP (approximately 90 genes) [80, 81]. In T cells, upon antigen receptor stimulation there is a rapid wave of tyrosine phosphorylation that involves the coordinated action of several protein tyrosine kinases that include members of the Src, Syk, Csk and Tec families [28, 82].

#### 1.3.2.1. Structure, function and regulation of Src family kinases

The first family of PTK to be recruited and activated after TCR engagement is the Src family kinases, namely Lck and Fyn. These two kinases have a domain design similar

to the other Src family kinases: N-terminal attachment sites for saturated fatty acids addition, a unique region, an SH3 domain, an SH2 domain, a tyrosine kinase domain, and a C-terminal negative regulatory domain [83]. Lck and Fyn, are inserted into the inner membrane leaflet through the cotranslational fusion of myristate to the glycine at position 2, after the start methionine is removed [84]. Besides being myristoylated these two Src family kinases also undergo reversible palmitoylation at cysteine 3 and at cysteine 5 in Lck or cysteine 6 in Fyn [85, 86]. The lipid modifications and consequent localization in lipid rafts, specialized plasma membrane microdomains enriched in glycosphingolipids, sphingomyelin and cholesterol (discussed below), seem crucial for the effective function of Lck, as a non-acylated form of the kinase failed to phosphorylate the TCR/CD3 complex [87].

The function of the unique domain of most Src kinases is still largely unknown with the exception of that of Lck and Fyn. In Lck, it is responsible for a specific interaction with co-receptors CD4 and CD8, through a dicysteine motif (CXXC) present in Lck unique N-terminal domain and two cysteines (CXCP motif) in the cytoplasmic tail of CD4 and CD8 [88]. Recent data suggest that these residues direct a high-affinity binding via coordination of a  $Zn^{2+}$  ion, which is critical for creating an ordered stabilized structure [89]. The unique domain of Fyn has been shown to be involved in the association of the kinase with subunits of the TCR/CD3 complex [90].

The SH3 and SH2 domains of the kinases that bind to polyproline motifs and to specific phosphotyrosine motifs, respectively, are implicated in both intramolecular and intermolecular regulation of kinase functions. Nevertheless, the kinase domain also as a role in the regulation of kinase activity, as it contains itself two critical residues, an autophosphorylation site, necessary for kinase activation, and a C-terminal tyrosine that negatively regulates the kinase activity [91]. After being phosphorylated mainly by Csk (C-terminal kinase), C-terminal tyrosine residues (Y505 on Lck and Y528 on Fyn) create an intramolecular binding motif for the SH2 domain, placing the kinase in a close/inactive conformation. Csk is recruited to the membrane by its interaction with the adaptor PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains; also known as Cbp for Csk binding protein) and becomes itself phosphorylated by the Src kinases. Upon TCR stimulation PAG is transiently dephosphorylated, releasing Csk from its membrane anchor and form the proximity of Lck, facilitating and/or sustaining the activation of Lck and Fyn [82, 92-94].

This mode of regulation likely applies to Lck and Fyn but, as mentioned above, the activity of these kinases also seems to depend on the phosphorylation status of the activation loop tyrosine (Y394 on Lck and Y417 in Fyn) on the kinase domain [82, 95, 96]. LYP/PEP, a potent phosphatase with a negative regulatory role in TCR signaling, binds to

the SH3 domain of Csk that through its interaction with PAG is brought to the vicinity of Lck and Fyn in lipid rafts, where it dephosphorylates Y394 on Lck and Y417 on Fyn. Thus, PEP dephosphorylation of activating tyrosine concomitantly with phosphorylation of the C-terminal inhibitory tyrosine by Csk are thought to synergize in inactivating Src family kinases [81, 97, 98].

The negative effect imposed by Csk and PEP is counterbalanced by the activity of a phosphatase, CD45. In this sense, it is known that in normal thymocytes and T cells, CD45 keeps the inhibitory tyrosine specifically dephosphorylated, rendering Lck in a primed state. In agreement with this evidence, it was proposed that an equilibrium state between conformationally different Lck molecules may exist: (1) open and activated (phosphorylated on Y394), (2) open and not activated or primed and (3) closed and not activated [99]. Together with several studies showing that CD45 was essential for tyrosine phosphorylation and PLC activation after TCR engagement, this phosphatase is viewed as a positive regulator of the Src family kinases function in T cell signaling [81].

The specific recognition of an antigen in the context of an MHC molecule groove by the TCR involves a further stabilization of the complex by the binding of CD4 and CD8 receptors to the nonvariable regions of MHC. This results in the recruitment of Lck via its noncovalent association with CD4 or CD8 to the complex, which positions the kinase to phosphorylate tyrosine residues within ITAMs [83]. This view has however been confronted with studies suggesting that it is Lck SH2 domain that mediates the recruitment of CD4 or CD8 to the TCR complex [100]. More recently, it was reported that the adaptor Unc119 associates with CD3 and CD4 and activates Lck and Fyn. Recombinant Unc119 binds to the SH3 domain of Lck and Fyn, perhaps facilitating an open and active conformation [101]. Accordingly, CD4 and CD8 possibly in concert with Unc119 may be involved in the recruitment and clustering of Lck and/or Fyn with  $\xi$  and CD3 ITAMs. However similar in structure, several lines of evidence support the idea that Lck and Fyn have distinct but yet overlapping functions. Both kinases function equally well in phosphorylating  $\xi$  chains in TCR-mediated naïve T-cell survival, however, only Lck is capable of TCR-dependent homeostatic proliferation of naïve T cells. Moreover, Lck seems to have a greater and more specific role throughout T cell development and function, while Fyn appears to be less critical (reviewd in [83]).

The function and the mechanism of activation of each kinase may also be regulated by their different lipid raft localization. As was recently suggested by Filipp and colleagues, after TCR and CD4 co-crosslinking, Lck is initially activated outside rafts and then translocates to lipid rafts where it activates the resident Fyn [102]. This concept is further supported by the observation that in resting cells the fraction of Lck found in the

rafts is in a close and inactive conformation, and upon cell activation the open/active form of Lck is most abundant in the rafts [103].

### 1.3.2.2. Structure, function and regulation of Syk family kinases

The Src family kinases have two major roles in the propagation and amplification of T cell receptor signaling: they phosphorylate ITAM domains in the TCR/CD3 complex and then they phosphorylate and activate Syk family kinases that are recruited to the ITAMs via their tandem SH2 domains. Syk family members expressed in T cells include ZAP-70, whose expression is restricted to T cells and natural killer (NK) cells [104] and Syk, that is widely expressed in the hematopoietic lineage [105]. The crystal structure of the tandem SH2 domains of Syk or ZAP-70 complexed with a dually phosphorylated ITAM peptide revealed that each of SH2 domains of Syk can function as an independent unit [106], whereas for ZAP-70, the two SH2 domains bind cooperatively [107].

Once recruited, ZAP-70 activation requires the phosphorylation of Y493 on its activation loop by the Src family kinases [108]. Upon phosphorylation at Y493, the bound ZAP-70 molecules are able to autophosphorylate, possibly *in trans*, to produce docking sites for SH2-containing signaling proteins [109]. Syk, on the other hand, is able to trans-autophosphorylate and activate itself, even though its activation is facilitated by Src kinases [110, 111]. Activation of the Syk kinases, mostly of ZAP-70 is an important step in instigating downstream signaling cascades [82]. In fact, a crucial substrate of ZAP-70 is the adaptor molecule LAT that, during T cell signaling, acts as a membrane scaffold, important for the assembly of numerous signaling complexes. Another adaptor that is also known to be a ZAP-70 substrate is SLP-76. Together these molecules couple the TCR to PLC $\gamma$ 1, Ca<sup>2+</sup> and Ras signaling [50, 112, 113]. Besides the substrates, several SH2-containing proteins bind phosphorylated ZAP-70 and Syk, these include Lck, Fyn, PLC $\gamma$ 1, Vav-1, Ras GAP and c-Cbl, and it is assumed these molecules produce a platform to create physical proximity and facilitate their tyrosine phosphorylation by the Syk and the Src family kinases [82].

Dephosphorylation of ZAP-70 and Syk is thought to be achieved by the action of two PTP: SH2-containing SHP-1 and LYP/PEP. SHP-1, probably the most studied PTP, is known to have a negative role in T cell activation. In fact, the importance of this phosphatase comes from the observation that thymocytes from mice with *motheaten* mutation [114], which results in a homozygous loss of SHP-1, are hyperresponsive to TCR stimulation. This negative regulation is at least in part mediated by a direct dephosphorylation of ZAP-70 [115]. LYP/PEP, in addition to its role in the regulation of

Src family kinase activity also dephosphorylates ZAP-70 possibly through its association with c-Cbl. The mechanisms that regulate LYP/PEP targeting to TCR associated PTK are illustrated on figure 3 but remain largely unknown [81].

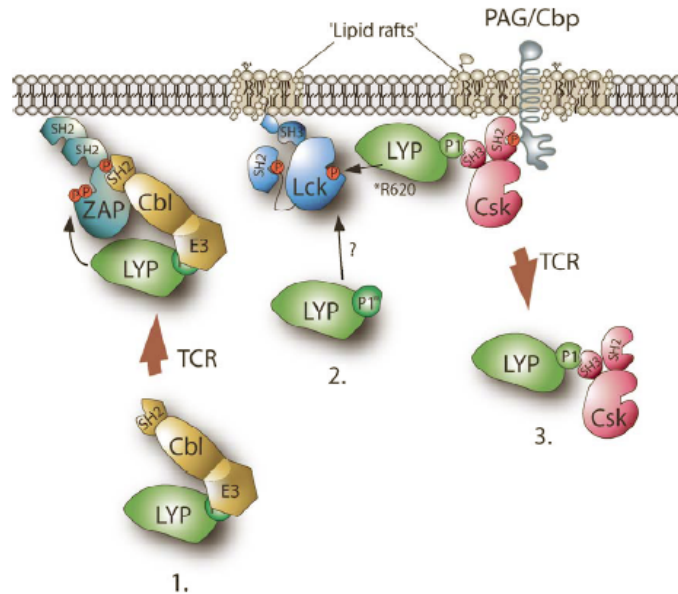


**Figure 3.** Schematic model for the three distinct ways that LYP (in green) can target TCR associated PTKs: (1) association with ZAP-70 through c-Cbl, (2) LYP may target PTKs directly, (3) after association with the SH3 domain of Csk is targeted to the PAG/Cbp, which is located in lipid rafts in the vicinity of Lck and other Src family PTKs. Taken from [81].

#### 1.3.2.3. Structure, function and regulation of Tec family kinases

The next step in TCR signal transduction is the activation of the Tec family kinases, namely three major found in T cells: Tec, Itk and Rlk. These Tec family members resemble Src family kinases in their domain organization, in the sense that they include a tyrosine kinase domain and a single SH2 and SH3 domains. However, the different subcellular localization of the Tec kinases is dictated by the presence of a pleckstrin homology (PH) domain responsible for their interaction with PI3K products. Following T cell activation, the phosphatidylinositol (3,4,5) triphosphate levels increase considerably targeting these kinases to the membrane. Rlk is an exception since it lacks a PH domain but in addition it contains a cysteine-string motif that after being palmitoylated targets Rlk to lipid rafts [116]. Once at the membrane, Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity by inducing autophosphorylation events [117].

In T cells, mice mutations affecting Itk or Itk and Rlk lead to mild perturbations of T cell responses namely decreased responses to TCR stimulation, including proliferation, IL-2 production, and production of effector cytokines. Nevertheless, these mice show severe defects in TCR-mediated phosphorylation and activation of PLC $\gamma$ 1 [116, 118, 119], establishing Itk as an intermediate in the pathway from the TCR to PLC $\gamma$ 1. Itk is able to directly associate with PLC $\gamma$ 1 [120] nevertheless, other mediators of this pathway have been elucidated. Specifically, Itk can directly associate with SLP-76 via cooperative binding of Itk-SH3 domain and Itk-SH2 domain that selectively binds SLP-76 in activated T cells. LAT, also known to be an essential mediator of this pathway, associates with Itk, although it is still not clear if this association is direct or indirectly mediated by Grb2 or SLP-76 [116]. ZAP-70 has been shown to be important in the regulation of Itk activation following TCR engagement through the phosphorylation of its substrate LAT [121]. All these findings are consistent with a role for Tec kinases in the formation of the trimolecular SLP-76-Gads-LAT complex described before in the regulation of PLC $\gamma$ 1 activity [48].

Besides the role of Tec family kinases as regulators of PLC $\gamma$ 1, recent data support a function for Tec kinase Itk in the regulation of polymerization and polarization downstream of TCR triggering. T cells from Itk-deficient mice, as well as Jurkat cells expressing mutant forms of Itk, are defective in actin polymerization, conjugate formation and localized activation of Cdc42 and WASP (a key actin regulatory protein) in response to antigen-pulsed APCs. It has been suggested that this function of Itk in TCR-mediated actin polarization is kinase-independent [122, 123]. Itk appears to be involved particularly in signaling through CD28 and CD2 [116].

A potential mechanism for downregulating Itk activity functions via its requirement to colocalize with the LAT/SLP-76 adapter complex. It was shown that overexpression of the tyrosine phosphatase CD148 leads to reduced phosphorylation of LAT and PLC- $\gamma$ 1 in activated Jurkat T cells and it is expected that this pathway downregulates Itk activity, further attenuating the signals downstream of the TCR [124].

#### 1.4. Accessory receptors involved in signal transduction in T cells

The first signal which confers specificity is provided by the interaction of the TCR with pMHC complex on APCs. This signal alone, although essential, is not sufficient to initiate transcriptional activation and mount an effective immune response [125]. The orchestration of accessory receptor-ligand interactions is required to direct, modulate and

fine-tune T cell receptor signals. These receptors may also play a role in the formation and stabilization of a highly organized supra-molecular signaling platform currently known as the immunological synapse (IS) (discussed in 2.1.).

#### 1.4.1. CD28 costimulatory receptor

It is well recognized that naive T lymphocytes require two distinct signals from APCs to be functionally activated: one provided by the TCR/CD3 complex and a second one generated by the engagement of costimulatory molecules [126, 127]. Several lines of evidence report that inhibition of costimulatory molecules at the cell surface or their intracellular signal transduction leads to T-cell unresponsiveness or anergy [128, 129].

Numerous costimulatory molecules have been described but CD28 is the classical illustrative example. CD28 is constitutively expressed on the surface of 95% of CD4<sup>+</sup> human T lymphocytes and of 50% of CD8<sup>+</sup> T cells [130], and on most mouse T cells [131]. It is a 44 KDa transmembrane protein that belongs to the IgSF (immunoglobulin superfamily) as do its ligands, the B7 molecules CD80 (B7-1) and CD86 (B7-2) [132, 133]. CD28 costimulation has been implicated in a wide variety of T-cell responses, including T cell proliferation, IL-2 production, prevention of anergy and induction of the antiapoptotic factor Bcl-xL [134, 135]. It has been suggested that CD28 induces cytokine gene transcription via the activation of the MAP kinase, JNK, and of the transcription factor NF- $\kappa$ B, however co-engagement of the TCR is still required for this activation [136]. Furthermore it is proposed that CD28 crosslinking reduces the number of TCRs that need to be engaged, as well as the time required for T cells to interact with antigen, and enhances the magnitude of the T cell response to low levels of TCR occupancy [137].

Although CD28 lacks enzymatic activity, it is capable of becoming tyrosine phosphorylated by Src family kinases after its crosslinking, resulting in an increase association with PI3K, Itk and Grb2 [138]. CD28 receptor engagement induces tyrosine phosphorylation and activation of Itk [116]. A study by Michel *et al.* suggests that Itk activation downstream of CD28 is involved in the amplification of TCR-mediated signals, such as PLC $\gamma$ 1 phosphorylation, leading to Ca<sup>2+</sup> mobilization [139]. Contrarily, and suggesting a negative regulatory role for Itk in CD28 signaling, is a different study showing increased responsiveness of Itk-deficient T cells to anti-CD28 stimulation [140]. Hence, the specific role of Itk in CD28 signaling awaits further elucidation. The roles of PI3K and Grb2 in CD28 signaling are also still not clear. The colligation of TCR and CD28 results in the synergistic and sustained phosphorylation and membrane localization of Vav [141]

that possible links to Gbr2 via SLP-76 and together will ultimately lead to IL-2 gene transcription [142].

Recently, CD28 was reported to accumulate at the IS by the selective recruitment of B7-2 [143, 144] and it was additionally suggested that CD28 recruits Lck to the synapse where it phosphorylates  $\xi$  chains of the TCR/CD3 complex and by this means sustains TCR signals [145-147].

The CD28 homologue ICOS, which is expressed on activated T cells and binds to a B7 family member named ICOSL, is upregulated on T cells after activation [148-150]. The ICOS and CD28 have unique and overlapping functions that synergize to regulate CD4<sup>+</sup> T cell differentiation. Like CD28, ICOS augments T-cell differentiation and cytokine production and provides critical signals for immunoglobulin production. ICOS signals are important for the regulation of cytokine production by recently activated and effector T cells: it can regulate both T helper 1 and T helper 2 effector cytokine production, and it has a particularly important role in regulating IL-10 production [151].

CTLA-4 is another member of the CD28 family and along with its homologue, CD28, is a B7-binding protein. CTLA-4 functions both by scavenging CD80/86 ligands away from CD28 and by direct negative signaling to T cells [134], thus, playing an important role in the downregulation of both CD28 and ICOS mediated signaling [152, 153]. Therefore, superimposition of inhibitory signals, such as those delivered by CTLA-4, leads to a complex network of positive and negative costimulatory signals, that will be integrated to modulate immune responses.

#### 1.4.2. CD4 and CD8

The assembly of a signaling competent complex involves the engagement not only of the TCR and pMHC but also of the CD4 or CD8 co-receptors and additional signaling molecules. CD4 and CD8 are expressed on mutually exclusive populations of T lymphocytes and recognize class II and class I MHCs, respectively, on target cells. The CD4 co-receptor is normally associated with helper T cells function whereas CD8 relates to cytotoxic T cells function [154, 155].

CD4 is a 55 KDa single chain type I transmembrane glycoprotein with four extracellular Ig-like domains, a single transmembrane region and a short cytoplasmic tail [156]. CD8 can be expressed as a disulphide-linked heterodimer ( $\alpha\beta$ ) or as a homodimer ( $\alpha\alpha$ ) at the surface of T lymphocytes. Both isoforms are differentially expressed and have distinct functional behaviours [157]. The CD8 $\alpha\alpha$  homodimer binds primarily to the  $\alpha_3$

domain of MHC molecule in an antibody-like fashion, but the position of CD8 $\alpha\alpha$ /MHC scaffold relative to the TCR is unresolved as the structure and relative conformation of the C- terminal stalk that attach the molecule to the T cell surface are still unclear [158, 159]. The crystal structure of CD8 $\alpha\beta$  heterodimer has not yet been resolved and the predicted models have used information from CD8 $\alpha\alpha$  mutational studies and take into account the different stalk lengths of the  $\alpha$  and  $\beta$  subunits. However, to understand the structural basis of the different isoforms functions, the determination of the crystal structure of CD8 $\alpha\beta$  in complex with pMHC is essential [6, 160]. CD8 was shown to be absolutely required for binding of weak agonist ligands and aided the binding of strong agonist ligands, moreover it has been suggested that CD8 molecules may directly facilitate TCR-pMHC binding, since certain anti-CD8 antibodies were shown to increase TCR-pMHC interactions [161].

In contrast to CD8 the extremely weak pMHCII/CD4 interaction does not stabilize TCR-pMHCII interactions or contribute to cognate tetramer binding. For this reason it is assumed that CD4 does not affect pMHC binding [162, 163]. This view has recently been challenged by the observation that some anti-CD4 antibodies block pMHCII tetramer binding and that these effects have a parallel in T cell activation assays [164].

#### 1.4.3. CD6

CD6, a monomeric 100-130 kDa type I cell surface glycoprotein, whose expression is largely restricted to peripheral T lymphocytes and medullary thymocytes, has been known to play a role in T cell activation [165]. CD6, like CD5, belongs to the scavenger receptor cysteine rich (SRCR) superfamily where these accessory receptors are the most closely related members, having similar domain organization including three extracellular SRCR domains [166]. The human *Cd6* gene is located in chromosome 11, region 11q13, contiguous to the *Cd5* gene, supporting the idea that both genes are likely to have arisen from the duplication of a common ancestral gene [167]. Human, rat and mouse *Cd6* genes contain thirteen exons: the first six exons code for most of the extracellular domain, exon 7 codes for the transmembrane domain and the last six code for the cytoplasmic tail. Different CD6 isoforms have been described arising from alternative splicing of exons coding for sequences within the cytoplasmic domain [167-170], but so far no specific physiological function has been attributed to any of these isoforms.

Although several studies point towards a role for CD6 as a costimulatory molecule in T cell activation, no precise function has been clarified up to now. Treatment of T cells with anti-CD6 antibodies can potentiate the outcome of TCR-induced signaling [171-173].

A role for CD6 in thymocyte maturation has also been suggested, as blocking CD6 reduces the adhesion of thymocytes to thymic epithelial cells [174]. Additionally, it was shown that a CD6 negative sub-population of T cells displayed a lower alloreactivity in mixed leukocyte reactions as compared to a normal CD6<sup>+</sup> T cell population [175], and, more recently, that soluble monomeric CD6 present in antigen-primed mixed leukocyte reactions inhibited antigen-specific T cell responses [176, 177]. On the other hand, it was shown that CD6 expression on immature thymocytes correlates with resistance to apoptosis, implying some inhibitory signaling properties for CD6 during selection [178]. CD6 may as well exert its function via association with CD5, as both receptors were shown to co-immunoprecipitate from Brij 96 lysates of normal and leukemic human T cells [179].

CD166/ALCAM is so far the only known ligand for CD6 on immune cells and the molecular basis of CD6-CD166 interaction has been comprehensively investigated [180]. CD166 is a widely expressed glycoprotein containing five immunoglobulin superfamily (IgSF) domains, of which the N-terminal domain has been shown to bind to the membrane-proximal domain (SRCR 3) of CD6 [181, 182]. Recently, thermodynamic analysis revealed that binding occurs with a relatively strong affinity ( $K_d = 0.4\text{--}1.0\ \mu\text{M}$ ) [177]. The resulting interaction between CD6 and CD166 occurs through lateral binding, an unusual type of interaction as most other T lymphocyte surface proteins mediating cell-cell interactions have a top-on-top binding manner. CD6 physically associates with the TCR/CD3 complex and is able to concentrate in the cSMAC after T cell activation in a Jurkat-Raji model system [176]. More recently, in a resting T cell/dendritic cell (DC) model, it was shown that ALCAM and CD6 are recruited to the IS and are involved in the stabilization of this structure which in turn contributes to both early and later stages of DC-induced T cell activation and proliferation, mimicking CD28 co-stimulatory behaviour [183].

The intracellular pathways elicited by CD6 are still largely unknown. Evidences have shown that CD6 is tyrosine phosphorylated after stimulation with CD3 alone or by crosslinking CD3 with CD2 or CD4 [184] suggesting that interactions with SH2 domain-containing intracellular mediators may occur. In addition, CD6 cytoplasmic tail contains multiple potential binding sites for SH3 domain-containing proteins, Casein Kinase II, and Protein Kinase C-consensus binding sites [173]. The rat homologue of CD6 has indeed been shown to associate with protein tyrosine kinases of different families, namely Src-family kinases Lck and Fyn, ZAP-70 of the Syk family, and the Tec-family kinase Itk [185]. Recently, the first intracellular partner for human CD6, syntenin-1, which functions as an adaptor able to bind cytoskeleton proteins and signaling effectors has been identified [186]. Despite the progress made, important issues regarding intracellular signaling partners and their functional roles remain elusive. Additional research focusing towards

the understanding of the molecular mechanisms underlying CD6-mediated signaling is necessary to establish an unambiguous role for CD6 in T cell activation.

#### 1.4.4. CD2

The human T cell adhesion molecule CD2 is one of the most studied accessory receptors. CD2 is a 45-58 KDa type I integral protein expressed on virtually all T lineage and natural killer cells [156]. The extracellular domain is composed of two immunoglobulin superfamily domains [187], of which the membrane-distal V-like domain is involved in the binding to the ligand [188]. Engagement of CD2 to CD58 in humans, or to CD48 in rodents, facilitates adhesion between T cells and antigen presenting cells (APC) and promotes the formation of an optimal intercellular membrane spacing (~140Å) suitable for TCR-pMHC recognition [189]. CD48 and CD58 are structurally related to CD2, as both molecules have two Ig-like domains and a short stalk and with the N-terminal domain mediating the interaction. The crystal structure of human and rat CD2 as well as of CD2 bound to CD58 have been determined [187, 189-191]. The interaction of CD2 with its ligands was shown to be of low affinity allowing transient multivalent contacts between cells expressing these molecules [192]. In fact, the interaction of rat CD2 with CD48 has a Kd of 65 µM that is largely outside the range values of 5-20 µM found for most leukocyte surface proteins interactions [193, 194]. It has been suggested that specificity depends largely on electrostatic complementarity at the binding surfaces without requiring increases in affinity [189, 195]. Studies on CD2-deficient mice have shown that the CD2-CD48 interaction is not essential for the development or function of an apparently normal immune system [196]. Instead, CD2 has been shown to have a subtle role in setting TCR-affinity thresholds for T cell activation, thus fine-tuning the T cell repertoire [197-199].

Along with its function as an adhesion molecule, CD2 also has a role in the process of signal transduction. Intracellular CD2 signaling pathways and networks have been a matter of extensive research. It has long been known that stimulation of CD2 with combinations of monoclonal antibodies or with the physiological ligand induces T cell proliferation in rats and human [200, 201]. Moreover, ligation of CD2 with CD58 augments the interleukin-12 (IL-12) responsiveness of activated T cells [202], and can also reverse T cell anergy induced by B7 blockage [203]. CD2-mediated activation depends on its cytoplasmic domain [204-207], but as it has no intrinsic enzymatic activity, it relies on physical interactions with other signaling molecules to propagate the stimulus received upon ligand binding. CD2 associates with the Src-family tyrosine kinases Lck and Fyn

[208-210], and through these kinases couples to downstream signal transduction pathways.

Proline-rich sequences of the cytoplasmic domain of CD2 have also been shown to establish interactions with the SH3 domains of several proteins. The interaction between CD2 and CD2AP, mediated by the first SH3 domain of CD2AP, is induced by T cell activation and is required for CD2 clustering and T cell polarization. CD2AP seems to function as a molecular scaffold for receptor patterning and cytoskeletal polarization [211]. Another CD2 cytoplasmic tail-binding protein, termed CD2BP1 has been identified: upon CD2 clustering, the SH3 domain of CD2BP1 binds CD2 and functions as an adapter to recruit cytosolic PTP-PEST, thereby down-regulating adhesion [212]. CD2 additionally binds to the GYF domain of CD2BP2, an interaction involved in cytokine production following CD2 stimulation. Fyn SH3 domain can compete with CD2BP2 as it binds specifically to the GYF domain-binding site of CD2 [213]. The same authors demonstrated that CD2BP2 binds to CD2 outside lipid rafts and is replaced by Fyn after CD2 is translocated into lipid rafts. In contrast to rat and mouse CD2 that are raft-resident molecules [214], human CD2 has been shown to translocate to lipid rafts only upon CD2-antibody crosslinking, or following binding to CD58 during conjugate formation [215]. CD2, like several other costimulatory molecules, has been shown to up-regulate TCR signals by enhancing the association of the TCR with lipid rafts [216].

## 2. Spatial organization

### 2.1. Immunological synapse

The orchestration of Ag-specific immune responses, elicited by the engagement of the TCR with pMHC on the surface of an APC, is performed in a localized environment, called the immunological synapse, and requires the coordinated activities of several TCR-associated molecules, including CD3, the co-receptors CD8 or CD4, and other accessory receptors.

Despite the knowledge gathered for years that T cell activation required physical interaction between the T cell and the APC, the first imaging studies that examined the molecular organization of the contact area were only performed in the late 90's [217-219]. Monks *et al.* (1998) first described the spatial segregation of LFA-1, talin, TCR and PKC- $\theta$  at the contact interface between T cells and antigen presenting B cells: TCR and PKC- $\theta$  are accumulated in the central region, the cSMAC (central supramolecular activation clusters) and are enclosed by a ring structure of LFA-1 and talin, the pSMAC (peripheral

supramolecular activation clusters). The authors also implied that TCR signaling was initiated and sustained by the cSMAC. One year later, Dustin and colleagues [217], termed the bull's eye pattern observed by Monks *et al.* and themselves as “immunological synapse”. Several studies that confirmed the formation of a cSMAC and a pSMAC have also helped discover other constituents of the cSMAC, such as CD2, CD28, Lck, Fyn, CD4 and CD8 (for review see [220]). The large molecule, CD45 shows a distinctive behaviour, it is recruited to the cSMAC at an early time point, possibly to activate Lck, and later moves to a more peripheral region than the pSMAC, termed distal SMAC (dSMAC) [221].

Although initially the IS was defined as a specialized structure composed of a cSMAC and a pSMAC, the term now refers to a more diverse range of structures that present an accumulation of TCR and related signaling molecules. In fact, several studies show diverse synapse morphologies and suggest that T cells can be activated in the absence of cSMAC formation [222]. Immature CD4(+)CD8(+) thymocytes form a decentralized synapse with multi-focal TCR clusters and do not form a cSMAC [223]. Also, the IS between T cells and dendritic cells was shown to be a multi-focal structure in the absence of Ag and when Ag is present, the T cells are activated without the formation of a cSMAC [224].

How is the kinetics of TCR signaling coupled to the dynamic nature of the immunological synapse formation? It has long been accepted that TCR signaling was initiated well before cSMAC formation and it has been hypothesized that the cSMAC was involved in sustaining signaling. In contrast, Lee and collaborators [225] have shown that early phosphorylation of Lck and ZAP-70 occurs in the peripheral region of the contact area and before the formation of a mature IS in normal T cells. Moreover, the first suggestion that T cell activation can be augmented in the absence of cSMACs came from studies using T cells from mice deficient in CD2AP. These T cells do not form cSMACs and show a defective TCR down-regulation [226]. These authors suggest that the cSMAC acts as an adaptive controller that enhances weak signals and attenuates signals by strong ligands.

It has long been recognized that small TCR clusters are formed prior to the IS, but it was only very recently that the precise behaviour of these microclusters (MCs) was analyzed. Using supported bilayer membranes and nanometer-scale structures to contain movement of molecules, Mossman *et al.* [227] demonstrate that the TCR MCs mechanically trapped in the peripheral regions of the synapse had long-lasting TCR signaling and calcium mobilization. Their observations suggest a three-step process to form a mature IS: TCR engagement of pMHC; TCR-pMHC assembly into MCs and transport of MCs to form the cSMAC. They suggest that the main role of cSMAC is the

internalization of the engaged TCRs while the pSMAC shows to have a role in initiating and sustaining TCR signaling.

The application of TIRF (total internal reflection fluorescence) microscopy to the IS led to the discovery that TCR-pMHC clusters are continuously forming at the periphery of the IS [228]. This study shows that microclusters, at the periphery of the contact interface between T cells attached to the pMHC-containing bilayer, include the TCR, ZAP-70 and SLP-76. Throughout the translocation of these initial MCs to the centre of the contact, to form the cSMAC, ZAP-70 and SLP-76 dissociate from the TCR MCs, supporting the idea that the initial MCs initiate and sustain T cell activation (figure 4). In the future, characterizing the influence of antigen quality in processes such as microcluster formation and the role of the synapse will be of extreme importance. Furthermore, the precise role of the cSMAC should be clarified.

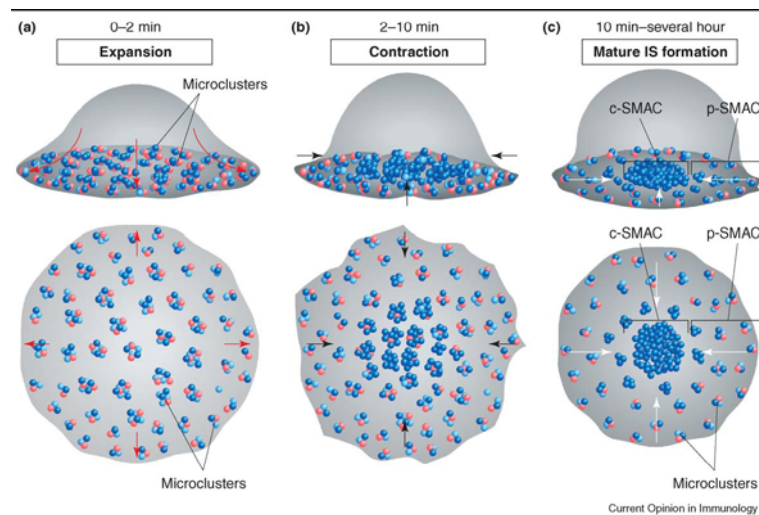


**Figure 4.** Dynamic formation of microclusters and immunological synapse during the processes of antigen recognition and T-cell activation. In the figure, MCs are represented by three components: the kinase, red; the receptor, dark blue; and the adaptor, light blue. (a) After a T cell is placed on a planar bilayer containing peptide-loaded MHCs and ICAM-1, TCR-MCs are generated at the contact interface, which assemble with kinases and adaptors that participate in TCR-proximal signaling. Following the first attachment, the T cell continues to spread on the bilayer, with new MCs generated on the edge (red arrows). (b) After the full expansion, the T cell begins to contract (black arrows) and MCs accumulate at the central region of the interface. During this process, the MCs fuse each other to form larger MCs, whereas kinases and adaptors dissociate from TCR-MCs. (c) Ten minutes after T cell-bilayer conjugation, TCR-MCs finally accumulate at the center of the IS to form 'c-SMACs'. The c-SMACs contain minimal levels of kinases and adaptors. New functional MCs are continuously generated on the peripheral edge, which are translocated to the c-SMACs to sustain TCR signals, which last for hours (white arrows). Taken from [222].

## 2.2. Protein crosstalk in lipid rafts

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### **Introduction**

The view that the T cell receptor (TCR) is part of a multimolecular complex that associates loosely at the surface of T cells and functions as an activation unit[229], is now widely accepted. More recently, the concept that the assembly of the TCR signaling machinery takes place in lipid rafts has been put forward[230, 231], and whereas the existence of these specialized lipid microdomains seems to be a general assumption, their role in the initiation of T cell signaling has been a matter of intense debate. Much of the controversy results from the experimental approaches used to differentiate these membrane structures and to trail the assemblage of signaling receptors and effectors at the cell surface. In this review we describe the current knowledge and the recent advances towards the understanding of the principles that dictate the organization of the signaling complexes as protein networks in the plasma membrane.

### **Lipid rafts definition: an overview**

Lipid rafts are membrane microdomains enriched in glycosphingolipids, sphingomyelin and cholesterol, embedded in the glycerophospholipid-rich fluid plasma membrane. In general, these lipid microdomains can also be designated as GEMs (glycolipid-enriched membranes) or DIGs (detergent-insoluble glycolipid domains), so called due to their property of insolubility in cold 1% Triton X-100. Non-transmembrane proteins with raft affinity undergo lipid modifications which renders them hydrophobic and thus able to anchor to the plasma membrane. These modifications could be either a covalent linkage to glycosyl-phosphatidylinositol (GPI) in the case of outer leaflet insertion, or the attachment of S-palmitoyl fatty acid, possibly added by myristoylation of amino-terminal glycine residues, for inner leaflet anchoring[232, 233]. A classical example of the latter is the Src-family protein tyrosine kinase Lck, which contains a myristoylation site at Gly2 and double palmitoylation sites at Cys3 and Cys5[87].

The first evidence for the existence of membrane microdomains came from the recognition of different levels or degrees of order in the membrane hydrophobic core,

detected by fluorescent dyes[234]. Further proof supporting this concept resulted from the observation that cholesterol and sphingolipid-rich domains were resistant to non-ionic detergent solubilization at low temperatures, and could be isolated as low-density fractions on sucrose gradient centrifugation[232, 235]. This is intrinsically related to the organizational levels of the lipids in the membrane; the tightly packed saturated lipid acyl chains and cholesterol, forming a liquid-ordered ( $L_o$ ) phase will be more resistant to solubilization than the more fluid, liquid-disordered ( $L_d$ ) phase. The cholesterol dependence of lipid rafts in order to form a liquid-ordered phase in the cell membrane has been demonstrated in studies using model membranes[236]. However, the principle of heterogeneity based on artificial models as well as the correlation of the lipid rafts concept with detergent insolubility has created much controversy in the field, leading to very critical opinions on the nature and function of lipid rafts[237].

### **Visualizing lipid microdomains: from models to live cells**

One property attributed to Triton X-100 is that it can partially solubilize the  $L_o$  phase, which may lead to loss of selected rafts components, such as weakly associated transmembrane receptors[238]. Results obtained by Simons and co-workers suggest that the composition of detergent-resistant membranes is both dependent on cell type and detergent solubilization properties, raising questions on lipid rafts definition based on a Triton X-100 insolubility criterion[239]. Nevertheless, most detergents do not induce artifactual ordered domains or promote association of solubilized components with  $L_o$  microdomains[236], therefore detergent-resistant lipid microdomains can at most diverge from “physiological” rafts in that some components may be selectively lost during lysis procedures.

Given that biochemical approaches produce controversial results, characterization of the lipid rafts and particularly the dynamics in living cells can best be achieved through the usage of advanced microscopy techniques. Contrarily to the immunological synapse, which can be readily followed by light microscopy, lipid rafts in resting cells, termed elemental rafts, are submicroscopic complexes ( $26 \pm 13$  nm) and cannot be visualized by classical microscopy methods[240]. However, crosslinking raft-resident proteins induces the coalescence of rafts into patches of hundreds of nanometres in diameter that can be viewed by fluorescence microscopy[241, 242].

Several novel technical approaches have been used in the attempt to clarify the raft concept by characterizing the size and structure, and the diversity and dynamics of these microdomains. Fluorescence resonance energy transfer (FRET) measurements

have provided evidence that raft markers exhibit an energy transfer pattern that is consistent with cholesterol-dependent clustering in small domains[243-245]. Using homotypic FRET, Sharma and colleagues have shown that a fraction of GPI-anchored proteins at the plasma membrane can be detected in small (4 nm) and dense clusters of as few as 4 GPI protein molecules[246].

Techniques such as fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT) were brought into play to address questions concerning the diffusion and dynamics of individual raft proteins or lipids. The FRAP technology showed that the diffusion rate of the non-raft mutant of influenza hemagglutinin (HA) was superior to the rate of lateral diffusion of the wild type molecule[247]. However, a different study using a similar approach showed that the dynamics of a set of raft and non-raft proteins were comparable, all molecules diffused freely over large areas[248]. One limitation of FRAP measurements is that the temporal resolution is below the residence time of a protein in the rafts.

With the SPT technique and the development of single fluorophore imaging[249] it is now possible to follow an individual molecule and measure its diffusion coefficient and confinement times within certain boundaries, allowing spatial and temporal information on the molecule's behaviour[250-252]. Using total internal reflection fluorescence microscopy (TIRF) to track the trajectory of single raft-associated Lck and LAT, as well as the non-raft molecule CD2, Douglass and Vale surprisingly observed, in non-activated cells, a much larger diffusion coefficient for raft molecules than for CD2[253], although it can be argued that the relative immobility of CD2 may be due to its association with the cytoskeleton[211]. The fact that any of these proteins can in some circumstances move in or out of lipid rafts still hinders any definitive conclusion on the mobility of lipid rafts based on protein markers, though.

### **T cell receptor signaling association with lipid rafts**

Upon productive engagement of the T cell receptor to peptide/MHC complexes presented at the surface of antigen presenting cells, co-receptor (CD4 or CD8) binding to non-polymorphic regions of MHC molecules results in the approximation of CD4/CD8-associated Lck to the TCR/CD3 complex. Lck is thus able to phosphorylate tyrosine residues within ITAM sequences present in the different CD3 chains, which become docking sites for the cytoplasmic kinase ZAP-70. Once ZAP-70 is targeted to the membrane, the TCR/CD3 complex is armed with a cocktail of protein tyrosine kinases,

also including Fyn, that extensively phosphorylate tyrosine residues in a number of receptors and adaptors.

The fact that several signaling molecules were found in association with lipid raft microdomains lead to the assumption that rafts were more than just a structural peculiarity. In unstimulated cells, important signaling proteins such as the adaptors LAT[254] or PAG[255], or the Src-like kinase Fyn[102] are resident in rafts, whereas most transmembrane receptors are found outside these platforms. However, the protein content of the rafts as well as the activity of raft-associated proteins changes with cell stimulation. Signaling effectors like Lck that are in equilibrium between the two phases can change their behaviour or activity upon TCR triggering; while in the resting cell the fraction of Lck found in the rafts is in a close and inactive conformation, upon cell activation the open/active form of Lck is most abundant in the rafts[103], being able to activate resident Fyn[102]. The lipid modifications and consequent localization in lipid rafts seem crucial for the effective function of Lck, as a non-acylated form of the kinase failed to phosphorylate the TCR/CD3 complex[87].

A recent study tackling the functional significance of membrane localization on the initiation of signal transduction, using mutant molecules engineered to not address to rafts, produced intriguing results on the function of the lipid moiety of the molecules. A LAT mutant (C26/29A) that cannot be palmitoylated, and a fusion protein consisting of the extracellular domain of LAX fused to intracellular LAT (LAX-LAT) were both detected outside rafts only. Just the LAX-LAT fusion could rescue T cell function both in vitro, as well as and in vivo in transgenic mice expressing LAT mutants in a null background[256]. So, whereas raft residency does not seem to be essential for the signaling activity, lipid modifications of raft-based molecules possibly tether membrane proteins stably to the membrane.

As many signaling effectors do concentrate in lipid rafts, these membrane microdomains are thus considered as signaling platforms where optimal conditions for cell activation are met. A further function suggested for lipid rafts is to link the signaling machinery with the cytoskeleton. The requirement of cytoskeleton reorganization for sustained signaling has been previously demonstrated[257], and the observation that filamentous actin and  $\xi$  chains are enriched in lipid microdomains following CD48/TCR co-ligation strengthens that view[258].

All these findings show or imply that the TCR itself associates with lipid rafts at some stage after selective stimulation of T cells, although there is some controversy on the kinetics of such an association: the TCR is either induced to associate with lipid rafts[230, 259], or it is already resident, at least a small fraction, in a subset of lipid rafts[260, 261], these disputes again falling in the general discussion on the correct

methodology. However, given that TCR engagement to MHC/peptide should precede all changes in cell behavior due to antigen recognition, including membrane and cytoskeleton reorganization, it is more appropriate to envision TCR association with lipid rafts from the receptor-centered point of view: T cell receptors are constrained to the immunological synapse, and lipid rafts/raft resident molecules move to these cellular interfaces[262]. Protein-protein interactions can thus be foreseen as the driving forces for redistribution of raft-associated proteins, as well as for the lipid phases (Figure 1).

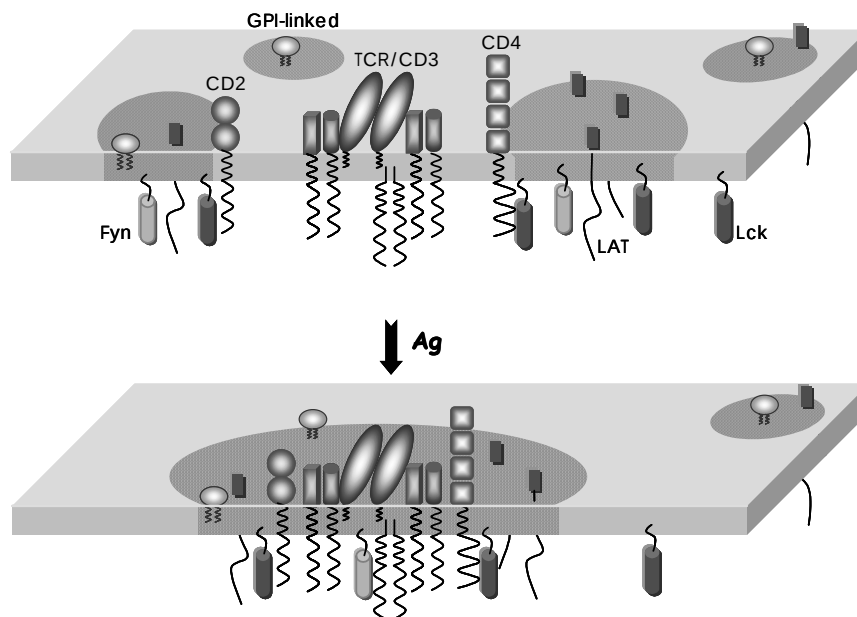


**Figure 1.** A model for membrane microdomain organization in T cell receptor signaling. For simplicity only illustrative molecules are represented. In resting cells, lipid rafts may exist as regions of heterogeneous composition and size. Some proteins already partition into these domains either by GPI-anchoring or by acylation, as is the case of the Src kinases Lck and Fyn as well as several adaptor proteins (eg. LAT). Most transmembrane proteins are found outside these platforms. Upon antigen stimulation rafts coalesce bringing together the TCR and the signaling machinery. The current view sustains the idea that lipid rafts are highly dynamic structures which is probably based on a high diffusion rate of lipid raft resident proteins. This mobility requires the ability of these proteins to interact with other proteins. When the proper protein-protein interaction is established this results in the creation of a signaling complex.

### Dynamics of protein interactions in lipid rafts

Results mainly from raft patching experiments suggest that the association of the TCR with lipid rafts increased when the TCR is crosslinked together with accessory proteins such as CD2, CD5, CD9 and CD28[214, 216, 263]. This has been suggested as a mechanism of co-stimulation, in the sense that the induced clustering of lipid rafts

resulted in increased tyrosine phosphorylation events[214, 263]. Additionally, CD28 co-stimulation induces the movement of actin cytoskeleton to the TCR engagement site[264].

Raft-controlled interactions, by their dynamic nature, should facilitate transient encounters between raft-resident proteins whereas the interactions of raft with non-raft proteins could be limited, because of the physical segregation in different membrane phases. This constraint is overcome if protein-protein interactions are prevalent over the raft/non-raft separation, and indeed no obstruction on the recruitment of non-raft molecules to the ordered lipid phase is evident from current experimental research. The co-receptor CD8 exists as a CD8 $\alpha\alpha$  homodimer or a CD8 $\alpha\beta$  heterodimer. Whereas the  $\beta$  subunit facilitates raft-association, the  $\alpha$  unit provides an extracellular binding site for MHC class I and an intracellular motif that binds to Lck. Since CD8 $\alpha\beta$ , but not CD8 $\alpha\alpha$ , partitions into rafts, it promotes the association with lipid rafts of a significant fraction of TCR/CD3 that binds to CD8 $\alpha$ /Lck[265]. Therefore, protein-protein interactions can dictate the localization at a certain moment of proteins within lipid raft microdomains.

Recently, Douglass and Vale were able to show that while non-raft CD2 single molecules are mainly immobile, Lck and LAT showed abrupt changes from highly mobile to a highly immobile state, the latter occurring when they spatially overlapped a signaling cluster[253]. Using a series of mutants that interfere either with lipid raft-targeting or with protein associations, they concluded that protein-protein interactions are determinant in selecting which non-raft molecules are retained or excluded from lipid rafts, rather than any type of raft-addressing label. As rafts were not found to be the primary factor in the clustering and diffusional immobility of CD2/Lck/LAT clusters, the authors propose that highly mobile signaling proteins diffusing in the membrane randomly encounter activated signaling complexes, and are captured by the emerging signaling complex provided the correct bonds can be established. CD2 and Lck have been shown to interact directly, and the recruitment of CD2 to lipid rafts relies on such interactions[208, 209, 266]. However, these specific protein interactions were not addressed in the study, so complementary work is required, with these and other molecules, before a solid model can be built.

## Conclusions and perspectives

Are lipid rafts central stages where multifactor signaling complexes are assembled, or are they platforms that carry the signaling apparatus where it is required, largely dependent on protein interactions, as recent advances seem to suggest? If so, what role is performed by the lipid content of rafts, is the lipid environment essential for signal transduction or is it merely the result of random or facilitated aggregation of proteins that are covalently connected to the plasma membrane?

Development of more refined techniques that allow a better understanding of protein movements within the plasma membrane is thus required. The development of new sets of fluorescent lipids, such as polyene-lipids that exhibit a similar distribution in respect to the membrane ordered and disordered liquid phases as their natural counterparts, will make it possible to trace the movement of individual proteins in membrane microdomains of diverse composition[267]. With the appropriate tools, the concepts of the immunological synapse and clustering of proteins in lipid rafts microdomains will undoubtedly be assessed.

## **Introduction references**

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## **II. Research work**

**OX52 IS THE RAT HOMOLOGUE OF CD6: EVIDENCE FOR AN EFFECTOR  
FUNCTION IN THE REGULATION OF CD5 PHOSPHORYLATION**

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## ABSTRACT

The MRC OX52 monoclonal antibody is a marker of rat T lymphocytes. We have cloned by PCR the rat homologue of CD6, and FACS analysis and immunoprecipitations using OX52 in COS7 cells transfected with rat CD6 cDNA showed that CD6 is the cell surface molecule recognized by OX52. Immunoprecipitation analysis showed that CD6 coprecipitated with CD5. CD5 in turn, was coprecipitated equivalently with CD2, CD6 and the TCR, but the fraction of CD5 associated with CD6 was highly phosphorylated in kinase assays, in marked contrast with the low level of phosphorylation of CD5 associated with either TCR or CD2. Examination of protein kinases associating with these antigens showed that, paradoxically, CD2 coprecipitated the highest amount of Lck and Fyn. CD6 also associated with Lck, Fyn, and ZAP-70, although at lower levels, but additionally coprecipitated the Tec family kinase Itk, which is absent from CD2, CD5 and TCR complexes. Lck together with Itk was the best combination of kinases effectively phosphorylating synthetic peptides corresponding to a cytoplasmic sequence of CD5. Overall, our results suggest that CD6 has an important role in the regulation of CD5 tyrosine phosphorylation, probably due to its unique feature of associating with kinases of different families.

## INTRODUCTION

CD5 is a surface antigen central to the regulation of signal transduction in thymocytes, T lymphocytes and B-1a lymphocytes [1]. The mature 67 kDa glycoprotein contains an extracellular region composed of three domains of the scavenger receptor cysteine-rich (SRCR) superfamily, a single transmembrane segment, and a cytoplasmic tail containing several serine/threonine phosphorylation sites and four tyrosine residues (numbering of the CD5 amino acid residues indicated in this text refers to the human sequence) - Y378, Y429, Y441 and Y463, putative docking sites for SH2 domain-containing signaling effectors [2].

Both co-stimulatory [3-5] and inhibitory [6-8] roles have been proposed for CD5, and these may correlate with the activity or nature of the effectors that upon stimulation bind to phosphorylated tyrosine residues of CD5. In T cells, Lck is the major kinase responsible for the phosphorylation of CD5, at residues Y429 and Y463 [9], the former being also the principal site of docking of Lck itself [10]. ZAP-70 and CD3- $\xi$  have also been shown to bind to CD5, although the exact mode of interaction is not fully understood: ZAP-70 does not bind directly to the pseudo-ITAM sequence containing residues Y429

and Y441 [11, 12], and moreover, there is no evidence of direct phosphorylation of CD5 by this kinase. Additionally, CD5 signaling activates a pathway involving phosphatidylinositol 3-kinase, whose two SH2 domains bind to phosphorylated Y441 and Y463 [5, 11].

Regarding the inhibitory effect of CD5, the molecular mechanism remains to be elucidated. Two of the tyrosine residues, Y378 and Y441, are within putative ITIMs, but the evidence of involvement of these residues in inhibitory effects is very slim. So far, no repressors have been shown to bind to Y441, and only in Jurkat T cells the phosphatase SHP-1 seems to dock to Y378 [7]. No evidence of such binding was found in B cells [8]. Curiously, it is residue Y429 that harbors molecules that down-modulate cellular activation, like Ras-GAP and Cbl in thymocytes [8, 13].

The exact mode of CD5 phosphorylation remains also to be fully deciphered. CD5 is rapidly phosphorylated following TCR stimulation [14]. However, as the TCR/CD3 complex does not include Lck, the interaction between CD5 and Lck is possibly promoted in assembled signaling micro-domains, namely lipid rafts. Indeed, crosslinking CD5 with the TCR targets both molecules to these specialized domains [15]. Paradoxically, stimulation of T cells with antibodies against CD2, a membrane antigen that does associate with both Lck [16, 17] and CD5 [18, 19], seems not to induce CD5 phosphorylation, but rather its dephosphorylation [18].

Given the complexity of the signaling mechanisms involving CD5, and the apparent contradiction between the outcomes of stimulation through the TCR versus CD2, we looked for alternative molecules and kinases that might regulate CD5 phosphorylation. Here we report on the rat T cell protein, OX52, which appears to have a role in mediating the phosphorylation of CD5, making use of its capacity to associate with tyrosine kinases of different families.

## **MATERIALS AND METHODS**

### **Antibodies and CD5 Peptide**

Monoclonal Ab [17, 19] used: CD2-OX34, CD5-OX19, OX52 [20], and human C3bi-OX21; TCR-R73, and CD28-JJ319 [21], gifts from T. Hünig (University of Würzburg); and anti-phosphotyrosine HRP-conjugated-4G10, purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-sera: anti-CD6 [22], raised against the extracellular domain of mouse CD6; anti-CD5, raised against a peptide of amino acids 451-471 of human CD5, a gift from D. Y. Mason (John Radcliffe Hospital, Oxford); anti-Lck, raised against a peptide of amino acids 39-64 of murine Lck, a gift from J. Borst (The

Netherlands Cancer Institute, Amsterdam); BL90, polyclonal anti-Fyn and BL12 [23], polyclonal anti-Itk were gifts from J. Bolen and M. Tomlinson (DNAX Research Institute, Palo Alto); polyclonal anti-ZAP-70 from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-mouse peroxidase conjugated was purchased from BD Biosciences (Heidelberg, Germany) and rabbit anti-mouse immunoglobulin (RAM) was purchased from Serotec (Kidlington). The N-terminal biotinylated peptide, >70% pure, corresponding to the sequence AASHVDNEYSQPPRNSRLSAYPALE-OH of rat CD5, was purchased from New England Peptide (Fitchburg, MA).

### **Cells and cell lines**

Cervical lymph node cells were from 12 week old Lewis male rats, Charles River Laboratories (Barcelona). Cell lines: W/FU(C58NT) [17], a rat thymoma, from A. Conzelmann (University of Fribourg); and COS7 [19], obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in RPMI with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml).

### **Cell stimulation and detection of tyrosine phosphorylation**

Approximately  $1 \times 10^7$  rat lymph node cells were used per sample. R73 and/or OX52 were added at 10 µg/ml to cells and incubated on ice for 15 min. Cells were washed with PBS, resuspended with 20 µg/ml RAM and incubated for 2 min at 37°C. Cells were lysed by the addition of NP-40 lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% (v/v) NP-40) for 30 min on ice. The nuclear pellet was removed by centrifugation at 12,000 x g for 10 minutes at 4°C. Cell lysates were denatured in 2 x SDS buffer, and subjected to SDS-PAGE. Immunoblotting and detection of tyrosine phosphorylation was performed as previously [18], using 4G10-HRP.

### **Cloning of rat CD6 cDNA**

Total RNA was isolated from Lewis male rat spleen using Trizol (Life Technologies, Barcelona). cDNA was obtained using the ThermoScript RT-PCR System (Life Technologies) from total RNA primed with oligo(dT). Rat Cd6 was amplified with primers designed based on the reported sequence of mouse Cd6 (GenBank U37543). The following primers were used: forward primer from 5' UTR of mouse Cd6 5'-GCACTAAGCTTGGGCAGGCGTGAGTAGCAGT-3' and reverse primer containing the stop codon sequence of mouse Cd6 5'-TACGATGACATCGGTGCAGCCTAGCCTCTAGACCCG-3'. The 2.0 Kb fragment obtained in the PCR reaction was then gel purified and cloned into TOPO 2.1-vector

(Invitrogen, Groningen). The sequence was confirmed by de-deoxy sequencing. The sequence of the 3' untranslated region was obtained by using 3'RACE (Life Technologies) following the manufacturer's instructions. Based on sequence information from the previous PCR, the primers designed were: forward 5'-GCTCCCAGAGACATCCCA-3' (within exon 6) and reverse universal adapter primer. The first strand for this reaction was obtained using oligo dT/adaptor primer to obtain the 3' end of the cDNA.

### **Transfection of COS7 cells**

A truncated form of rat CD6 (M6.1) lacking part of the cytoplasmic domain (residues 524 to 665) was used for transient transfection of COS7 cells and was obtained as follows: rat CD6 cDNA cloned in TOPO 2.1 vector was used as a template for the amplification PCR reaction using as forward primer a sequence derived from 5'untranslated region of mouse CD6 5'-GCACTAAGCTTGGGCAGGCGTGAGTAGCAGT-3' and a reverse primer designed within CD6 cytoplasmic tail 5'-CTCTTCAAGTCATTCTCCAAGGGTGGCATCCG-3', containing a STOP codon. The PCR product obtained was blunt-end sub-cloned into the expression vector pEF-BOS [24]. The orientation of the insert was confirmed by colony-PCR. Transient transfection in COS7 cells was performed as follows: cells were grown in 80 cm<sup>2</sup> flasks in CO<sub>2</sub> incubator, 37°C, 5% CO<sub>2</sub>, until they were 50-75% confluent. Following washing with RPMI a transfection mix containing 0.4 mg/ml DEAE-Dextran, 100 µM chloroquine and 1 µg/ml DNA, in RPMI, was added to the cells for 2 hrs, at 37°C, 5% CO<sub>2</sub>. Cells were washed with PBS, and incubated in DMSO, 10% in PBS, for 2 min followed by two washings with PBS. Cells expanded for 48 hrs in RPMI/10% FCS + antibiotics, at 37°C in 5% CO<sub>2</sub>. COS7 cells were detached from flasks 24 hrs post-transfection, with Trypsin-EDTA solution (Sigma-Aldrich, Sintra), washed with PBS, and replated in complete RPMI medium.

### **Flow cytometry**

48 hours after transfection, COS7 cells were removed from flasks with PBS/EDTA (0.2 g/L) and resuspended in PBS containing 0.2% BSA and 0.1% NaN<sub>3</sub> (PBS/BSA/NaN<sub>3</sub>), at a concentration of 1 x 10<sup>6</sup> cells/ml.

Staining was performed by incubation of 2 x 10<sup>5</sup> cells/well with mAbs (20 µg/ml) for 30 min on ice, in 96-well round-bottom plates (Greiner, Nürtingen). Cytometric analysis was as previously described [18].

### **Cell surface biotinylation and immunoprecipitations**

Cell surface biotinylation, immunoprecipitations and reprecipitations were performed as previously described [18].

### **Immune complex kinase assays**

Kinase assay buffer (30  $\mu$ l) containing 10 mM  $\text{MnCl}_2$ , 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, and 50  $\mu\text{Ci}$  (185 KBq) of  $[\gamma\text{-}^{32}\text{P}]$  ATP (>5000 Ci/mmol, Amersham), was added to the beads containing the immune complexes, and in vitro kinase reactions were allowed to occur for 15 min at 25°C. When indicated, a biotinylated peptide containing the CD5 pseudo-ITAM sequence (Biot-AASHVDNEYSQPPRNSRLSAYPALE-OH) was also included in the reaction mix at a final concentration of 0.5  $\mu\text{g}/\mu\text{l}$ , and in this case incubated at 30°C for 10 min. Reactions were stopped with 30  $\mu\text{l}$  of 2x SDS buffer and boiling for 5 min, and the products separated by SDS-PAGE. Gels were dried and exposed at -70°C to Biomax MR-1 films. Biotin-labeled CD5 peptide was recovered using avidin beads (Pierce, Illinois) and the incorporated  $[\gamma\text{-}^{32}\text{P}]$ -ATP measured in a Beckman liquid scintillation counter. Results are expressed in counts per minute (cpm).

## **RESULTS**

### **Enhanced tyrosine phosphorylation of cellular substrates upon crosslinking the TCR with the OX52 antigen**

We have looked for molecules involved in the regulation of CD5 phosphorylation. Upon TCR triggering the 67-kDa antigen CD5 becomes tyrosine phosphorylated [14]. Using rat lymph node cells, we detected phosphorylation of substrates in cells stimulated with the mAb OX52 or an anti-TCR mAb, R73 (Fig. 1). Using a combination of both mAb, proteins of 100 kDa, 85 kDa, and 38-40 kDa had an increased level of phosphorylation when compared to cells stimulated with R73 or OX52 alone. However, we particularly noticed that a protein of 66 kDa phosphorylated upon R73 triggering was also phosphorylated upon OX52 crosslinking alone. Co-ligation of OX52 and R73 increased the phosphorylation only slightly. We therefore decided to further investigate the OX52 antigen, and to see whether this molecule was involved in the regulation of CD5 phosphorylation.

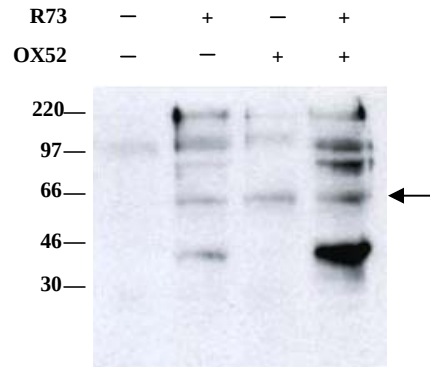


**Figure 1. Coligation of OX52 antigen and TCR enhances tyrosine phosphorylation.** Rat lymph node cells were left unstimulated or stimulated with either R73 (10 $\mu$ g/ml), OX52 (10 $\mu$ g/ml), or with a combination of both, and cross-linked with RAM. Cells were lysed in 1% NP-40 and equal amounts of cleared lysates were resolved on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with 4G10-HRP. Phosphorylation of a 66-kDa protein is indicated by an arrow.

### CD5 associates with the OX52 antigen, and is highly phosphorylated in kinase assays

We performed immunoprecipitations on cell lysates from surface biotinylated C58 cells. Following cell lysis using Brij 96, CD2, CD5, CD28, TCR and the OX52 antigen were precipitated using specific antibodies. OX21 was used as a negative control (Fig. 2A). Immune complexes were subjected to SDS-PAGE and transferred onto nitrocellulose membranes, and biotinylated proteins were detected by streptavidin-peroxidase/enhanced chemiluminescence. CD2, the  $\alpha\beta$ TCR chains, CD5 and CD28 could easily be detected at their expected sizes in the respective lanes. In the lane of OX52, a broad smear was detected above 100 kDa. Common to all lanes, except those of CD28 and OX21, was a protein of 66 kDa, presumed to be CD5. To prove that this protein was CD5, we disrupted the immune complexes and performed reprecipitations using polyclonal anti-CD5. The amount of CD5 precipitating with OX52 was comparable to that associating with either CD2 or with the TCR (Fig. 2B).

In parallel, immunoprecipitates of CD2, CD5, TCR, CD28 and OX52, were subjected to in vitro kinase assays. Very strong phosphorylation was detected in both CD5 and OX52 immunoprecipitations (Fig. 2C). Again, the OX52 precipitate contained a protein of molecular weight above 100 kDa, and additionally the 66-kDa protein. Conversely, CD5 coprecipitated a high molecular weight phosphoprotein with the same mobility as that present in OX52 immune complexes. Reprecipitation of CD5 confirmed that OX52 was associated with phosphorylated CD5 (Fig. 2D).

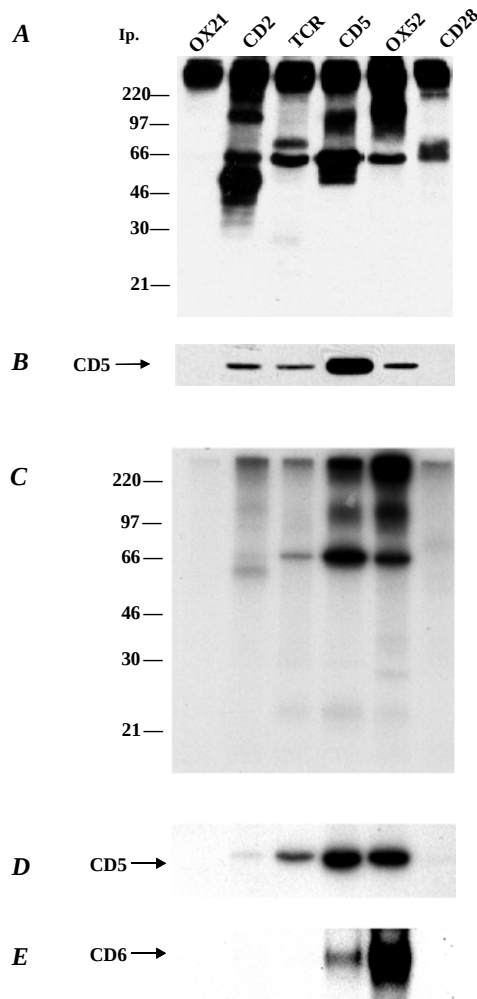


**Figure 2. CD5 associates at similar levels with CD2, TCR and OX52, but is highly phosphorylated when associating with OX52.** A, C58 cells were biotinylated, lysed in 1% Brij 96 and subjected to immunoprecipitations with mAb specific to CD2, TCR, CD5, CD6, CD28 and OX21 (negative control). Immune complexes were resolved on 10% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. The membrane was incubated with streptavidin-HRP and visualised by enhanced chemiluminescence. B, Biotinylated CD5 was reprecipitated from the immune complexes shown in A, run in SDS-PAGE and visualised as above (indicated by an arrow). C, *In vitro* kinase reactions were performed in immune complexes of the molecules indicated. Following SDS-PAGE, phosphorylated products were visualised by exposure of the dried gel at  $-70^{\circ}\text{C}$  to Biomax MR-1 films. Immune complexes were disrupted and CD5 (D) and CD6 (E) were reprecipitated with the use of polyclonal antibodies, subjected to SDS-PAGE and exposed to autoradiography.

Interestingly, although CD5 did coprecipitate with CD2 and TCR, the fraction of CD5 associating with either CD2 or the TCR was not highly phosphorylated. In fact, phosphorylated CD5 could hardly be detected in CD2 immunoprecipitations. These data mean that in our experimental conditions CD2, TCR and OX52 all coprecipitate CD5 equivalently, but only the fraction of CD5 associating with OX52 is highly phosphorylated.

Based on the molecular weight and tissue distribution of OX52 [20], we hypothesized that the OX52 antigen was the rat homologue of CD6. Using a polyclonal anti-mouse CD6 serum, we attempted to reprecipitate CD6 from the immune complexes. Reprecipitation of CD6 yielded a phosphoprotein of 110 kDa present in OX52 immune complexes, and to a lesser extent in CD5 immune complexes, but not in those of CD2, TCR or CD28 (Fig. 2E).

**PCR cloning of the rat homologue of CD6**

For the purpose of cloning rat Cd6, we obtained cDNA by RT-PCR from RNA isolated from splenocytes of Lewis male rats. Primers were designed based on the cDNA sequence of mouse Cd6 (GenBank U37543). The 5' primer is 17 bases upstream of the start codon, and the 3' primer overlaps with the stop codon. The PCR amplified DNA fragments ranging from 1.4 to 2.0 Kb, probably corresponding to different CD6 isoforms that result from alternative splicing. We gel-purified the heaviest DNA product, and sub-cloned it into the vector TOPO 2.1. Sequencing of this product revealed an open reading frame of 1998 bases. With this sequence information, the gene-specific primer for 3'-RACE was designed (see Material and Methods). The 3'-RACE reaction produced a fragment that included a further 893 bp from the stop codon until the poly (A) tail.

The coding sequence of rat Cd6 (GenBank accession no AF488727) corresponds to a 665 amino acids long polypeptide (Fig. 3). It has 89% identity with mouse CD6 and 71% identity with the human molecule (GenBank U34625). Domain 2 shares the highest identity, 95%, with the mouse homologue, when compared with domains 1 and 3, which have 54% and 88% identity, respectively. The highest level of identity with the human sequence (85%) is found for domain 3.

Analysis of the rat CD6 amino acid sequence reveals only one feature different from the mouse sequence, an additional putative N-glycosylation site at position N92. Comparing to the human sequence, major differences lie within the cytoplasmic domain. Although all tyrosine residues and PKC phosphorylation sites are conserved, only five of the eight putative casein kinase II phosphorylation sites are shared among man, rat and mouse CD6. One relevant difference between the human and the rodent sequences is that the mouse and rat sequences contain three putative SH3-binding sequences, whereas the human has only two, the membrane-proximal diproline motif 468PRVP being changed to 472APPP.

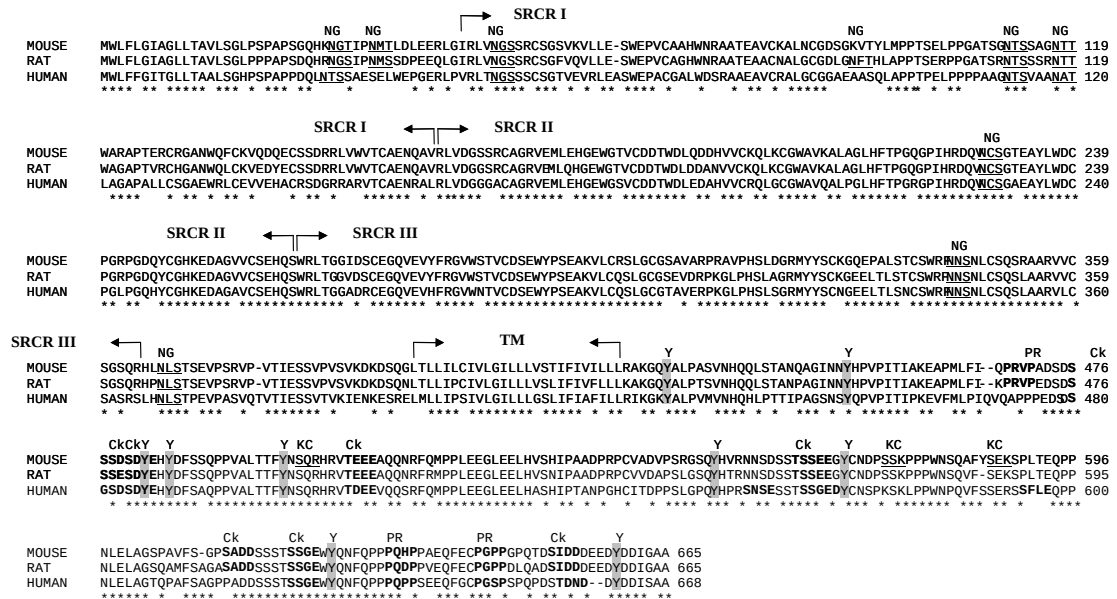


**Figure 3. Comparison of the predicted amino acid sequence of rat CD6 with mouse and human sequences.** Identical residues are denoted by asterisks. SRCR domain boundaries and the transmembrane region are indicated by arrows. Potential N-glycosylation sites are NG's. Tyrosine residues are highlighted at grey. The following intracellular motifs are indicated: three proline-rich sequences in rat and mouse contrasting with only two in human are denoted by PR's. The motifs containing the casein kinase II phosphorylation site consensus sequence are indicated by Ck. PKC phosphorylation consensus sequence containing motifs are indicated by KC. The accession number of rat CD6 is AF488727 at the database of the National Center of Genome Resources.

### OX52 is the rat homologue of CD6

The proof required to assign OX52 as rat CD6 was that this antibody specifically recognizes expressed CD6, and we performed these experiments in a non-lymphoid system. As the OX52 epitope lies within the extracellular domain, we produced a truncated form of rat CD6, which removes part of the cytoplasmic domain (see Material and Methods). We transfected the CD6 cDNA into COS7 cells, and 48 hours post-transfection cells were surface biotinylated, lysed with Brij 96, and subjected to immunoprecipitations using the OX52 mAb. As control, we used COS7 cells transfected with the empty vector. OX52 mAb was able to precipitate a labeled protein expressed only in the CD6 transfected cells, not in the control cells (Fig. 4A). Moreover, we additionally used a polyclonal antibody raised against the extracellular domain of mouse CD6 to immunoprecipitate the expressed rat CD6. Given the high homology between the rat and mouse molecules, it was not surprising that this anti-serum precipitated a protein with the same mass as that precipitated by OX52.

The identity of OX52 as rat CD6 was also confirmed by FACS analysis. OX52 mAb did not detect any proteins on the surface of mock-transfected COS7 cells. However, in CD6 transfected cells, a sub-population was clearly positive for staining with OX52 (Fig. 4B).

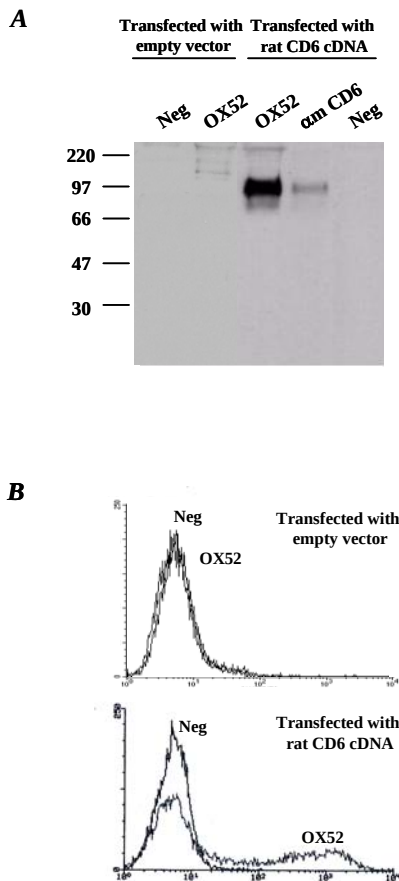


**Figure 4. OX52 mAb recognizes the rat homologue of CD6.** (A) COS7 cells were transiently transfected with cDNA containing a truncated form of rat CD6 (M6.1) lacking most of the cytoplasmic domain, inserted in pEF-BOS, or transfected with the empty vector (pEF-Bos). 48 hours post-transfection cells were biotinylated and lysed in Brij 96. OX52 mAb and polyclonal anti-mouse CD6 antiserum were used to immunoprecipitate the expressed rat CD6. As negative control (Neg), we used the OX19 mAb. Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with streptavidin-HRP and biotinylated CD6 visualised by enhanced chemiluminescence. (B) Rat CD6 expression was analysed by flow cytometry. Both mock-transfected (upper histogram), as well as rat CD6 (M6.1 cDNA)-transfected COS7 cells (lower histogram) were stained with OX52, or with OX19 as negative (Neg), followed by RAM-FITC.

### Rat CD6 associates with protein kinases of different families

The high phosphorylation detected in kinase assays in OX52 immune complexes possibly meant that rat CD6 associates with proteins with kinase activity. From immune complexes of CD2, CD5, CD28, TCR and CD6, obtained from Brij 96-prepared C58 lysates and subjected to in vitro kinase assays, we attempted to identify kinases responsible for the kinase activities. We used antibodies against the Src-family kinases, Lck and Fyn, against the Tec-family kinases Tec, Itk and Txk, and against the Syk-family kinase ZAP-70. Reprecipitations where we detected specific signals are shown in Figure 5. CD2 coprecipitated the most Lck and Fyn, although the amount coprecipitated by TCR and CD6 was also significant. ZAP-70 could be seen in association with CD5, and to a lesser extent with the TCR and CD6. Finally, Itk associated specifically with CD6, and was not detected in CD2, TCR, CD5 or CD28 immune complexes. The associations between

the surface proteins and the kinases, except ZAP-70, were also detected using other detergents such as NP-40 and Triton X-100 (data not shown). CD6 could coprecipitate Lck, Fyn and Itk, in all detergents, whereas ZAP-70 associations were lost when using other detergents than Brij 96.

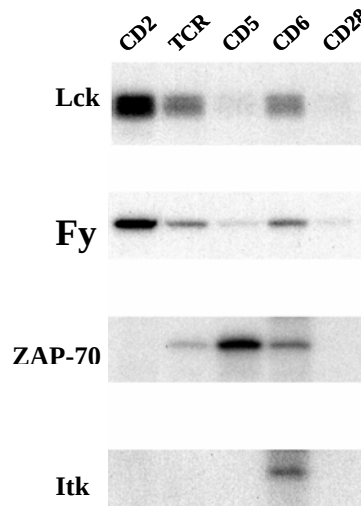


**Figure 5. CD6 associates with Src kinases Lck and Fyn, with ZAP-70 and with the Tec family kinase Itk.** C58 cells were lysed in Brij 96 and the indicated surface proteins immunoprecipitated. Immune complexes were subjected to kinase reactions, following which the reaction products were denatured, and Lck, Fyn, ZAP-70 and Itk reprecipitated using specific polyclonal Abs. Precipitates were ran on SDS-PAGE, transferred to nitrocellulose and the  $^{32}\text{P}$  incorporation detected by autoradiography.

### **Lck, together with Itk, efficiently phosphorylates CD5 peptides**

The previous data suggested that a kinase or a combination of kinases associating with rat CD6 has the capacity to induce a high level of phosphorylation of tyrosine residues within the CD5 cytoplasmic domain. Therefore, we decided to test the phosphorylation efficiency of individual or combinations of kinases towards a synthetic peptide comprising two tyrosine residues within the ITAM-like motif of CD5. C58 cells were lysed with Triton X-100, and Lck, Fyn, Itk, and Zap-70 were immunoprecipitated, either alone or in pairs, using specific polyclonal anti-sera. Kinase assays of the immune complexes were performed at 30°C for 10 min in the presence of the CD5 peptide, biotinylated at the N-terminal residue. Phosphorylated peptide was recovered from the solution through precipitation with streptavidin beads, and radioactive counts associated with the peptide were measured in a  $\beta$  counter. From the upper panel on Figure 6, it can be observed that Lck alone is able to efficiently phosphorylate the peptide, whereas Fyn has a much lower efficiency, and Itk or ZAP-70 phosphorylate the peptide only residually. When testing combinations of pairs of kinases (Fig. 6, lower panel), we observed that the

best combination effectively phosphorylating the peptide was that of Lck with Itk. All other combinations had a significantly lower capacity of inducing peptide phosphorylation.

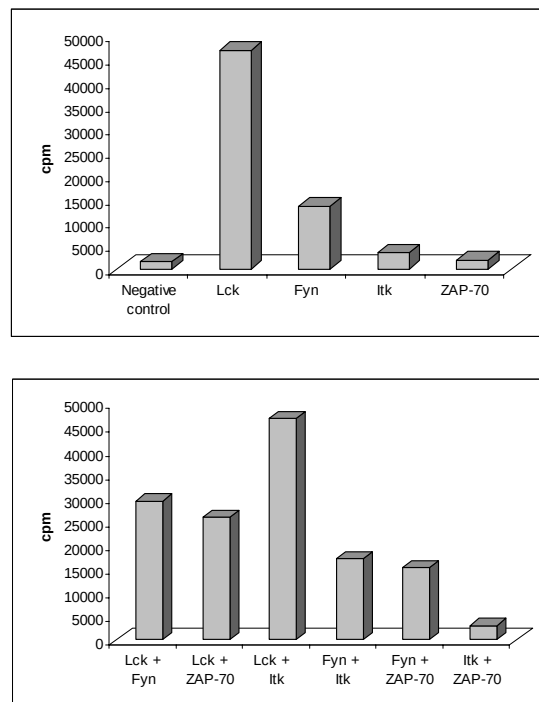


**Figure 6. Lck together with Itk is the most efficient combination for the phosphorylation of an exogenous CD5 peptide.** C58 cells were lysed in Triton X100 and Lck, Fyn, ZAP-70 and Itk immunoprecipitated alone (upper panel) or in pairs (lower panel) using specific polyclonal Abs. As negative control, we used rabbit pre-immune serum. Immune complexes were submitted to kinase assays in the presence of exogenous biotinylated CD5 peptide. The reaction was 10 min at 30°C, following which the complexes were denatured and the peptide recovered by streptavidin beads. Incorporated radioactivity was measured in a  $\beta$  scintillation counter. The results are expressed in counts per minute (cpm).

## DISCUSSION

T cell activation results from a combination of signals delivered by the TCR and other signaling molecules. Upon TCR/CD3 stimulation, CD5 is phosphorylated on tyrosine residues [14] and binds intracellular SH2 domain-containing signaling effectors. Conversely, CD5 triggering augments TCR responses [3, 4]. In the last years, however, in vivo studies have challenged the view that CD5 solely mediates activation signals. CD5-null mice display enhanced reactivity of thymocytes towards antigen and increased positive selection, consistent with a down-modulatory role for CD5 [6]. These studies have rapidly obtained the support of in vitro data, in T and B-1a cells, showing that CD5 binds repressor molecules such as SHP-1, Cbl and Ras-GAP [7, 8, 13, 25].

Surface receptors other than the TCR may also exert some of their activities through CD5. CD2 falls in this category, firstly because stimulation via CD2 induces the dephosphorylation of CD5, possibly through the induction of SHP-1 activity [18], and secondly because CD2 synergizes with CD5 in the regulation of thymocyte selection. CD2 null-mice also display enhanced thymocyte reactivity, an effect highly augmented in the CD2/CD5 double knock-out, suggesting that CD2 and CD5 are functionally associated in restraining thymocyte activation [26]. It is possible that engagement of different T cell antigens to the counter-receptors on APC originate different signals that may converge at the level of CD5 and thus regulate CD5 usage in signaling pathways. We propose that, a third T cell surface protein, CD6, is involved in the regulation of CD5 phosphorylation.

The mAb MRC OX-52 was described in 1986 by Mason and co-workers, and was found to label an antigen mainly confined to the T cell areas of the lymphoid organs [20]. It has since been considered a marker for the T cell population in rat tissues or cell suspensions. We proved by cloning, cellular expression, cytometric analysis and molecular characterization that this protein is the rat homologue of CD6.

Having established OX52 as rat CD6, we focused on the role of CD6 in the regulation of CD5 phosphorylation. In our assays, equal amounts of CD5 were obtained in immunoprecipitations of CD2, TCR and OX52. CD6, on the other hand, could only be coprecipitated with CD5 but not with CD2 or with the TCR, suggesting a close interaction between CD5 and CD6. Interestingly, whereas CD5 in OX52 immunoprecipitates could be highly phosphorylated in kinase assays, the same amount in CD2 precipitates could hardly be detected (Fig. 2).

The obvious explanation, that CD2 did not associate with kinases capable of phosphorylating CD5, had to be discarded, since CD2 precipitated the largest amount of Lck. The hypothesis that Lck is not responsible for CD5 phosphorylation is equally improbable, given the evidence for this activity collected in the past [9, 10, 27]. Nevertheless, we tested this hypothesis by incubating Lck directly with a synthetic peptide containing the site of phosphorylation in the cytoplasmic domain of CD5, Y429, preferentially used by Lck. As expected, Lck efficiently phosphorylated the peptide (Fig. 6).

We have used tyrosine phosphatase inhibitors throughout our experiments. Nevertheless, it cannot be excluded that phosphatases may play a role in the inhibition of phosphorylation of the fraction of CD5 that is coupled to CD2. Most of the phosphatase activity associated with CD2 derives from CD45 [28, 29]. CD45 could dephosphorylate CD5 directly, or alternatively, dephosphorylate Lck and thus render it inactive [30]. Additionally, SHP-1 may as well dephosphorylate Lck at its activating residue Tyr-394 [31].

Although it remains unclear why CD2-associated CD5 is not readily phosphorylated, it appears easier to explain the strong phosphorylation observed in the fraction of CD5 that associates with OX52/CD6. Our studies show that tyrosine kinases of different families bind to CD6. In fact, rat CD6 coprecipitated Lck, Fyn, ZAP-70 and Itk, something not generally observed for the other cell surface proteins studied, CD2, TCR, CD5 and CD28. It is conceivable that the capacity of CD6 to induce the phosphorylation of CD5 may derive from the fact that it associates with different kinases, which may increase each other's activities. Indeed, Lck and Fyn synergize with ZAP-70 to produce sustained responses [32], and a functional Lck is required for Itk phosphorylation and activation to occur [33]. Previous studies, where the levels of CD5 phosphorylation observed in Lck-deficient cells were significantly lower when compared to Lck<sup>+</sup> cells, clearly indicated a major role for Lck, but nevertheless suggested that other kinases whose activity may depend on Lck, potentially have a role in CD5 tyrosine phosphorylation [10].

In individual terms, Lck ranked as the most efficient kinase phosphorylating a CD5 peptide, as can be depicted from Figure 6. Fyn is significantly less efficient, and Itk and ZAP-70 phosphorylate the CD5-peptide only residually. However, when we used pairs of kinases, the combination of Lck with Fyn was not the most effective. Rather, Lck in conjunction with Itk gave the best incorporation of radioactive ATP in the CD5 peptide. Moreover, we noticed that the Itk kinase was phosphorylated only in the presence of Lck, but not of Fyn or ZAP-70 (not shown). Therefore, Lck and Itk may have a combined role in promoting the efficient phosphorylation of CD5, and thus explain the exceptional capacity of CD6 in supporting the high level of CD5 phosphorylation observed. Alternatively, an independent inhibitory effect of both Fyn and ZAP-70 on Lck, possibly through the phosphorylation of the C-terminal inhibitory tyrosine residue of Lck, may exist, thus explaining why CD5 is less susceptible to tyrosine phosphorylation in the presence of the TCR or CD2.

## ACKNOWLEDGMENTS

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**HUMAN CD6 POSSESSES AN ALTERNATIVELY SPLICED EXTRACELLULAR  
DOMAIN: IMPLICATIONS ON CD6 IMMUNOLOGICAL SYNAPSE  
ARRANGEMENT AND T CELL ACTIVATION**

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## ABSTRACT

The great majority of mammalian genes yield multiple transcripts arising from differential mRNA processing, but in very few instances have alternative forms been assigned distinct functional properties. We have cloned and characterized a new isoform of the accessory molecule CD6 which lacks the CD166-binding domain. The novel isoform, CD6 $\Delta$ d3, results from exon 5 skipping and consequently lacks the third scavenger receptor cysteine-rich (SRCR) domain of CD6. Differential expression of the SRCR domain 3 resulted in a remarkable functional difference: whereas full-length CD6 targeted to the immunological synapse, CD6 $\Delta$ d3 was unable to localize at the T cell:APC interface during antigen presentation. Strikingly, whereas full-length CD6 is the predominant polypeptide species in resting cells, CD6 $\Delta$ d3 is markedly up-regulated upon activation of T lymphocytes, partially substituting full-length CD6, as evaluated by immunoblotting, and by flow cytometry using antibodies recognizing SRCR domains 1 and 3 of human CD6. Analysis of expression of CD6 variants showed that, the CD6 $\Delta$ d3 isoform is significantly expressed in a significant number of thymocytes, constituting the sole species in a small percentage of cells; suggesting that CD6 switching between full-length and  $\Delta$ d3 isoforms may be involved in thymic selection as well. This elegant mechanism controlling the expression of the CD166-binding domain may help regulate signaling delivered by CD6, through different types of extracellular engagement.

## INTRODUCTION

T cell receptor recognition of peptide-MHC complexes expressed on antigen presenting cells (APC) involves the formation of a tight cell-cell contact area, the immunological synapse, where individual proteins are selectively partitioned [1, 2]. The model of synapse formation predicts an initial stabilization of the contact by engaged integrins, which anchor the region of the contact and allow the TCRs to scan MHC-peptide complexes [3]. With time, stably engaged TCR complexes coalesce into a central area (cSMAC, central supramolecular activation clusters), surrounded by a ring (pSMAC, peripheral supramolecular activation clusters) enriched in the integrin LFA-1 [2, 4]. In the mature synapse, cSMAC are also enriched in the co-stimulatory molecule CD28 and the CD4 or CD8 co-receptors [3, 5]. It has been suggested that the formation of the synapse is favoured by receptor-ligand interactions of small auxiliary molecules such as CD2-CD58 and CD28-CD80 that stabilize the cell contact by the formation of low-affinity interactions, in a size dependent-manner. According to this model, the small adhesion pairs cluster at a

tight membrane region, thus segregating larger glycoproteins such as CD43 or CD45 [1, 6].

CD6 is a 100-130-kDa surface glycoprotein expressed primarily on medullary thymocytes and mature T lymphocytes [7]. CD6 has been regarded as a co-stimulatory molecule as antibody-mediated CD6 crosslinking can potentiate proliferative T cell responses [8-10]. Moreover, a sub-population of CD6 negative T cells displayed lower alloreactivity in mixed leukocyte reactions compared to normal CD6<sup>+</sup> T cell populations [11]. CD6 could additionally play a role in thymocyte maturation, as CD6 antibodies have been shown to partially block the adhesion of thymocytes to thymic epithelial cells [12]. This observation also provided the first evidence that CD6 had a cell surface ligand. A requirement for ligand engagement for costimulatory effects of CD6 was shown by inhibition of antigen-specific and CD3 mAb induced T cell responses by soluble CD6 [13, 14]. Recently, CD6 has been reported to accumulate at the immunological synapse, mediating early and late T cell-APC interactions required for immunological synapse maturation and cell proliferation [13, 15].

Upon CD3 engagement CD6 becomes phosphorylated on tyrosine residues suggesting that interactions with SH2 domain-containing intracellular effectors may occur [16]. An interaction with the SH2 domain of SLP-76 has been shown to be critical for the costimulatory effects of CD6 [17]. The cytoplasmic domain of CD6 is unusually long, and contains additionally two well-conserved proline-rich sequences, potential binding sites for SH3 domain-containing proteins well-suited for signal transduction [16, 18]. Indeed, the rat homologue of CD6 has been shown to associate with protein tyrosine kinases of different families, namely Src-family kinases Lck and Fyn, ZAP-70 of the Syk family, and the Tec-family kinase Itk [19]. CD6 has additionally been shown to associate at the surface of T cells with CD3 [13] and with the structurally related receptor CD5 [19, 20]. However, there are still no data proposing a functional role for these interactions.

By contrast, the molecular basis of the interaction between CD6 and the extracellular physiological ligand, CD166, has been comprehensively investigated in human and murine models [21]. CD166 is a widely expressed glycoprotein containing five immunoglobulin superfamily domains, of which the N-terminal domain has been shown to bind to the third Scavenger Receptor Cysteine-Rich (SRCR) type domain of CD6, the membrane-proximal domain [22, 23]. The residues of CD6 involved in the contact with CD166 are located in the E-F loop, a region that in the particular case of the third domain of CD6 is most divergent from all known SRCR domains [24]. The resulting interaction between CD6 and CD166 is therefore unusual, as most other T cell surface proteins mediating cell-cell interactions bind through their N-terminal domains. However, affinity and kinetic measurements revealed that, in solution, binding occurs with a  $K_d$  of 0.4-

1.0  $\mu$ M [14], relatively strong compared to most other leukocyte adhesion pairs, albeit in the range of low affinity interactions characteristic of cell surface receptors [25-27].

The human, rat and mouse *Cd6* genes contain thirteen exons, of which the last six code for the cytoplasmic tail. Numerous CD6 isoforms have been reported that result from alternative splicing of the exons coding for the cytoplasmic domain [18, 28-30], but no specific physiological function has been attributed to any of these isoforms. Exon 7 contains the sequence coding for the transmembrane domain, and the first six exons code for most of the extracellular domain. Alignment of the CD6 extracellular sequence with that of the scavenger receptor domain of Mac-2 binding protein, for which a crystal structure has been obtained [24], predicts that each scavenger-type domain of CD6 is encoded by a single exon, and that removal of each of these exons would result in a protein only devoid of the correspondent domain. We have obtained from thymocytes and peripheral T cells four alternatively spliced isoforms of human CD6 cDNA lacking sequences encoding extracellular exons. In particular, one isoform omits exon 5, whose corresponding translated polypeptide lacks the SRCR domain 3. This CD6 isoform, CD6 $\Delta$ d3, is up regulated upon activation, paralleling a decline in the expression of full-length CD6. The failure of CD6 $\Delta$ d3 to bind to the CD6-ligand, CD166, highlights the role of domain 3 in addressing CD6 to the immunological synapse, and the down-modulation of domain 3 induced upon T cell activation reveals a rare mode of positional control of cell surface receptors dependent on alternative mRNA splicing.

## MATERIALS AND METHODS

### Cells and cell lines

Human primary T cells were isolated from peripheral blood by using the RosetteSep human T cell enrichment cocktail (StemCell Technologies, Grenoble, France). All cell lines: E6.1, JKHM, B-CLL, J.CaM1 [31], P116 and Raji [32] were maintained in RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50  $\mu$ g/ml). Thymocytes were isolated from human thymic biopsy specimens (derived from children under 2 years of age undergoing corrective cardiac surgery).

### Antibodies and reagents

Human monoclonal antibodies were OX126 [17], specific for human CD6 domain 3, FITC-labeled UMCD6 [9], anti-domain 1 of CD6, from Ancell (Bayport, MN), MEM-98 [33] (anti-CD6 domain 1, a kind gift from Václav Hořejší, Academy of Sciences, Prague, Czech

Republic), CD166-3A6 [34] (BD Pharmingen, San Diego, CA), CD71 (Dako, Glostrup, Denmark) and CD3-OKT3. Polyclonal antibodies: goat anti-mouse peroxidase-conjugated was from Molecular Probes Europe (Leiden, The Netherlands), rabbit anti-mouse FITC-labeled from Dako (Glostrup, Denmark), rabbit anti-mouse Ig, RAM from BD Biosciences (Heidelberg, Germany), anti-phosphotyrosine HRP-conjugated-4G10 from Upstate Biotechnology (Lake Placid, NY) and Rhodamine Red-X-conjugated anti-mouse IgG secondary antibody from Jackson ImmunoResearch Laboratories (WestGrove, PA) were also used.

### cDNA cloning and plasmids

Total RNA was isolated from JKHM, E6.1, human PBMC and human thymocytes using RNA extraction Kit (Qiagen, Venlo, The Netherlands). cDNA was obtained using the ThermoScript reverse transcriptase-polymerase chain reaction (RT-PCR) system (Life Technologies, Barcelona, Spain) from total RNA primed with oligo(dT). Human *Cd6* was amplified with primers designed based on the reported sequence (GenBank U34625). The following primers were used in the cloning of the isoforms: forward primer spanning the initiation codon 5'-CGCGGATCCTCTAG**ATGT**GGCTCTTCTTCGGGATCACTGGAT TG-3' and reverse primer in the exon 7 (TM-coding sequence) of human *Cd6* 5'-GGAGCATTAGCTCCCGAGATTCCTTG - 3'. The PCR fragments were cloned into pCR2.1-TOPO vector. The sequences were confirmed by de-deoxy sequencing. Human CD6FL/pEGFP-N1 was produced by amplifying full-length sequence from the clone CD6/pBJ-neo [18] by PCR using as the forward primer 5'-CGCGGATCCTCTAG**ATGT**GGCTCTTCTTCGGGATCACTGGATTG-3' and the reverse primer 5'-CTACTAGAATCCGCTGCGCTGATGTCATCG -3'. The PCR product was then cloned into the BamHI restriction site of pEGFP-N1 vector (Clontech Laboratories, Palo Alto, CA) producing CD6FL/pEGFP-N1. The CD6Δd1/pEGFP-N1 construct was obtained by digestion of CD6Δd1/TOPO 2.1 with XhoI and SmaI and the product obtained was subcloned into CD6WT/pEGFP cut with the same enzymes. The isoform CD6Δd3 was amplified from the vector pCR2.1-TOPO using the primer M13-R from the vector as the forward primer, and a reverse primer in the exon 7 of human CD6 including a naturally occurring EcoRI restriction site 5'-CTACTAGAATTCCCAGAACGATGGAGG GGATGAGGAGCATTAGCTCCCGAGATTCCTTG-3'. The PCR product was inserted in the CD6FL/pEGFP-N1 producing CD6Δd3/pEGFP-N1. A cytoplasmic deletion mutant of human CD6 fused to GFP was produced by PCR from CD6/pBJ-neo with the forward primer 5'-CTCCAGAC**ATGT**GGCTCTTCTTCGG-3' and the reverse primer 5'-GCCTTCATCCTCTTGAGAATTAAAGGAAAA-3', terminating just before the first tyrosine residue codon of the cytoplasmic domain. The PCR product was cloned into the TOPO

cloning site of pcDNA3.1/CT-GFP-TOPO, using GFP Fusion TOPO TA Expression Kits (Invitrogen, version I), to produce CD6CY5/pcDNA3.1/CT-GFP-TOPO.

### **Cellular activation**

Human primary T cells were activated by incubating  $3 \times 10^6$  cells/ml with Phytohemagglutinin (PHA-P) at 5  $\mu$ g/ml in RPMI, at 37°C, 5% CO<sub>2</sub>, or left untreated. After 72h cells were collected and analyzed by flow cytometry or immunoblotting. For phosphotyrosine detection, approximately  $5 \times 10^6$  cells were used per sample. The different mAb used were added at 2-10  $\mu$ g/ml to cells and incubated on ice for 15 min. RAM at 20  $\mu$ g/ml was then added to cells and incubated at 37°C for the times indicated. No crosslinking was required when cells were stimulated with OKT3. Cells were briefly pelleted and lysed for 30 min on ice in NP-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% NP-40), containing 2 mM NaF, 2 mM PMSF and 2 mM Vanadate. Nuclei and cellular debris were removed by centrifugation at  $12,000 \times g$  for 10 min at 4°C.

### **Immunoblotting and flow cytometry**

Immunoblotting and flow cytometry were performed as described previously [35].

### **Cell Transfections**

Primary T lymphocytes ( $5 \times 10^6$  cells) were transiently transfected with 5  $\mu$ g of CD6FL/pEGFP-N1, CD6 $\Delta$ d3/pEGFP-N1 or CD6CY5/pcDNA3.1/CT-GFP-TOPO plasmids using the Human T cell Nucleofector kit (Amaxa, Köln, Germany), and T cells were used 24 h post-transfection.

### **Conjugate formation and fluorescence analysis**

Raji B cells were incubated with a mix of superantigens (rSEE, SEA, SEB and SEC3, 200 ng/ml each, Toxin Technologies, Sarasota, FL) and plated on poly-D-lysine-coated glass coverslips for 30 min at 37°C. T cells were added to APCs and then incubated at 37°C for 45 min. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed several times with PBS before analysis. Where indicated, T cells were pre-incubated, for 30 min at 4°C, with the mAbs OX126 or UMCD6-FITC, and Raji B cells pre-incubated with 3A6 (CD166), all at 10  $\mu$ g/ml, or left untreated. Immunofluorescence and transmission light images were acquired on an Eclipse TE300 inverted microscope (Nikon, Melville, NY) equipped with a cooled CCD camera (CoolSNAPFx, Roper Scientific, Evry, France). Images were acquired and analyzed using the Metamorph software (Roper Scientific). Conjugate formation and synapse localization of CD6 or

CD166 were quantified with blind scoring, counting a minimum of 50 productive conjugates in each of two or more experiments, and each experiment was observed by two to three examiners.

### **Single-cell $\text{Ca}^{2+}$ and fluorescence video imaging**

Raji cells were prepared and pulsed with Ag as described above and plated on glass coverslips mounted on 30-mm petri dishes. T cells were pre-incubated for 30 min at 4°C with the blocking mAb against d3 of CD6, OX126, at 10 µg/ml or left untreated. The T cells were then loaded with 1 µM Fura-2AM (Molecular Probes) in 1 ml of Hepes buffer 10 mM, pH 7.5 containing 140 nM NaCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 1 mM  $\text{Na}_2\text{HPO}_4$  and 1 mg/ml glucose and added to APC at 37°C. After T cells were added, Fura-2 fluorescence was followed with a TE300 inverted microscope and the Metafluor software (Universal Imaging, West Chester, PA) was used. Averaged  $\text{Ca}^{2+}$  responses were calculated in cells showing a  $\text{Ca}^{2+}$  increase >150 nM. Transmission light images were taken every 12 s in turn with fluorescence images.

### **Phosphotyrosine accumulation measurements at the immunological synapse**

Raji and T cells were prepared as described for conjugate formation. After cells were fixed, phosphotyrosines were labeled with the anti-phosphotyrosine, 4G10 at 5µg/ml followed by labeling with Rhodamine Red-X-conjugated anti-mouse IgG secondary antibody. For synaptic phosphotyrosine signal we calculated a ratio of the signal inside the synapse divided by the signal outside the synapse measured on two regions of same area. Data were averaged from 40 cells analyzed for each condition.

### **RNAse Protection Assays (RPA)**

Total RNA from E6.1 cells was analyzed for quantifying the different human CD6 mRNA species. The two RPA probes and the regions they span in human *Cd6*, as well as the expected sizes of the protected fragments are shown in figure 3A. Probe 1 cDNA template was designed to include 46 bp of exon 1, exon 2, exon 3 and 311bp of exon 4. An RPA with this probe could generate three different patterns of bands: one with a band sized at 777 bp, the full-length isoform; the other with two bands at 115 and 311 bp corresponding to the CD6Δd1 isoform and the third pattern with also two bands of 46 and 311 bp generated by the CD6ΔEx2d1 isoform. Probe 2 comprises 79 bp of exon 4, exon 5, exon 6 and 77 bp of exon 7. In the same manner, this probe can give rise to three patterns: one band at 525 bp, the full-length isoform; another with two bands at 79 and 143 bp corresponding to the CD6Δd3 isoform and the last pattern with two bands of 79

and 77 bp generated by the CD6 $\Delta$ 3Ex6 isoform. RT-PCR was used to obtain a cDNA template for the antisense RNA probe 1 and the fragment was cloned into pCR4-TOPO. The cDNA for probe 2 was produced by restriction of the plasmid pBJneo-CD6 with HindIII and EcoRI. The fragment was cloned into pDP-20 using the same restriction enzymes. Probe 1 and 2 were linearized using NotI and HindIII respectively, purified and complete digestion was checked on agarose gel. <sup>32</sup>P-radiolabeled UTP (NEN) was incorporated into probes by in vitro transcription using T3 polymerase for probe 1 and T7 polymerase for probe 2.

E6.1 cells total RNA (30  $\mu$ g) was hybridized with excess amounts of each riboprobe. After overnight hybridization at 65°C and at 75°C, the samples were digested with RNase A/T1 for 90 minutes at 37°C. As a digestion control (D), of RNase A/T1, the same amount of probes was directly digested. The fragments protected from RNase digestion were resolved on 8% 8M urea denaturing acrylamide gels and visualized using X-ray films. pBR322 DNA-MspI digest (New England Biolabs, ) was used as molecular weight marker.

## RESULTS

### Four novel human CD6 extracellular isoforms arise from alternative splicing

Different CD6 isoforms, both from human and mouse, have been described arising from alternative splicing of exons coding for sequences within the cytoplasmic domain [18, 28-30]. Cloning of the rat homologue of CD6 [19] allowed us to notice that an additional isoform, CD6 $\Delta$ 3, resulting from alternative splicing of an exon coding the third SRCR domain, the CD166-binding domain, was expressed on different tissues of lymphoid origin (Castro *et al.* 2007, unpublished results). We thus investigated whether a similar isoform, or eventually additional isoforms, were expressed in human cells as well.

The first cDNA coding for human CD6 was originally obtained from a peripheral blood acute lymphocytic leukemia expression cDNA library [7], although, as was acknowledged later, it lacked a substantial portion of the cytoplasmic tail coding sequence. In the subsequent work, polymerase chain reaction-based methods were unsuccessfully employed to re-clone the entire molecule. This was partly due to the length of the first intron (35 kb), but also to the fact that the 5' coding region contains large GC-rich sequences. Therefore, the cytoplasmic isoform-coding transcripts that were being found by different research groups were grafted onto the original HPB-ALL cDNA containing the extracellular coding sequences [18, 30]. All succeeding work addressing the function of different mRNA-encoded cytoplasmic isoforms thus contained the same

extracellular domain, and no further research on possible extracellular variants of CD6 has been performed since.

Having improved amplification efficiency with the use of DMSO and the choice of an efficient polymerase, we were successful in obtaining from human peripheral blood mononuclear cells (resting and activated), human thymocytes, and the leukemic T cell lines JKHM and E6.1, cDNAs coding for the full-length molecule and also several PCR products with sizes ranging from 2000 down to 1600 bp (Figure 1A, results shown are from amplification using PBMC). We then tested for the existence of extracellular isoforms, performing RT-PCR reactions amplifying just the extracellular domain-coding sequences. Amplification products of 1200 bp, and 300 bp shorter, were clearly detected (Fig. 1A, right lane).

The several cDNA species obtained from each cell type, displaying distinct mobility on agarose gels, were gel-purified and sub-cloned into the TOPO 2.1 vector. Sequencing showed that the longest product was full-length CD6 in all cases. Four different isoforms lacking extracellular-coding exons were revealed, and confirmed by sequencing (Figure 1B-C). Alignment, from the N-terminus up to the transmembrane domain, of the corresponding amino acid sequences with that of wild-type human CD6 shows that these cDNAs code for novel isoforms. The boundaries of the SRCR domains are indicated by arrows, according to Hohenester *et al.* [24] (Figure 1B). The scheme of the structure obtained for the five CD6 protein isoforms is shown in Figure 1C.

The isoform corresponding to CD6 $\Delta$ d3, that we had previously identified in rat, lacking just exon 5 which codes for the SRCR domain 3, was acknowledged. A further mRNA species skipped exons 5 and 6, the latter coding for a linker between the membrane proximal SRCR domain and the transmembrane stretch. Two additional isoforms were unveiled, one missing exon 3 which codes for the SRCR domain 1, and one other lacking exons 2 and 3. These isoforms are referred to as CD6 $\Delta$ d3 (GenBank accession no. DQ786329), CD6 $\Delta$ d3Ex6 (GenBank accession no. DQ786330), CD6 $\Delta$ d1 and CD6 $\Delta$ Ex2d1, respectively, and were obtained from the tissues and cell lines indicated in Figure 1C. However, at this stage we did not perform an extensive study to address the frequency, or even the existence, of each isoform in all different tissues and cells.

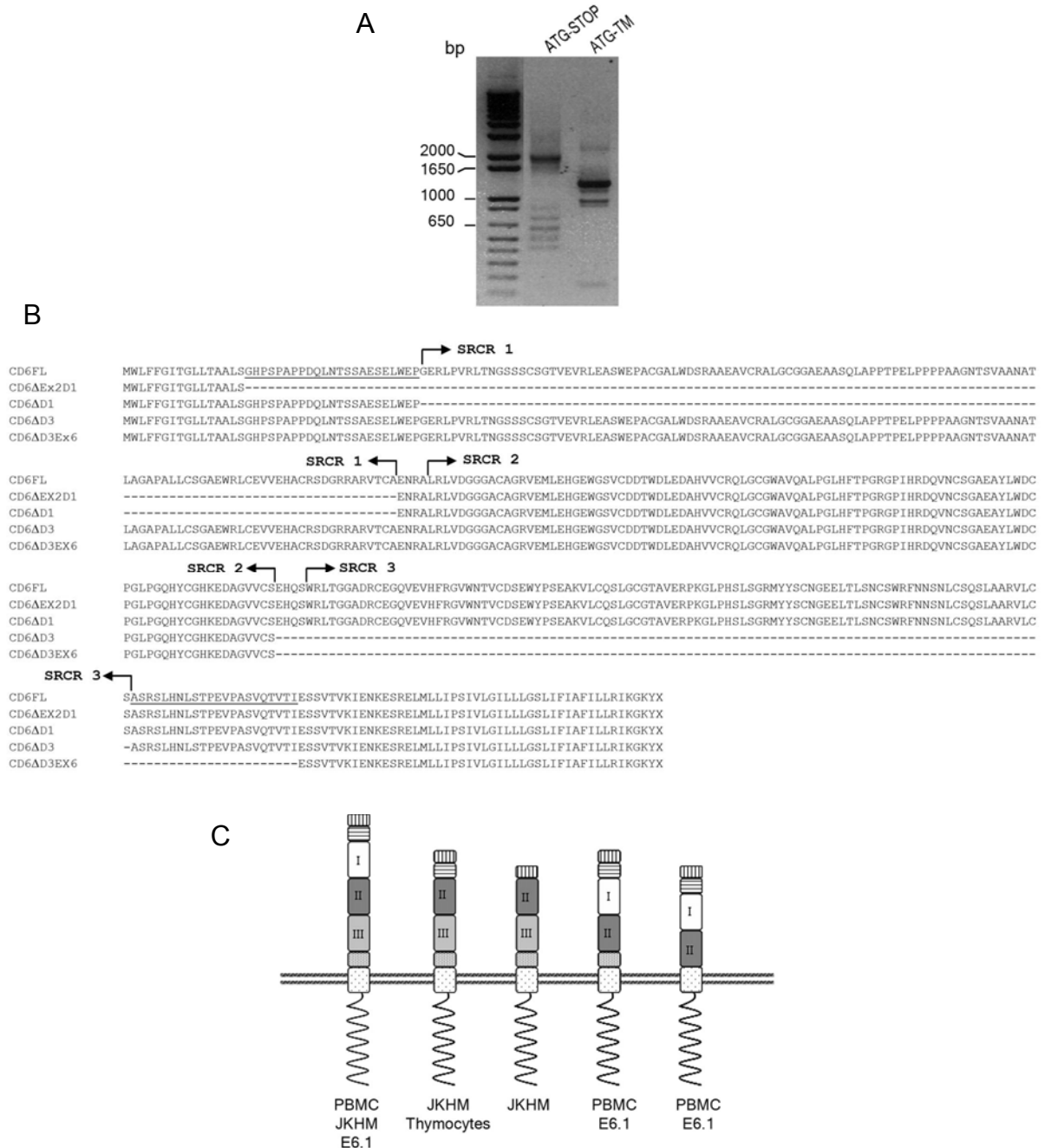


**Figure 1. Cloning of four novel CD6 isoforms lacking extracellular exons.** A) Total RNA from human PBMC was used for RT-PCR and amplified from the ATG up to the transmembrane region (TM) or the STOP codon. The cDNA products were analyzed on gels, isolated and sequenced. In the middle lane the amplification of the full-length cDNA is shown, clearly visible at 2071 bp, and additional shorter products between 2000 and 1600 bp. As can be seen on the right hand sided lane, products of amplification of the extracellular region were obtained, including the full-length isoform of 1200 bp and a smaller band of 900 bp. B) Alignment of CD6FL and smaller extracellular isoforms peptide sequences from the N-terminus up to the transmembrane domain. The boundaries of the SRCR domains are indicated by arrows, according to Hohenester *et al.* [24]. Residues missing in the sequence are indicated by dashed lines. C) Scheme of the structure obtained for the extracellular human CD6 protein isoforms and cells where they were found.

### **T cell activation controls expression of the human SRCR domain 3 of CD6**

The previous results suggested that, as in the rat, multiple CD6 polypeptide isoforms co-exist in human T cells. Since in rat cells we had observed a partial substitution of CD6FL by CD6 $\Delta$ d3 upon cellular stimulation, we compared the expression of the putative CD6 extracellular isoforms in resting T cells, and upon cell activation with PHA-P, using for detection of CD6 monoclonal antibodies specific for CD6 domain 3, OX126, or CD6 domain 1, UMCD6 [22]. In purified resting T lymphocytes, both SRCR domains 1 and 3 of CD6 are expressed at similar levels, a result consistent with resting T cells expressing mostly CD6FL (Fig. 2A, left panel). In contrast, 3 days following stimulation with PHA-P, the expression of domain 3 was significantly down-modulated, compared to the relatively unchanged labeling of domain 1 (Fig. 2A, right panel).

We have also analyzed the changes on CD6 isoform expression upon cell activation by immunoblotting. Non-activated human T cells expressed a major CD6 species of 130 kDa. However, three days post activation with PHA-P, an additional CD6 isoform with a MW of 97 kDa was clearly detected by immunoblotting, while the expression of the larger CD6 species was decreased to a level equivalent to that of the smaller isoform (Fig. 2B). Together, the cytometry and western blotting analysis suggest that following activation of human T lymphocytes, the expression of full-length CD6 is partially down-regulated with the concomitant appearance of the CD6 $\Delta$ d3 isoform.

### **Human CD6 targeting to the immunological synapse: analysis of binding to CD166**

Using rat CD6, we had established that during antigen presentation, the full-length CD6 molecule was translocated to the immunological synapse between T cells and antigen presenting cells, provided that the ligand, CD166, was expressed in the APC, and that domain 3 of CD6 was present at the surface of the T cell. We proceeded to confirm that human CD6 isoforms behaved similarly to those analyzed in rat, regarding translocation to the immunological synapse upon antigen recognition. Human T lymphocytes were induced to express full-length human CD6 (CD6FL), the CD6 $\Delta$ d3 and CD6 $\Delta$ d1 isoforms, or the CD6CY5 mutant, all fused to GFP. Following incubation of T cells with superantigen loaded Raji cells, which expressed endogenous CD166 at high levels on the surface (not shown), conjugate formation and CD6 translocation to the synapse was evaluated in all conditions. Both CD6FL, CD6 $\Delta$ d1 and CD6CY5 localized very efficiently to the immunological synapse in approximately 80% of total conjugates, whereas CD6 $\Delta$ d3 was typically dispersed throughout the cell surface (Fig. 3A and B).

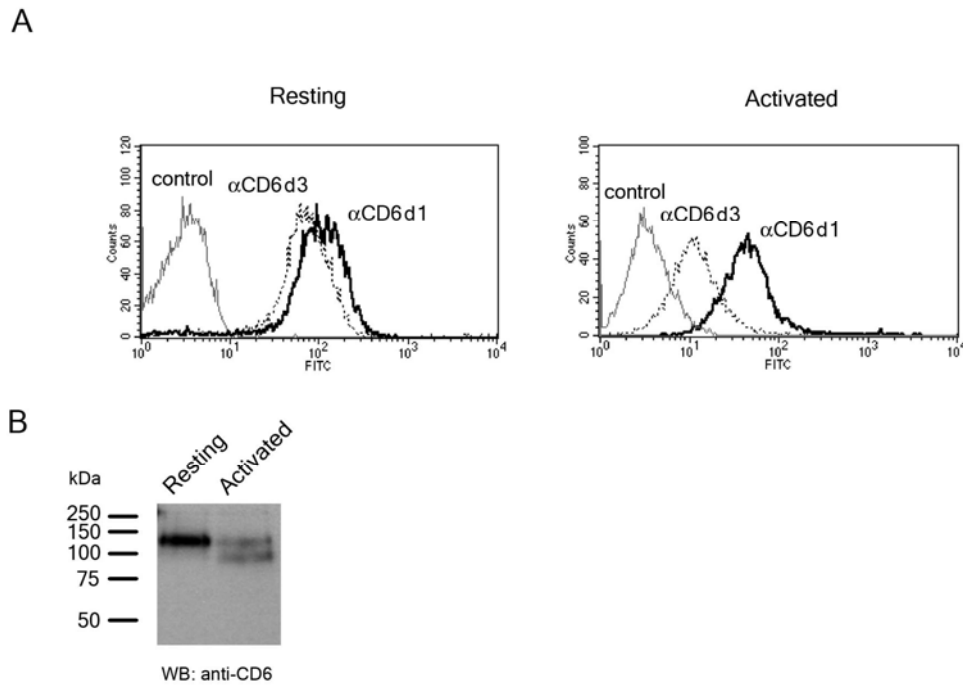


**Figure 2. Increased expression of CD6 $\Delta$ d3 in human cells, upon activation.** A) Human primary T cells isolated from peripheral blood, using the RosetteSep human T cell enrichment cocktail, were analyzed by flow cytometry for the expression of CD6 extracellular isoforms by using specific antibodies recognizing either domain 1, UMCD6, or domain 3, OX126. The expression is compared to the fluorescence of the control secondary antibody FITC-conjugated (left panel). Following 3 days stimulation with PHA-P, a significant decrease in the level of expression of the isoform lacking domain 3 could be detected, as can be seen by using the same antibodies (right panel). PHA-P activated cells were gated based on the expression of the activation marker CD71 (not shown). B) The expression of CD6 in resting cells, and in cells three days post-activation with PHA-P, was analyzed by Western blotting with anti-CD6 MEM-98 mAb. A decrease in the level of the higher molecular weight isoform and the appearance of a lower molecular weight species can be seen after activation.

In order to test whether non-engineered endogenous CD6 expressed in T lymphocytes was still capable of targeting to the synapse, and that this effect was due to the binding to endogenous Raji-expressed CD166, we evaluated, through immunofluorescence, the localization of CD6 in T:Raji interfaces using blocking, as well as non-blocking antibodies. Characteristically, CD6 confined to the synapse in 75% of unblocked T cell:Raji conjugates, a figure that did not significantly change when T cells had been previously incubated with UMCD6, an antibody recognizing domain 1 of CD6, and not able to block CD6-CD166 interactions (Fig. 3C and D). However, when T cells had been incubated with the blocking, anti-CD6 domain 3, mAb OX126, CD6 localization at the synapse dropped dramatically to residual levels (Fig. 3C and D). Similarly, previous incubation of Raji with the CD166 mAb, described to obstruct the interaction with CD6 [34], resulted in the noticeable reduction of the translocation of CD6 to the synapse (Fig. 3C and D), in agreement with a previous study using blocking CD166 antibodies in T cell

– dendritic cell conjugates [15]. Taken together, the results demonstrate that the direct interaction between CD166 and the SRCR domain 3 of CD6 is the driving force for the translocation of CD6 to the immunological synapse.

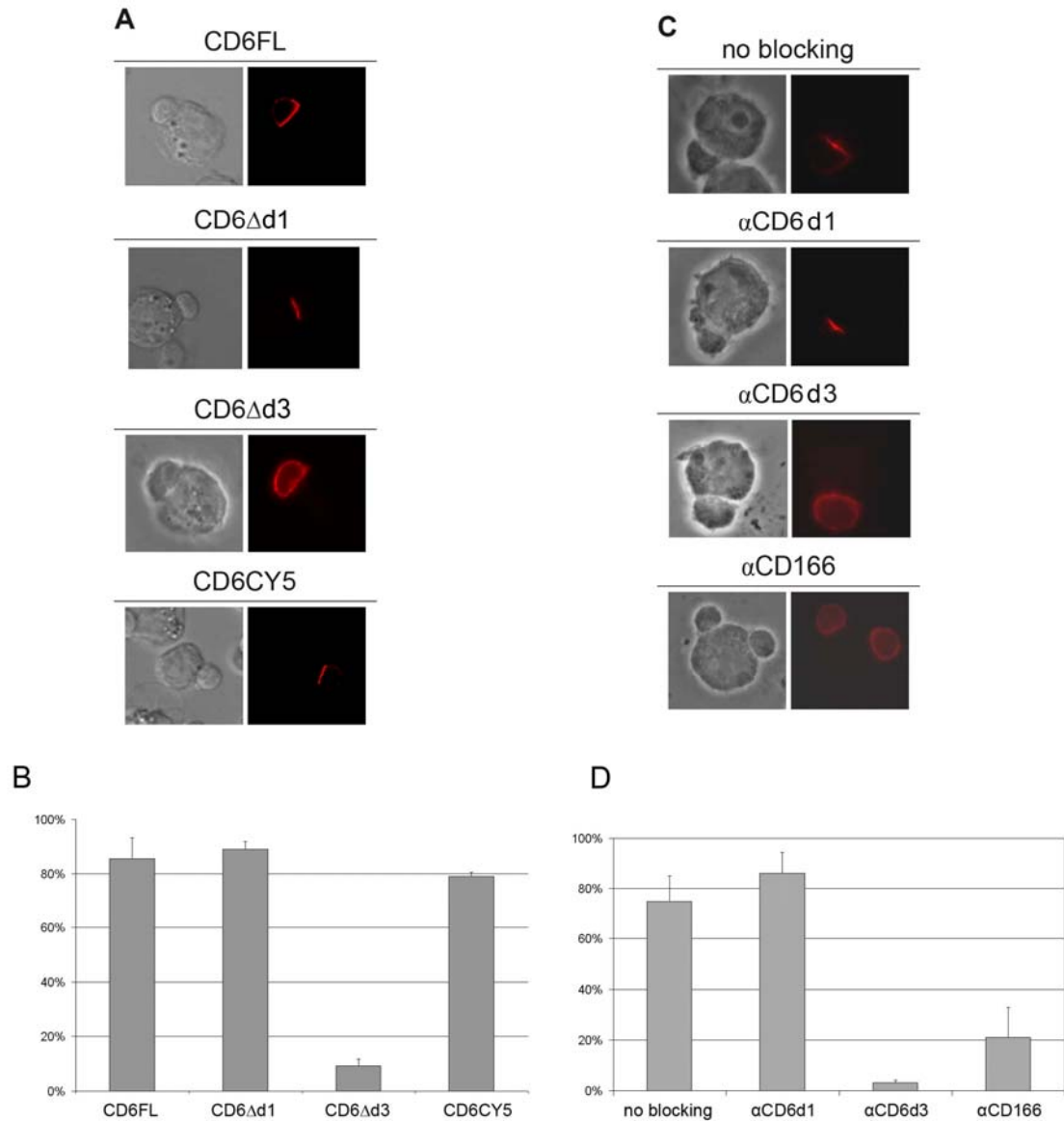


**Figure 3. Human CD6 localizes at the synapse depending on the binding of the CD6 SRCR domain 3 to endogenous CD166 expressed at the surface of Raji cells.** A) T cells expressing CD6FL-GFP, CD6 $\Delta$ d1-GFP, CD6 $\Delta$ d3-GFP, or CD6CY5-GFP (shown in red) were incubated with Raji B cells pre-pulsed with 1  $\mu$ g/ml of a superantigen mix and analyzed for localization at the immunological synapse. B) Bar chart representing CD6 accumulation at the immunological synapse in T cells expressing the different CD6 isoforms. Results are from three independent experiments. C) Antibody blocking interference of CD6-ligand interaction on the localization of CD6 at the immunological synapse. Prior to incubation with superantigen-pulsed Raji B cells, T lymphocytes were incubated with the mAbs UMCD6, recognizing CD6 domain 1 ( $\alpha$ -CD6d1), or OX126, recognizing CD6 domain 3 ( $\alpha$ -CD6d3), or left untreated. Alternatively, Raji B cells were treated with anti-CD166 mAb ( $\alpha$ -CD166). The localization of CD6 was performed by immunolabeling with UMCD6-FITC, or with RAM-FITC in the case of blocking with  $\alpha$ -CD6d3. D) Quantification of the number of cell conjugates displaying CD6 at the immunological synapse.

### Blocking CD6-CD166 binding changes CD6 localization and inhibits tyrosine phosphorylation but does not affect $\text{Ca}^{2+}$ responses on live T lymphocytes

When T lymphocytes expressing endogenous CD6 were pre-treated with the OX126 blocking antibody and then incubated with Raji B cells, as shown in Figure 3, or when using CD6FL/GFP T cells pre-blocked with OX126 (not shown), there was a significant decrease in CD6 clustering at the IS: just a few of the conjugates had CD6 accumulated at the synapse compared to close to 80% obtained without any treatment or using irrelevant antibodies. This result suggests that the interaction with CD166, blocked by OX126, is necessary to target CD6 to the synapse. This treatment additionally resulted in a small reduction, from 87% to 79%, of the number of T cell-APC conjugates actually formed (data not shown). Due to the lack of appropriate antigen presenting cells negative for the expression of CD166, we used this system where T cells were either pre-incubated with the OX126 or left untreated, to further evaluate the role of CD6 in T cell signaling.

The use of primary T lymphocytes interacting with superantigen-loaded APC, and its transient genetic manipulation have proven to be useful tools in the determination of critical parameters for T cell activation [36], such as calcium signals and phosphorylation of tyrosine residues quantification. The measured calcium signals of live T cells treated with OX126, interacting with CD166-expressing Raji cells, were not significantly different from those obtained with untreated T cells (Figure 4).

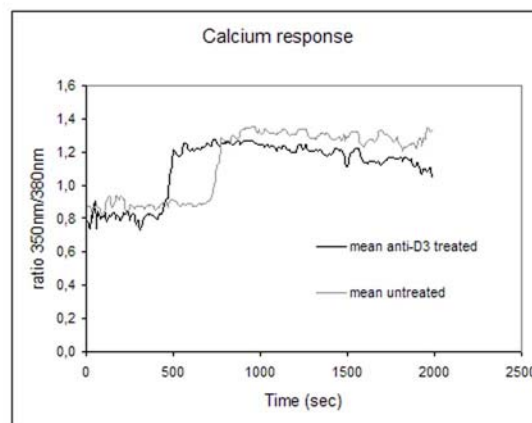


**Figure 4.  $\text{Ca}^{2+}$  responses are not affected by blocking CD6-CD166 binding.** Superantigen-pulsed Raji B cells were allowed to interact with Fura-2 loaded T lymphocytes that express endogenous CD6. Intracellular calcium was measured sequentially every 10s in T cells interacting with Raji B cells. Black line represents mean values obtained with T cells previously treated for 30 min at 4°C with the blocking antibody OX126 and the grey line, the values obtained with untreated T cells.

Using the same approach, tyrosine phosphorylation was measured with 4G10 (anti p-Tyr) immunolabeling in treated and untreated T cells interacting with CD166-expressing Raji cells. Figures 5A and 5B show typical conjugates obtained 30 min after cell-cell contact. The amount of p-Tyr at the IS was significantly lower in cells treated with OX126 blocking antibody (Figure 5B) when compared with untreated cells (Figure 5A). To further analyze this inhibition, we showed that the majority of OX126 treated cells have low levels of p-Tyr at the synapse, with the average mean of 1000 arbitrary units (AU) for p-Tyr intensity while the untreated cells have more distributed levels of p-Tyr intensity, with the mean of 1500 AU (Figure 5C). Hence, we observed a 33% inhibition of tyrosine phosphorylation at the IS when the contact between CD6 and CD166 was blocked and consequently CD6 was not clustered at the immunological synapse.

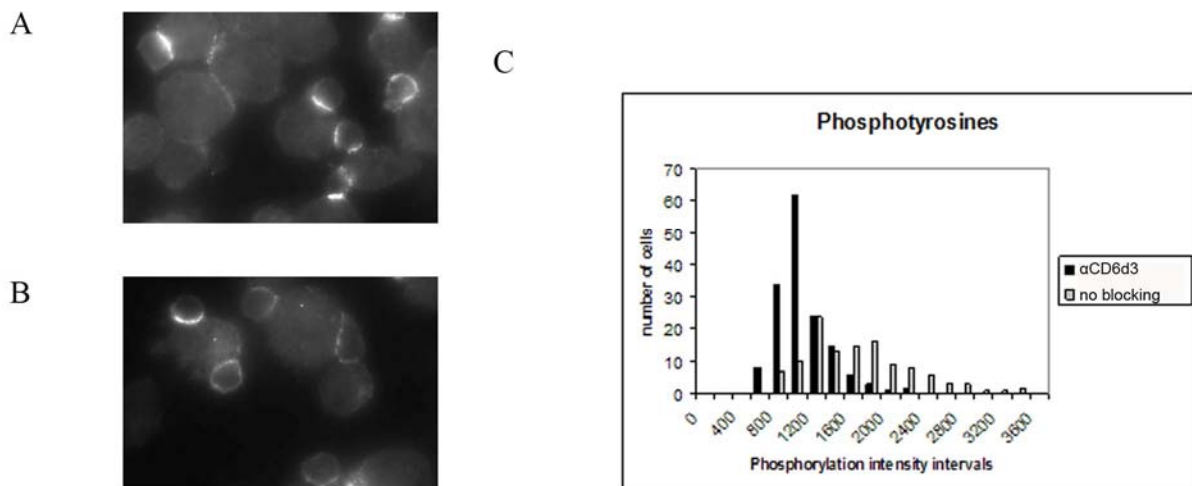


**Figure 5. Tyrosine phosphorylation inhibition after blocking CD6-CD166 binding.** A and B) Superantigen-pulsed Raji B cells were allowed to interact with T lymphocytes expressing endogenous CD6, either left untreated (A) or pre-incubated for 30 min at 4°C with the blocking mAb against d3 of CD6, OX126, at 10 µg/ml (B). After conjugates formed, cells were fixed and phosphotyrosines were labeled with the anti-phosphotyrosine, 4G10 at 5µg/ml, followed by labelling with Rhodamine Red-X-conjugated anti-mouse IgG secondary antibody. C) The graph shows the number of cells presenting different levels of a synaptic accumulation of phosphotyrosine above background. The signal represents a ratio of the signal inside the synapse divided by the signal outside the synapse measured on two regions of same area. Data were averaged from 40 cells analyzed for each condition.

### CD3 and CD6 co-stimulation enhance the overall tyrosine phosphorylation pattern – a role for Lck and ZAP-70

We followed our initial results obtained with rat CD6, demonstrating that CD6 could interact with protein tyrosine kinases of different families: Src-family kinases Lck and Fyn,

the Tec-family kinase Itk, and the Syk-family kinase ZAP-70. As CD6 synergized with the TCR/CD3 complex in promoting the phosphorylation of the SRCR-family molecule CD5, we had shown that Lck together with Itk was the best combination of kinases effectively phosphorylating synthetic peptides corresponding to a cytoplasmic sequence of CD5 [19].

We have further studied the involvement of tyrosine kinases in human CD6 signaling using wild type Jurkat cells, as well as Jurkat cells deficient of either Lck or ZAP-70. As shown in Figure 6, in wild type Jurkat cells, JKHM, CD6 crosslinking could enhance the phosphorylation of CD5 (68 KDa, under reducing conditions). CD3 stimulation as well could promote CD5 phosphorylation, however, the effect was greatly improved with a combination of CD6 and CD3 antibodies.

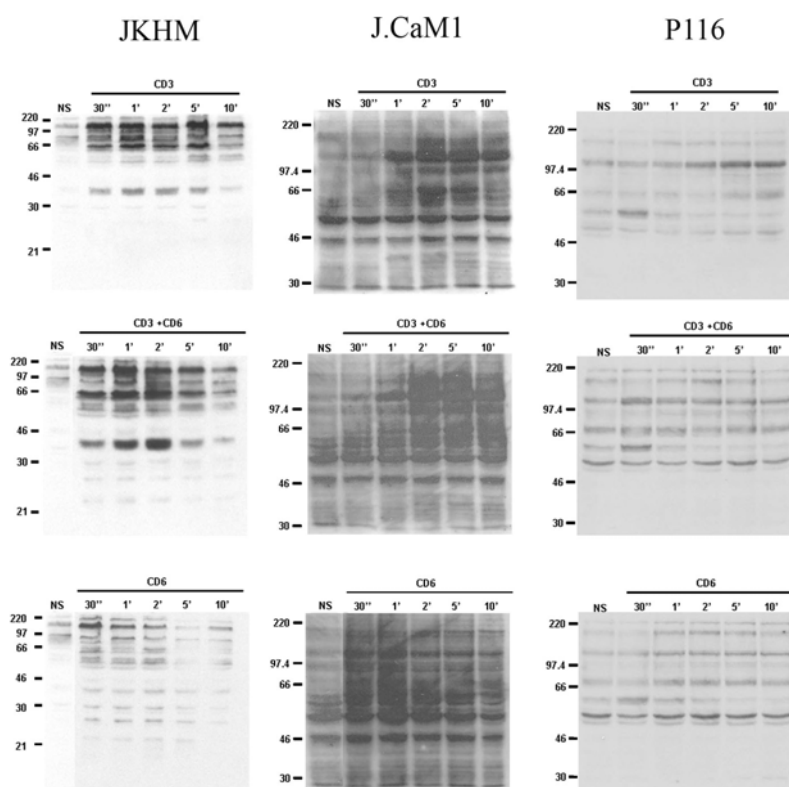


**Figure 5. Tyrosine phosphorylation pattern after CD3 and CD6 crosslinking.** JKHM, J.CaM1 and P116 were left unstimulated or stimulated with CD3 (2 $\mu$ g/ml), CD6 (10 $\mu$ g/ml) or a combination of both and were crosslinked with RAM. Cells were lysed in 1% NP-40 and equal amounts of cleared lysates were resolved on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with 4G10-HRP.

Apart from CD5 phosphorylation, the CD3 + CD6 stimulation resulted in the high level of phosphorylation of a cellular protein ~40 kDa, which we identified as Linker for Activation of T cells (LAT). Using the same protocol in J.CaM1 cells (Lck deficient) and P116 cells (ZAP-70 deficient), we noticed that CD6 signaling was greatly impaired in both

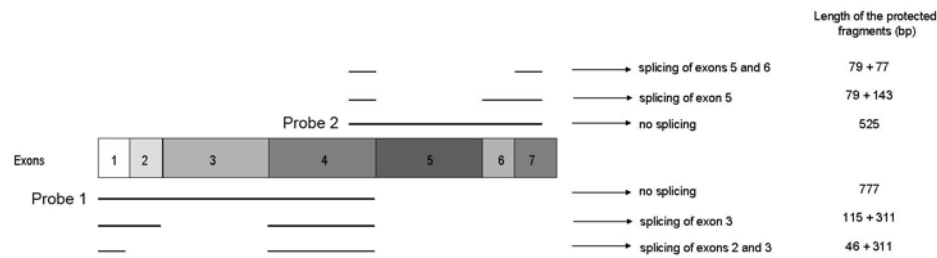
instances. In ZAP-70 deficient cells, no increased phosphorylation could be observed following CD3, CD6 or CD3 + CD6 stimulation. In Lck deficient cells, however, some phosphorylation of CD5 could be obtained, but with a long delay of 2 minutes, in the CD3 and CD3 + CD6 stimulations. No phosphorylation of LAT was observed, though.

### **Quantification of specific mRNA CD6 species using RNase Protection Assays**

While initially setting-up the experimental polymerase chain reaction based-methods for cloning CD6 extracellular-coding mRNA isoforms, we noticed that the frequency of cDNA clones corresponding to CD6 $\Delta$ d1 exceeded, and were easier to obtain than, CD6 $\Delta$ d3 cDNA clones. We thus compared the amounts of the different extracellular isoform-coding CD6 transcripts from resting E6.1 Jurkat cells by RNase Protection Assays, a powerful tool for detecting and quantifying specific RNAs based on the hybridization in solution of a labeled anti-sense probe RNA with the RNA pool being tested. Complementary portions of labeled probe and mRNA from the pool form hybrids that are resistant to digestion with single stranded-specific RNase T1 and RNase A.

Two RPA probes were designed, probe 1 to discriminate isoforms CD6 $\Delta$ d1 and CD6 $\Delta$ Ex2 and probe 2 to discriminate CD6 $\Delta$ d3 and CD6 $\Delta$ d3Ex6 (Figure 7A). As shown in Figure 7B, the unspliced transcripts protected the full-length of both probes, 777 bp for probe 1(★) and 525 bp for probe 2 (✦). The higher bands, on top of the full protected probes, correspond to the undigested probes. Using probe 2, bands corresponding to the splicing of exons 5 and/or 6 were absent. The intensity of the RPA bands obtained was low, but acceptable for the analysis of endogenous expressed mRNA, and did suggest that for non-activated cells there is no major production of CD6 $\Delta$ d3 or CD6 $\Delta$ d3Ex6 mRNAs. However, in line with our initial cloning assessment, transcripts excluding exon 3 gave rise to protected fragments of 311 bp (◀) and 115 bp (●), whereas it was not possible to determine if the simultaneously splicing of exons 2 and 3 occurred as the fragment (46 bp) was too small to be spotted. Given that we were able to detect, by two different methods, the production of mRNA species that lack exon 3, or exons 2 and 3, but have no evidence from cytometry analysis (Figure 2) that either resting or activated cells can express CD6 proteins lacking domain 1, it is possible that there is a block in the translation of these particular transcripts, or that these specific mRNAs are not further processed to become messenger RNA, being possibly regulatory RNAs.

A



B

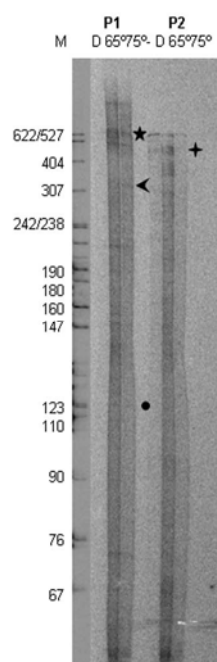


**Figure 7. Quantification of specific mRNA CD6 species using RNase Protection Assays.** A) Schematic representation of CD6 extracellular and transmembrane exons. The two probes designed for the RPA and the expected protected fragments are indicated. B) Total RNA from E6.1 cells was analyzed for quantifying the different human CD6 mRNA species. RT-PCR was used to obtain a cDNA template for the antisense RNA probes. Probe 1 and 2 were linearized, purified and complete digestion was checked on agarose gel.  $^{32}\text{P}$ -radiolabeled UTP (NEN) was incorporated into probes by in vitro transcription using T3 polymerase for probe 1 and T7 polymerase for probe 2. E6.1 cells total RNA (30  $\mu\text{g}$ ) was hybridized with excess amounts of each riboprobe. After hybridization at 65°C and at 75°C, the samples were digested with RNase A/T1 and the protected fragments were resolved on 8% 8M Urea denaturing acrylamide gels and visualized using X-ray films. The full-length of both probes protected the unspliced transcripts, with 777 bp for probe 1 (★) and with 525 bp for probe 2 (+). The higher bands, on top of the full protected probes, correspond to the undigested probes. Transcripts excluding exon 3 gave rise to protected fragments of 311 bp (◀) and 115 bp (●) and, it was not possible to determine if the simultaneously splicing of exons 2 and 3 occurred as the fragment (46 bp) was too small to be detected. Unexpectedly, with probe 2 bands corresponding to the splicing of exons 5 and/or 6 were absent. pBR322 DNA-MspI digest (New England Biolabs) was used as molecular weight marker. D represents the digestion control of each probe.

However, given that they can be produced, we investigated in other cell types whether CD6 polypeptides devoid of domain 1 could be expressed at a given stage. We analyzed T lymphocytes from healthy donors, a B-CLL derived line, and human thymocytes from four different subjects. Using two color FACS analysis addressing the expression of domains 1 and 3 of CD6, in most of the cell populations analyzed we found no evidence of cell populations expressing CD6 molecules lacking domain 1, although in two of the normal T lymphocyte samples, a minute number of cells (less than 0.5%) appeared to be in fact domain 3 positive, and domain 1 negative. Interestingly, a significant number of thymocytes from all four different samples were CD6d1<sup>+</sup>d3<sup>-</sup>, or had the domain 3 expressed at low levels. Therefore, adding to the down-regulation of domain 3 induced in activated T lymphocytes, the suppression of CD6 domain 3 expression is significantly more evident in a considerable number of thymocytes.

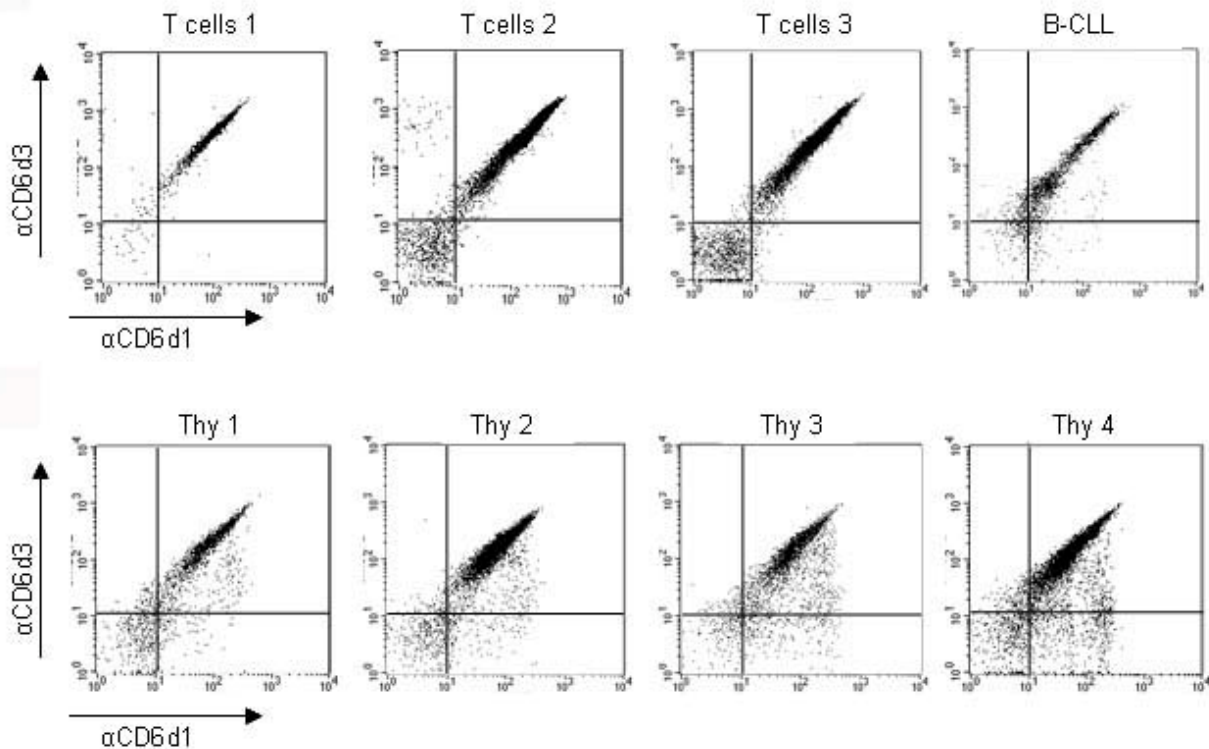


**Figure 8. FACS analysis of CD6d1 and CD6d3 expression.** Human primary T cells, from three healthy donors were isolated from peripheral blood, using the RosetteSep human T cell enrichment cocktail; human thymocytes (Thy), obtained from four human thymic biopsy specimens; and a B-CLL derived line were analyzed by flow cytometry for the expression of CD6 extracellular isoforms by using specific antibodies recognizing either domain 1, UMCD6, or domain 3, OX126. Most cell populations do not express CD6 $\Delta$ d1 isoform, although samples 1 and 2 from T cells have less than 0,5% that appear to be CD6d1<sup>+</sup>d3<sup>+</sup>. All four samples of thymocytes do cells that are CD6d1<sup>+</sup>d3<sup>-</sup>.

## DISCUSSION

The great majority of mammalian genes yield multiple mRNA transcripts arising from diverse promoter selection, alternative splicing and/or differential polyadenylation [37]. In spite of the frequency and potential impact of this phenomenon, particularly relevant in the immune system, the cases in which distinct functional properties have been assigned to different mRNA isoforms of the same molecule are scarce. In T lymphocytes, just a few examples of naturally occurring alternatively spliced isoforms of transmembrane proteins have been reported, and only in three cases with clear distinct functional consequences: CTLA4 and CD95 can become transmembrane molecules instead of being secreted [38, 39], as a consequence of the inclusion of membrane spanning domains following cell stimulation; and CD45, upon T cell differentiation, is produced with a shorter N-terminal extracellular domain, which increases the dimerization capacity of the molecule [40]. Alongside this general trend, multiple isoforms resulting from alternative splicing of cytoplasmic domain-coding exons have been described for CD6, but with no distinct functions assigned [18, 28-30]. In this work we describe a rare example of a functional consequence resulting from alternative splicing in molecules of the immune system: the localization of CD6 with respect to the immunological synapse depends on the regulated expression of the CD166-binding domain by means of alternative mRNA splicing.

Targeting of CD6 to the synapse can be driven by simple lateral diffusion and does not require cytoskeletal reorganization, as deletion of the cytoplasmic tail did not affect the ability of CD6 to localize to the synapse. Therefore, the positioning of CD6 within the synapse must be largely determined by molecular interactions established with the contacting cell. Human CD6 molecules targeted very efficiently to the immunological synapse when expressing the CD166-binding domain, provided that the antigen presenting cells expressed the ligand, CD166, and that no blocking using antibodies occurred. However, when using T cells expressing CD6 $\Delta$ d3, synaptic localization of that isoform could still be attained in a small percentage of conjugates. A putative role for domain 2 of CD6, now substituting the positioning of domain 3 in CD6 $\Delta$ d3, or even for the membrane juxtaposed stalk region, could be considered, given that previous studies had suggested a minor participation of these domains in the binding to CD166 [22, 23]. Nevertheless, it is the presence of domain 3 that largely determines CD6 synaptic localization, and thus regulation of its expression is the key event establishing the positioning of CD6 upon conjugate formation and T cell activation.

The mode of action of CD6 appears to be significantly different from that of other accessory molecules that influence signaling at or near the immunological synapse. CD2

binding to its ligand CD58 (CD48 in rodents) contributes to enhanced signaling, as it associates with intracellular positive mediators [41]. CD28 and CTLA-4 have devised a scheme whereby sequential expression of each molecule, combined with binding to one of the alternative ligands, CD80 and CD86 (themselves also sequentially expressed during T cell-APC interactions), drives responses from co-stimulation to inhibition [42]. Differences in the affinity and avidity of binding determine the selective recruitment of CD28 or CTLA-4 to the immunological synapse upon recognition of CD86 or CD80 [43].

Meanwhile CD6, which was reported to deliver co-stimulatory signals as strongly as CD28 [15], mimics CD28 distribution with respect to synaptic localization, in that it is recruited to the immunological synapse at the onset of activation, and, according to the present study, is excluded from the cellular interface at later stages. However, it may achieve this through a completely different mechanism: expressing the SRCR domain 3, CD6 is directed to the synapse where it can control signaling; choosing not to express the SRCR domain 3, it is no longer restricted to the cellular interface and its regulatory function may be diluted.

The reason why CD6 does not simply switch off its expression is not immediately evident. However, the existence of an alternative ligand for CD6 raises new perspectives for its overall function [44]. If the new ligand can interact with a different part of CD6, switching from full-length to  $\Delta$ d3 may allow CD6 to shift from the synapse established with the APC to either an interaction with a second adjacent cell, or again to the initial interface, provided that the second ligand is present. Differential molecular interactions could then modulate the behaviour of CD6. A role for domain 1 in the binding to this putative alternative ligand is worth consideration, given that domain 1 itself could be, albeit very rarely, excluded.

The apparent steadiness in expression of the alternative CD6 mRNAs in different mature sub-populations in the rat may suggest that CD6 $\Delta$ d3 can define small subsets of thymocytes and mature T cells (Castro *et al.* 2007, unpublished results), however, the switch to the expression of the shorter isoform observed upon stimulation of T cells is undoubtedly a consequence or a product of activation. In the resting state, the majority of the cells express mainly the full-length CD6 form; upon productive T cell stimulation, there is a partial substitution of the full-length isoform by a substantial number of CD6 molecules per cell that no longer express the CD166-binding domain and thus are not restricted to the immunological synapse. This type of regulation of splicing is not unique, as it has long been known to control the expression of CD45 isoforms [45, 46]. The novel CD6 isoform reported here seems to have a more obvious function as it disables the interaction of the protein with its ligand. Productive TCR recognition of antigen may signal for the shorter

CD6 isoform to be produced, and this may reduce the activity of CD6 at the synapse, possibly inducing a different behaviour.

The expression of the CD6FL isoform seems to be highly favoured in the DP stage in the thymus (Castro *et al.* 2007, unpublished results). Previous observations suggest that CD6 can be involved in positive selection where it correlates with the expression of CD69 in DP thymocytes [47]. Moreover, an inverse correlation between thymocyte CD6 expression and the rate of apoptosis has been demonstrated. Our results may be indicative of a mechanism of regulation where the expression of CD6FL capable of interacting with the ligand, CD166, expressed by thymic epithelial cells, is favoured at the double positive stage and bypassed later on during thymic selection.

Although preliminary, our findings show that the inhibition of CD6-ALCAM binding, results in a negligible effect on  $\text{Ca}^{2+}$  signals but in a 33% inhibition of tyrosine phosphorylation at the IS. Our previous observations on rat CD6 (unpublished results), indicating that it can down-modulate calcium signals initiated upon T cell interaction with superantigen-primed Raji cells are not in disagreement with the current results. In rat  $\text{CD6}^+$  cells establishing contacts with Raji cells, expressing or not rat CD166, no differences in the registered  $\text{Ca}^{2+}$  profiles were observed, clearly indicating that the localization at the IS is not crucial for CD6 to down-modulate signaling. This may be the reason why we don't see any difference in the  $\text{Ca}^{2+}$  profile after blocking CD6 engagement. It is tempting to speculate that CD6 expression can result in a general inhibitory outcome but the engagement of CD6 with ALCAM and subsequent accumulation at the synapse, point towards a positive or co-stimulatory function for CD6, as demonstrated by our p-Tyr and crosslinking results and also previous studies [8-11, 13, 14]. It is possible, however, that the suppression of activation signals is due to the overall interference that the soluble reagents cause on the cell-to-cell contact, rather than to a very specific effect blocking just the CD6-CD166 ligation.

Some of the early reports on the co-stimulatory properties of CD6 relied on the use of monoclonal antibodies as the source of external stimuli [8-10]. Also, in our experiments crosslinking human CD6 together with the TCR, with the use of monoclonal antibodies, we observed a very strong phosphorylation of LAT, an important mediator of T cell signaling. However, such type of stimulation can very likely induce the indiscriminate aggregation of protein tyrosine kinases that associate non-covalently with CD6 [19], possibly originating effects very dissimilar from those obtained through physiological stimulation. CD5, a highly homologous molecule also expressed in T lymphocytes, was originally accepted as a co-stimulatory molecule for equivalent reasons [48-50]. It turned out to be a clear inhibitor of T-cell activation and thymocyte selection [51].

An inhibitory role for CD6 on thymocyte development has been previously suggested [47, 52]. Our earlier unpublished results with rat CD6 provide biochemical evidence that CD6 can indeed modulate signals triggered upon antigen recognition. Whether this role is intrinsic, or dependent on regulated interactions with other surface molecules, is still to be determined. For example, the IgSF surface glycoprotein CD2 can transduce mitogenic signals due to its association with the tyrosine kinases Lck and Fyn [41], however, through its interaction with the inhibitory molecule CD5 it can also induce or amplify a negative response [35, 53]. Regarding CD6, it shares with CD5 the attribute to inhibit signaling upon T cell-APC interactions, not requiring ligand binding to do so [54]. Since CD5 and CD6 also associate at the surface of T cells and one major effect of CD6 stimulation is the phosphorylation of CD5 [19], it is feasible that CD5 can integrate or transform the signals triggered through CD6. The physiological mode of regulation exerted by CD6 on cellular behaviour could therefore depend on the levels of expression, or most likely and given that very few T cells do not express CD6, on the isoforms of CD6 produced at a certain stage of cellular activation or differentiation.

This interpretation can nevertheless make the case that CD6 assists in T cell activation as well: given that, as we suggest, the CD6-associated enzymatic activity is kept constant regardless of the cellular localization of CD6, if CD6 localizes to the immunological synapse and binds to CD166, these interactions are likely to increase cellular adhesion and thus support a stimulatory role for CD6, albeit indirect.

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**PROTEIN INTERACTIONS BETWEEN CD2 AND LCK ARE REQUIRED  
FOR THE LIPID RAFT DISTRIBUTION OF CD2**

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## ABSTRACT

In T lymphocytes, lipid rafts are preferred sites for signal transduction initiation and amplification. Many cell membrane receptors, such as the T cell receptor, co-receptors, and accessory molecules associate within these microdomains upon cell activation. However, it is still unclear in most cases whether these receptors interact with rafts through lipid-based amino acid modifications, or whether raft insertion is driven by protein-protein interactions. We have addressed the mechanisms that control the localization of rat CD2 at the plasma membrane, and its redistribution induced upon activation. Following incubation of rat CD2-expressing cells with radioactive-labeled palmitic acid, or using CD2 mutants with cysteines 226 and 228 replaced by alanine residues, we found no evidence that rat CD2 was subjected to lipid modifications that could favor the translocation to lipid rafts, discarding palmitoylation as the principal mechanism for raft addressing. On the other hand, using Jurkat cells expressing different CD2 and Lck mutants, we show that the association of CD2 with the rafts fully correlates with its capacity to bind to Lck. As CD2 physically interacts with both Lck and Fyn, preferentially inside lipid rafts, and reflecting the increase of CD2 in lipid rafts following activation, CD2 can mediate the interaction between the two kinases and the consequent boost in kinase activity in lipid rafts.

## INTRODUCTION

CD2 is a 45-58 KDa type I integral protein expressed on virtually all T lineage and natural killer cells [1]. The extracellular domain of CD2 is composed by two immunoglobulin superfamily domains [2], of which the membrane-distal V-like domain is involved in the binding to the ligand [3]. Engagement of CD2 to CD58 in humans, or to CD48 in rodents, facilitates adhesion between T cells and antigen presenting cells (APC) and is proposed to promote the formation of an optimal intercellular membrane spacing ( $\sim 140\text{\AA}$ ) suitable for TCR-pMHC recognition [4].

Concomitantly with its function as an adhesion molecule, CD2 has a role in the process of signal transduction. It has long been known that stimulation of CD2 with combinations of monoclonal antibodies or with the physiological ligand induces T cell proliferation in rats and human [5, 6]. Moreover, ligation of CD2 with CD58 augments the interleukin-12 (IL-12) responsiveness of activated T cells [7], and can also reverse T cell anergy induced by B7 blockage [8].

The cytoplasmic domain is required for CD2-mediated activation [9-12], but as it has no intrinsic enzymatic activity, it depends on physical interactions with other signaling

molecules to propagate the stimulus received upon ligand binding. CD2 associates with the Src-family tyrosine kinases Lck and Fyn [13-15], and through these kinases couples to downstream signal transduction pathways. Proline-rich sequences of the cytoplasmic domain of CD2 have also been shown to establish interactions with the SH3 domain of the adaptor protein CD2AP [16], and thus connect CD2 to the cytoskeleton, and with the SH3 domain of CD2BP1 [17], resulting in the down-regulation of activation-dependent CD2 adhesion. CD2 additionally binds to the GYF domain of CD2BP2, an interaction involved in cytokine production following CD2 stimulation [18].

The T cell plasma membrane contains regions of distinct lipid composition, enriched mainly in sphingolipids and cholesterol immersed in a phospholipid-rich environment, and termed lipid rafts [19]. In unstimulated cells, key signaling proteins such as the adaptors LAT [20] or PAG [21], or the Src kinase Fyn [22], are resident in the rafts, whereas most integral proteins are found outside these platforms. Localization and targeting of signaling molecules to lipid rafts is largely dependent on post-translational acylation modification of proteins, one of the most important being membrane-proximal cysteine palmitoylation. These cysteine residues are usually present within a conserved motif consisting of cysteines and hydrophobic residues, CVRC in LAT, GCVC in Lck and Fyn.

The composition of rafts-associated proteins may, nevertheless, change upon cell stimulation. Membrane compartmentalization and partitioning of essential T cell-activating components in lipid rafts were shown to be involved in the initial stages of T cell activation [23]. Several co-stimulatory molecules on the T cell surface, such as CD2 [24], CD5, CD9 [25] and CD28 [26] can up-regulate TCR signals by enhancing the association of the TCR with lipid rafts. Also, whereas the fraction of Lck found in the rafts is in a close inactive conformation, upon cell activation the open/active form of Lck accumulates in the rafts [27], where it activates resident Fyn [22].

Human CD2 has been shown to translocate to lipid rafts upon CD2-antibody crosslinking, or following binding to CD58 during conjugate formation [28], and the shift between phases may result in the replacement of CD2BP2 by raft-resident Fyn [29], which competes for the same proline-rich sequence of the cytoplasmic tail of CD2. In contrast to human CD2 which is reported to be entirely non-raft resident in resting cells [28], mouse CD2 contains a CIC motif at the membrane-proximal region that can putatively address CD2 to lipid rafts [25]. We have investigated the localization and transition of rat CD2 between different phases at the T cell surface, and show that a significant fraction of rat CD2 is constitutively present in lipid rafts, and that this proportion increases upon CD2-stimulation. However, membrane-proximal cysteine palmitoylation is not the decisive tag addressing rat CD2 to lipid rafts, but rather the interaction with the Src family kinase Lck.

## MATERIALS AND METHODS

### Cells and transfections

Splenocytes were obtained by maceration of spleens from 3-months old Wistar male rats in ice cold PBS. The rat thymoma cell line W/FU(C58NT)D [30], referred to in the text as C58 cells, was given by A. Conzelmann (University of Fribourg, Switzerland).

Jurkat E6.1 cells [31] were obtained from A. Weiss (UCSF, CA); E6.1 cells expressing full-length rat CD2, E6.1-CD2(CY whole) (referred to in this text as E6.1-CD2), or expressing CD2 cytoplasmic deletion mutants, E6.1-CD2(CY 6), (CY 40), (CY 66), (CY 81) and (CY 97), that possess respectively the first 6, 40, 66, 81 and 97 amino acids of the cytoplasmic tail [12, 32] were kindly provided by M. Puklavec (University of Oxford, UK).

pKG5-CD2-2C, a plasmid where the codons for Cys<sup>226</sup> and Cys<sup>228</sup> are mutated to encode alanine residues, was constructed by PCR using pRCD2-11, a plasmid containing the cDNA sequence of full-length rat CD2 [12], as template with the forward primer 5'-GGGGCGCTGTTTATTTTCGCTATCGCCAAGAGGAAAAACGGAAC-3' and the reverse primer 5'-GTTCCGTTTTTCTCTTGGCGATAGCGAAAATAAACAGCGCCCC-3'. The changed codons are underlined. The PCR product was digested with Dpn I and used to transform competent cells. CD2 positive mutants were digested with Bam HI and replaced the equivalent restriction fragment containing wild type CD2 subcloned in pKG5. pKG5-CD2-2C was used for transfecting E6.1 cells to handle E6.1-CD2(CIC/AIA). Briefly, cells at  $1.0 \times 10^7$  cells/ml in ice cold PBS were transferred into 0.4 cm electroporation cuvettes, mixed at 4°C with plasmid at a final concentration of 0.1 mg/ml, and pulsed with 0.62 kV and 25  $\mu$ F using a Gene Pulser II electroporator from Bio-Rad (Hercules, CA). Cells were transferred to culture flasks containing RPMI with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50  $\mu$ g/ml), all from Invitrogen (Barcelona, Spain). After forty-eight hours, the selection antibiotic was added to the cultures (G418 at 1 mg/ml). Following antibiotic selection, cells were analyzed for expression by flow cytometry and immunoblotting.

The Lck deficient J.CaM1 cell line [33] was transfected with pRCD2-11, and G418-resistant cells that expressed high levels of rat CD2 were selected and named J.CaM1-CD2. To generate J.CaM1/Lck(KD)-CD2 cells, the newly obtained J.CaM1-CD2 cells were co-transfected with pSR $\alpha$ -Lck-KD, a plasmid containing Lck cDNA carrying a mutation at the ATP-binding site (K273A) [34]. J.CaM1 cells reconstituted with wild-type Lck, J.CaM1/Lck(WT), and with an Lck molecule having Cys<sup>3</sup> mutated to Ala<sup>3</sup>, J.CaM1/Lck(C3A) [35], were obtained from P. Kabouridis (Queen Mary, University of London, UK), and transfected with pRCD2-11, to handle J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells, respectively.

### **Antibodies**

The mAbs used were rat CD2-OX55 [6] and rat CD2-OX34 [36]. Polyclonal antibodies and conjugates were: rabbit anti-LAT, from Upstate Biotechnology; rabbit anti-Lck, raised against a peptide of amino acids 39-64 of murine Lck, a gift from J. Borst (The Netherlands Cancer Institute, Amsterdam); BL90, polyclonal anti-Fyn, a gift from J. Bolen and M. Tomlinson (DNAX Research Institute, Palo Alto, CA); goat anti-mouse peroxidase conjugated, purchased from Molecular Probes (Eugene, OR); and goat anti-rabbit peroxidase conjugated from Zymed (San Francisco, CA).

### **Metabolic labeling of cellular proteins with [<sup>3</sup>H]palmitate**

Aliquots of  $1.5 \times 10^7$  cells were collected from culture, resuspended in 1.5 ml RPMI complete medium supplemented with 300  $\mu$ Ci/ml [<sup>3</sup>H]palmitic acid and incubated for 16 h at 37°C. Cells were harvested and lysed in lysis buffer (10 mM Tris.Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% (v/v) Triton X-100). The nuclear pellet was removed by centrifugation at  $12,000 \times g$  for 10 min at 4°C and the lysate run on SDS-PAGE under non-reducing conditions. Immunoprecipitations were performed by incubating the lysate with antibodies (5  $\mu$ g IgG or 2  $\mu$ l anti-serum) and 100  $\mu$ l of 10% protein A Sepharose CL-4B beads (Amersham Biosciences), for 90 min at 4°C by end-over-end rotation. The beads containing the immune complexes were washed three times in 1 ml lysis buffer, denatured and loaded on an SDS-PAGE gel. The gel was soaked for 30 min in fixing solution (25:65:10 isopropanol/H<sub>2</sub>O/acetic acid) and additionally treated for 30 min with “Amplify” (Amersham Biosciences), dried and exposed to film for 6 weeks.

### **Flow cytometry**

Cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% NaN<sub>3</sub> (PBS/BSA/NaN<sub>3</sub>), at a concentration of  $1 \times 10^6$  cells/ml. Staining was performed by incubation of  $5 \times 10^5$  cells/well with mAbs (20  $\mu$ g/ml) for 15 min on ice, in 96-well round-bottom plates (Greiner, Nürtingen). Cytometric analysis was as previously described [37].

### **Sucrose gradient centrifugation**

Approximately  $1.5 \times 10^8$  cells were used per sample. Cells were washed, resuspended in 1 ml RPMI medium and maintained at 37°C for 15 min. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 30 min on ice in 1 ml MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5), containing 1% Triton X-100, 1 mM PMSF and cocktail protease inhibitors (1 mM AEBSF, 0.8  $\mu$ M aprotinin, 50  $\mu$ M bestatin, 15  $\mu$ M E-64, 20  $\mu$ M leupeptin and 10  $\mu$ M pepstatin A, Calbiochem, La Jolla, CA). The lysates were

homogenized by brief sonication for ten pulses on ice, using a Heat Systems/Ultrasonics sonicator (model W-375) equipped with a microtip and set to 50% duty cycle, output 3. To obtain the rafts fraction, cell lysates were mixed with an equal volume of 85% sucrose in MBS buffer and transferred to the bottom of Sorvall ultracentrifuge tubes. The samples were then overlaid with 6 ml of 35% sucrose followed by 2 ml of 5% sucrose. After centrifugation at  $200,000 \times g$  for 17 h at 4°C in a TST41.14 swing-out rotor (Sorvall), 10 fractions of 1 ml were collected from the bottom of the tube and analyzed by Western blotting. The fractions are labeled from the top of the gradient.

### **Western blotting**

Proteins were separated by SDS-PAGE in non-reducing conditions and then transferred to Hybond-C-extra membranes by electroblotting. Membranes were blocked in TBS, 0.1% (v/v) Tween 20 (TBS-T), containing 5% (w/v) non-fat dried milk, probed with unconjugated primary antibody for 1 hour and revealed with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (1:20,000 dilution). Immunoblots were developed using ECL-plus reagents (Amersham Biosciences, Carnaxide, Portugal) and exposed to CL-XPosure films (Pierce, Rockford, IL).

### **Densitometry**

Densitometric analyses were performed on autoradiographs of the SDS-PAGE gels, using a GS-800 Densitometer (BIO-RAD) and Quantity One software. All densitometry values obtained were calculated from non-saturated signals.

### **Capping and immunofluorescence microscopy**

All procedures were performed at 4°C unless otherwise described. Cells were washed in RPMI, resuspended at  $2 \times 10^6$  cells/ml in RPMI/5% FCS and incubated for 10 min with the mAbs OX34 at 5 µg/ml. Cells were washed twice with PBS containing 0,2 % BSA, followed by incubation with RAM-Alexa 568 conjugate (Molecular Probes, Leiden, The Netherlands) for 10 min. After washing, cells were incubated with Cholera-toxin subunit B Alexa 488 conjugate (Molecular Probes, Leiden, The Netherlands) for another 10 min, and washed again. Cells were resuspended in RPMI/5% FCS and incubated for 15 min at 37°C to induce antibody capping. For fixation, cells were treated with 4% para-formaldehyde in PBS for 30 min at RT. Fixed cells were plated onto glass coverslips and mounted in Vectashield media (Vector Laboratories, Burlingame, CA). Stained preparations were observed with a Zeiss Axioskop microscope, and images acquired with a SPOT2 camera (Diagnostic Instruments). Images were processed with Photoshop 6.0 (Adobe Systems). The percentage of co-localization represents the counts of CD2 caps

that also had GM1 caps, with the mean and standard deviation of counts of at least 40 cells from different experiments.

### **Cell activation**

For lipid rafts analysis, approximately  $1.7 \times 10^8$  cells were used per sample. Cells were washed and resuspended in 1 ml RPMI medium containing OX34 at a 1:100 dilution of ascitic fluid. After 5 min incubation on ice, cells were crosslinked with RAM at 20  $\mu\text{g/ml}$  and maintained at 37°C for 15 min. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS), lysed and fractionated by sucrose gradient centrifugation as described above.

### **Immunoprecipitations and in vitro kinase assays**

Immunoprecipitations, in vitro kinase assays, and reprecipitation of phosphorylated substrates were performed as previously described [13].

## **RESULTS**

### **A significant proportion of CD2 associates with plasma membrane lipid rafts in resting rat T cells and in a rat thymoma cell line**

Previous work has shown that in human resting T lymphocytes the transmembrane glycoprotein CD2 is virtually absent from membrane lipid rafts, as defined based on a Triton X-100 insolubility criterion [28]. However, in mouse cells a considerable fraction of CD2 molecules are raft resident [25]. We have investigated the membrane localization of the rat homologue of CD2 in resting primary cells as well as in the rat thymoma cell line C58. Non-activated splenocytes or C58 cells were solubilized in a lysis buffer containing 1% Triton X-100, and the lysates were subjected to sucrose gradient centrifugation. The localization of the molecules within or outside lipid rafts was evaluated by immunoblotting with the monoclonal antibody OX55, using as marker for the rafts fractions immunoblottings for the resident palmitoylated molecule LAT [20]. As measured by densitometry of immunoblots in different experiments, between 7 and 15% of rat CD2 is constitutively confined to the rafts in unstimulated splenocytes, and the majority of CD2 molecules are recovered from the “soluble” phase (Fig. 1). Using non-stimulated C58 cells, we could observe that CD2 is over represented in the rafts (approximately 75%), compared to the non-raft fractions.

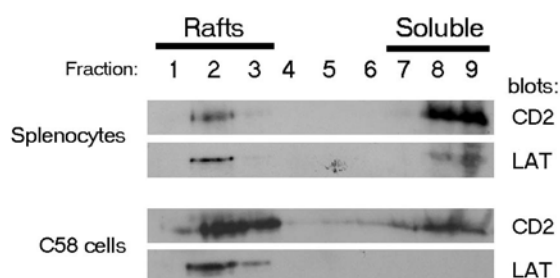


**Figure 1. CD2 associates with lipid rafts in resting rat T cells and in a rat thymoma cell line.** Approximately  $1.5 \times 10^8$  cells rat splenocytes and a rat thymoma cell line, C58 cells, were lysed and total cell lysates fractionated by sucrose density centrifugation as described in Material and Methods. Fractions were recovered, and numbered 1 to 9 from the top of the gradient. Equal volumes of each fraction were separated by SDS polyacrilamide gel electrophoresis (SDS-PAGE) and immunoblotted with OX55 ( $\alpha$ -CD2), followed by horseradish peroxidase-labeled goat anti-mouse. LAT was visualized with a polyclonal antibody followed by horseradish peroxidase-labeled goat anti-rabbit. The preferential distribution of LAT into the rafts allows for the identification of raft fractions. Densitometry of the bands reveal that in the experiment shown, 15% of rat CD2 molecules from splenocytes were localized in lipid rafts, as were approximately 75% of CD2 from C58 cells.

### Rat CD2 targeting to lipid rafts is not fully determined by palmitoylation of Cys<sup>226</sup> and Cys<sup>228</sup>

The difference in raft-localization between human and mouse CD2 has been attributed to two membrane-juxtaposed cysteine residues, present in the mouse amino acid sequence and absent from the human molecule, that could be palmitoylated and thus address the molecule to lipid rafts. We examined whether the membrane-juxtaposed cysteine residues Cys<sup>226</sup> and Cys<sup>228</sup>, also present in rat CD2, might have a role in targeting the molecule to the rafts, using a mutant where these residues were replaced by alanines. The mutant CD2(C1C/A1A) was stably expressed in E6.1 Jurkat cells, and surface expression levels were confirmed by flow cytometry (not shown). E6.1-CD2(C1C/A1A) cells, as well as E6.1 cells expressing full-length rat CD2, E6.1-CD2, were lysed in 1% Triton-based lysis buffer, lysates were subjected to sucrose gradient separation, and analyzed by immunoblotting. As can be seen in Figure 2A, wild-type CD2 is largely concentrated in lipid rafts (74% as measured by densitometry), however CD2(C1C/A1A) is still able to localize very significantly to the rafts (49%), indicating that the cysteines 226 and 228 are not fully accountable for the lipid raft-targeting of rat CD2.

We next examined whether rat CD2 could in fact be palmitoylated in the cysteine residues. In these studies we used E6.1-CD2 cells, and cells expressing a cytoplasmic deletion mutant, E6.1-CD2(CY6), containing only the first six amino acids of the cytoplasmic domain, but still retaining the two cysteine residues. Cells were incubated in medium containing [<sup>3</sup>H]palmitate, for 16 hrs at 37°C. Following cell lysis, rat CD2 was

immunoprecipitated from E6.1-CD2 and E6.1-CD2(CY6) cells, and samples from the lysates as well as from the immunoprecipitates were run on SDS-PAGE under non-reducing conditions, since the presence of 2-mercaptoethanol is recognized to interfere with S-ester and hydroxyester linkages, as can be the case for the covalent linkage of palmitate with the cysteine residues [38]. As a positive control for the experiment, we immunoprecipitated Lck, previously shown to incorporate [ $^3$ H]palmitate [39].

Fluorography analysis of radiolabeled products in SDS-PAGE showed that a number of cellular proteins did incorporate [ $^3$ H]palmitate, as can be seen in the lysate lanes (Fig. 2B). Lck was easily detected in the respective lane. However, we were not able to confirm that rat CD2 could be subjected to lipid modification, as no labeled protein bands could be perceived in the lanes of rat CD2. The failure to detect palmitoylated rat CD2 could not be attributable to a low cellular expression of the molecule, or to a low affinity of the antibodies. As seen in Figure 2C, rat CD2 immunoprecipitated from E6.1-CD2 and E6.1-CD2(CY6) cells is clearly evident by immunoblotting. Moreover, rat CD2 was well expressed at the cell surface as confirmed by FACS analysis (not shown).

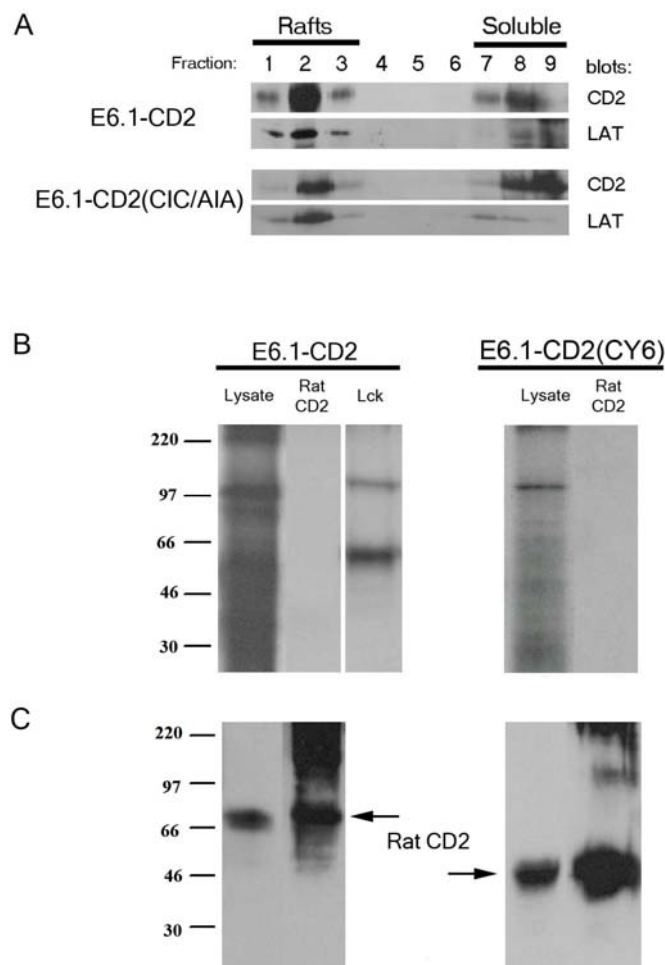


**Figure 2. Analysis of the role of Cys<sup>226</sup> and Cys<sup>228</sup> in raft targeting of rat CD2.** A) E6.1-CD2 cells, expressing rat CD2, or E6.1-CD2(CIC/AIA), expressing a rat CD2 mutant where Cys<sup>226</sup> and Cys<sup>228</sup> are mutated to alanine residues, were lysed in 1% Triton X-100-containing buffer and lysates fractionated by sucrose density centrifugation. Equivalent amounts of each fraction were resolved by 10% SDS-PAGE and immunoblotted with OX55, and with a LAT polyclonal. B-C) E6.1-CD2 and E6.1-CD2(CY6) Jurkat cells were labeled with [ $^3$ H]palmitate for 16 h, lysed in Triton X-100 and immunoprecipitated with OX34 ( $\alpha$ -rat CD2), or with Lck sera, as indicated. Immunoprecipitates and cell lysates were separated on 10% SDS-PAGE under non-reducing conditions and analyzed by fluorography (B), or western blotting with OX34 (C).

### A physical association of rat CD2 with Lck is sufficient for the raft targeting of CD2

To determine the molecular basis for the constitutive presence of a large fraction of rat CD2 in the rafts, we focused on the cytoplasmic domain, determining which segments could play a role. We used previously characterized cells where rat CD2 mutants are expressed in E6.1 Jurkat cells [12, 13, 32, 40]. E6.1 Jurkat cells stably expressing cytoplasmic domain deletion mutants of rat CD2 possessing 97, 81, 66, 40 and 6 amino acids of the cytoplasmic region were used. All CD2 variants were expressed at similar levels at the cell membrane (not shown). The location of each mutant was analyzed following sucrose gradient centrifugation and subsequent immunoblotting of rat CD2 with OX55. As shown in Figure 3, the deletion mutants CD2(CY97), CD2(CY81), CD2(CY66), and CD2(CY40) are mostly raft-resident like the full-length molecule, whereas rat CD2(CY6) is totally excluded from lipid rafts. This suggests that the first 40 N-terminal amino acids of CD2 cytoplasmic tail are sufficient for the localization of CD2 in lipid rafts. Also, the fact that E6.1-CD2(CY6) does not address to lipid rafts while still possessing the two membrane-proximal cysteine residues further strengthens the argument that these residues do not serve as anchors for the raft lipids.

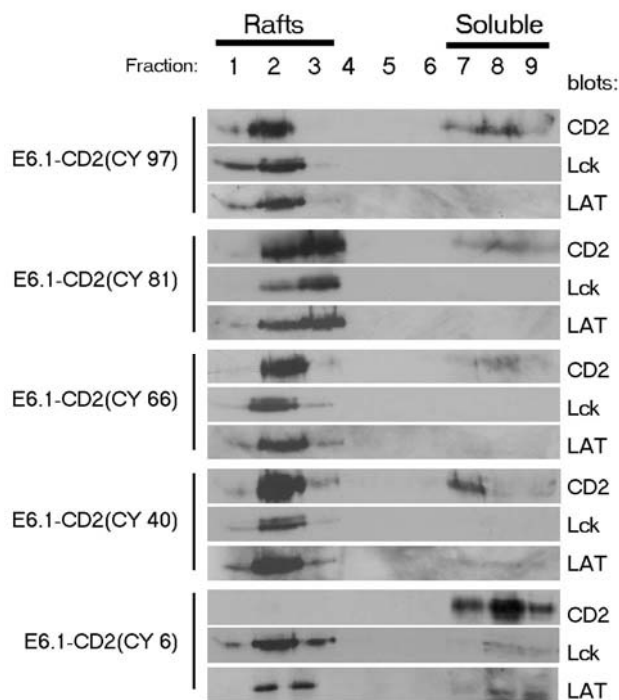


**Figure 3. Analysis of raft localization of different rat CD2 cytoplasmic deletion mutants.** E6.1 Jurkat cells stably expressing different rat CD2 cytoplasmic tail deletion mutants were lysed, separated by sucrose gradient density centrifugation and the collected fractions analyzed by immunoblotting for the localization of CD2, Lck and LAT. The mutants analyzed were E6.1-CD2(CY 97), E6.1-CD2(CY 81), E6.1-CD2(CY 66), E6.1-CD2(CY 40) and E6.1-CD2(CY6), expressing the membrane proximal 97, 81, 66, 40 or 6 amino acid residues, respectively. All mutants except E6.1-CD2(CY6) still partition within lipid rafts fractions.

As the pattern of association of rat CD2 mutants with lipid rafts correlated entirely with the pattern of binding to the tyrosine kinase Lck [13], we raised the possibility that the interaction between rat CD2 and lipid rafts could be mediated via Lck. Moreover, as seen

from Figure 3, all CD2 mutants except CD2(CY6) are in general confined to the same fractions as Lck. To test this hypothesis, rat CD2 was stably expressed in the Lck deficient J.CaM1 cell line and its inclusion in the membrane fractions analyzed. Supporting our prediction, rat CD2 expressed in J.CaM1 cells localizes to the “soluble” fractions, whereas the kinase is absent in these cells as expected (Fig. 4A). Further evidence that Lck could couple rat CD2 to lipid rafts was obtained through co-capping experiments. Capping of rat CD2 molecules, labeled by Alexa Fluor 568-conjugated antibodies, was visualized by fluorescence microscopy, in red, and the localization of rafts detected by Alexa Fluor 488 (green) conjugated to Cholera-toxin subunit B that binds to raft-imbedded GM1. In E6.1-CD2 cells, capping of CD2 induced very clear co-capping of GM1 labeled rafts in the majority of cells, nearly 80% (Fig. 4B and C). By contrast, in the Lck-deficient J.CaM1-CD2 cells, co-capping was always less well-defined in the fewer cells (32.5%) where we could observe some co-localization of CD2 and GM1.

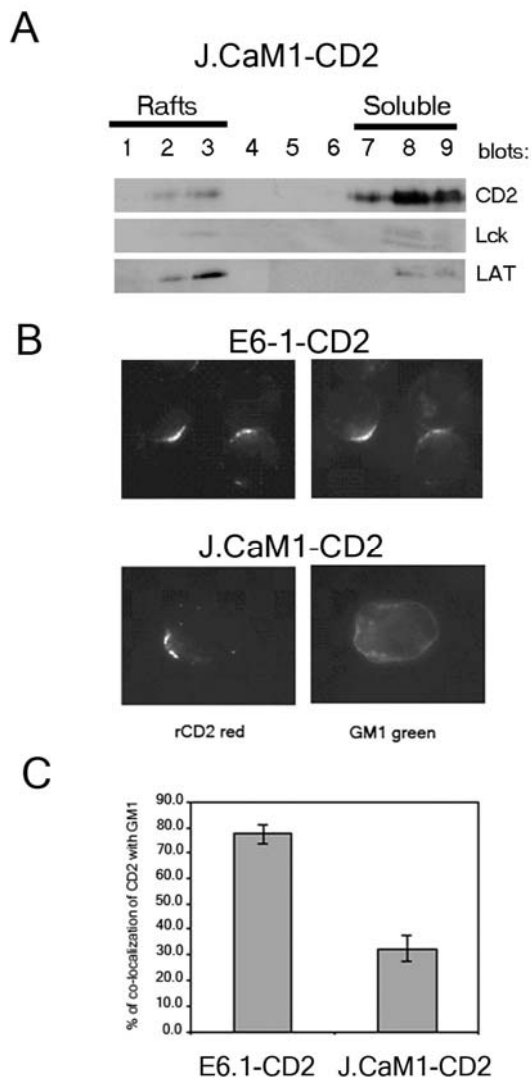


**Figure 4. Lck is required for the translocation of CD2 into lipid rafts.** A) Sucrose density centrifugation and immunoblotting with OX55 confirm the exclusion of most rat CD2 from the raft fractions of J.CaM1-CD2, an Lck-deficient cell line stably expressing rat CD2. Immunoblots of Lck attest the lack of expression of Lck in these cells, and LAT immunoblots confirm that these cells display normal raft behavior. B-C) Analysis of co-capping of rat CD2 molecules with lipid rafts in Jurkat E6.1-CD2 and J.CaM1-CD2 cells. Rat CD2 was labeled with OX34 followed by RAM-Alexa Fluor 568, and the raft marker GM1 was detected by Cholera-toxin subunit B Alexa 488 conjugate. Cells were incubated at 37°C for 15 minutes to induce capping. After fixation, cells were visualized by fluorescence microscopy (B). GM1 co-capped very clearly with CD2 in nearly 80% of E6.1-CD2 cells. By contrast, in J.CaM1-CD2 cells, co-capping was less well-defined and detected in approximately 32.5% of cells, as assessed from three independent counts of different experiments (data shown as mean counts  $\pm$  SE).

Our hypothesis that Lck couples CD2 to the rafts was further strengthened when J.CaM1-CD2 cells were reconstituted with Lck, and targeting of CD2 to the lipid rafts fractions, co-localizing with the re-expressed Lck, was by large restored (Fig. 5A). In order to determine whether a functional Lck kinase was required for addressing CD2 to the rafts, we transfected J.CaM1-CD2 cells with an Lck cDNA encoding a mutation at the ATP-binding site (K273A), and so referred to as Kinase Defective (KD). J.CaM1/Lck(KD)-CD2 cells were lysed, subjected to sucrose gradients, SDS-PAGE and immunoblotting with OX55. As can be seen in the respective panels in Figure 5A, both Lck(KD) as well as rat CD2 still localize in lipid rafts, discarding the Lck activity as required for the lipid raft-CD2 localization.

It was apparent that the gross majority of rat CD2 molecules would localize with Lck when a physical interaction could be established, and that the site of co-localization were lipid rafts, as that is the membrane phase where Lck resides in Jurkat cell lines. We thus inquired whether CD2 would still localize in the rafts if Lck was excluded, and for this we used J.CaM1 cells reconstituted with an Lck mutant which has had cysteine 3 replaced by an alanine residue. Lck contains a double palmitoylation site at Cys3 and Cys5 [41], and in contrast to an Lck mutant C5A or the double mutant C3,5A, which are mainly or exclusive cytoplasmatic, respectively, Lck(C3A) confines to the plasma membrane. However, as can be seen in the lower panel of Figure 5A, it no longer addresses to the lipid raft fractions, neither does exogenous rat CD2 expressed in these cells, thus proving that CD2 homing to lipid rafts is dependent on a physical association with Lck.

It did not become clear, however, whether in J.CaM1/Lck(C3A)-CD2 cells rat CD2 associated with “soluble” phase Lck(C3A), and through this association was dragged out of lipid rafts, or whether it was free and kept out from the rafts by default, with the theoretical possibility of being recruited through the association of another raft resident molecule. We tested these hypothesis through the analysis of molecular associations of rat CD2, expressed in J.CaM1/Lck(WT)-CD2 as well as in J.CaM1/Lck(C3A)-CD2 cells, thus addressing the interaction with raft-resident Lck versus raft-excluded Lck molecules.

Non-activated cells were lysed in Triton X-100 lysis buffer, Lck or CD2 were immunoprecipitated from the lysates, and immune complexes were subjected to in vitro kinase assays. CD2 immunoprecipitates from J.CaM1/Lck(C3A)-CD2 cells displayed very little kinase activity, when compared to CD2 immunoprecipitates from J.CaM1/Lck(WT)-CD2 (Fig. 5B). Since raft-resident Lck(WT) and raft-excluded Lck(C3A) displayed comparable kinase activities and autophosphorylation efficiency, also indicating that no major differences on the activity of the kinase depend on its localization, this suggested that in non-activated cells the association between CD2 and Lck is preferentially held in the rafts, and that association in the “soluble” phase is much reduced.

Given that the T cell specific Src-like kinase Fyn has also been shown to contribute to some of the CD2-associated kinase activity, we tested whether Fyn could also associate with CD2 in J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells. The primary CD2 immune complexes were disrupted by heat and SDS, and Fyn as well as Lck were re-precipitated. As can be seen from the lower panels of Figure 5B, both Lck and Fyn are clearly present in CD2 complexes from J.CaM1/Lck(WT)-CD2 cells, but absent from CD2 immunoprecipitates from J.CaM1/Lck(C3A)-CD2 cells, indicating that in non-activated cells CD2 associates with both kinases mostly within lipid rafts.

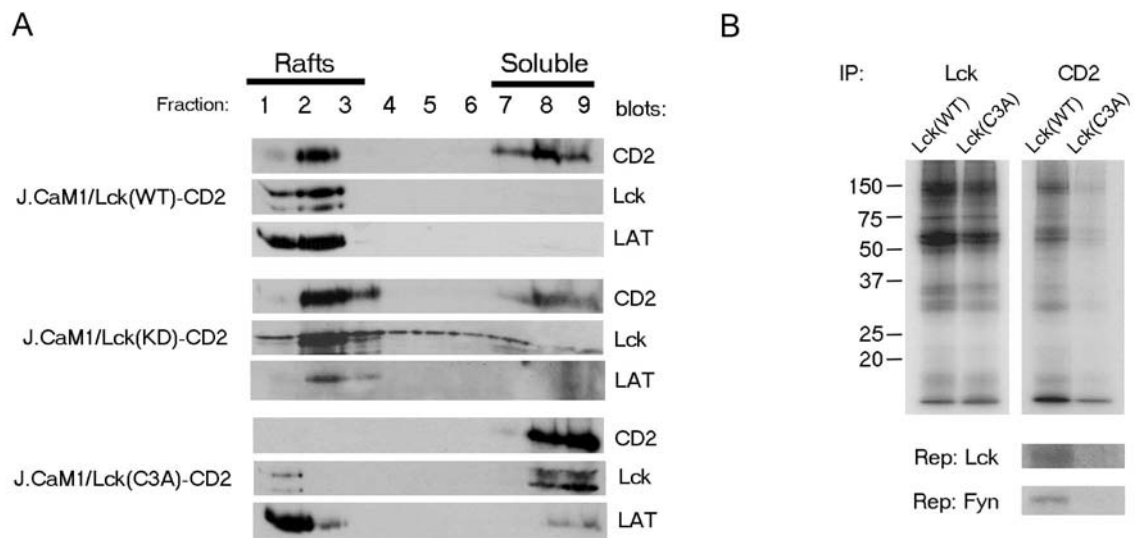


**Figure 5. CD2 localization in lipid rafts is dependent on a physical association with Lck but independent of the kinase activity.** A) J.CaM1-CD2 cells expressing either Lck(WT), Lck(KD), a kinase defective mutant carrying a mutation at the ATP binding site, or Lck(C3A), an Lck mutant which has cysteine 3 replaced by an alanine but still localizes to the plasma membrane were analyzed for CD2 rafts localization. Cell lysates were fractionated on sucrose gradients, separated by SDS-PAGE, and immunoblotted with CD2, Lck and LAT antibodies. As can be seen in the Lck detection, the mutation on cysteine 3, Lck(C3A), results in the nearly total exclusion of the kinase from the rafts, whereas Lck(WT) and Lck(KD) still confine to the rafts. CD2 partitioning in the different fractions follows the same pattern as Lck. B) J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells were lysed and Lck and CD2 were immunoprecipitated. Immune complexes were subjected to kinase assays, run on SDS-PAGE and exposed to autoradiography. On lower panels, Lck and Fyn were reprecipitated from the original CD2 immunoprecipitations and exposed to autoradiography. IP, immunoprecipitation; Rep, reprecipitation.

### The traffic of CD2 in the membrane of rat T cells upon CD2 activation

We established that rat CD2 localizes to lipid rafts dependently of its association with Lck and that within rafts also interacts with Fyn, both protein tyrosine kinases deeply involved in signal transduction in T cells. Thus, we investigated whether upon cell activation CD2 could shift through different membrane microenvironments, and what

would be the role of CD2 reallocation in the regulation of kinase activity in the different membrane phases.

Splenocytes from 3-month old Wistar rats were stimulated through CD2 crosslinking for 15 min at 37°C, or left unstimulated, cells were lysed in Triton-based lysis buffer and lysates subjected to sucrose gradient. The different recovered fractions were analyzed for the presence of CD2, Lck and Fyn. In resting cells, the majority of CD2 molecules, but also of Lck, are outside lipid rafts, whereas virtually all Fyn resides in the rafts (Fig. 6A and B). Following CD2 stimulation, the proportion of Lck and Fyn in and outside rafts remained unchanged, however a significant fraction of CD2 was translocated to lipid rafts. Quantification of the immunoblots by densitometry indicates that the percentage of CD2 in the rafts increased over 4 fold, from 8 to 36% (Fig. 6B).

In order to analyze the kinase activity associated with CD2 in the different fractions, we selected fraction 2 as representative of rafts, fraction 9 as a “soluble” fraction, and also a control fraction 6, where no CD2, Lck or Fyn are present (selected fractions are indicated by arrows on Fig. 6A). CD2 was immunoprecipitated from fractions 2, 6 and 9, both from resting as well as from activated cells, and immune complexes subjected to in vitro kinase assays. Immune complexes of Lck were processed in parallel. As can be seen in Figure 6C, phosphoproteins of 55-60 kDa were present in CD2 immune complexes already in resting cells, and were better defined in the rafts than in the “soluble” fraction, with no signal in the control fraction 6. Upon cellular activation, increased phosphorylation of the CD2-associated 55-60 kDa proteins was detected in the rafts and the soluble fractions. These phosphoproteins corresponded mostly to phosphorylated Lck and also Fyn, as can be seen in the reprecipitations of the kinases from the original CD2 immunoprecipitates.

Careful analysis of the kinase assays suggested that CD2 could precipitate more active Lck and Fyn from the rafts fraction than the “soluble” one. Whereas more Fyn is expected to associate with CD2 in the rafts, given that 99% of Fyn is raft-resident, the fact that more active Lck was also co-precipitated with CD2 from the rafts fraction clearly confirms that the association of CD2 and Lck is favored within lipid rafts, despite the fact that the majority of CD2 and Lck are not resident in lipid rafts, neither in resting nor in activated cells.

In resting cells, the kinase activity of Lck reflects the proportion of the protein in and out of rafts, however there is a clear enhancement of the activity of raft-based Lck upon CD2 stimulation that does not require any translocation of Lck to the rafts (Fig. 6D, compare to Fig. 6A and B). Interestingly, Fyn, which is not known for associating specifically to Lck, co-precipitates with Lck only in rafts, and displays higher activity following CD2 stimulation, as can be seen in the lower panel of Figure 6D. This suggests

that Lck and Fyn may interact in lipid rafts of activated cells, and that this association may be mediated by CD2.

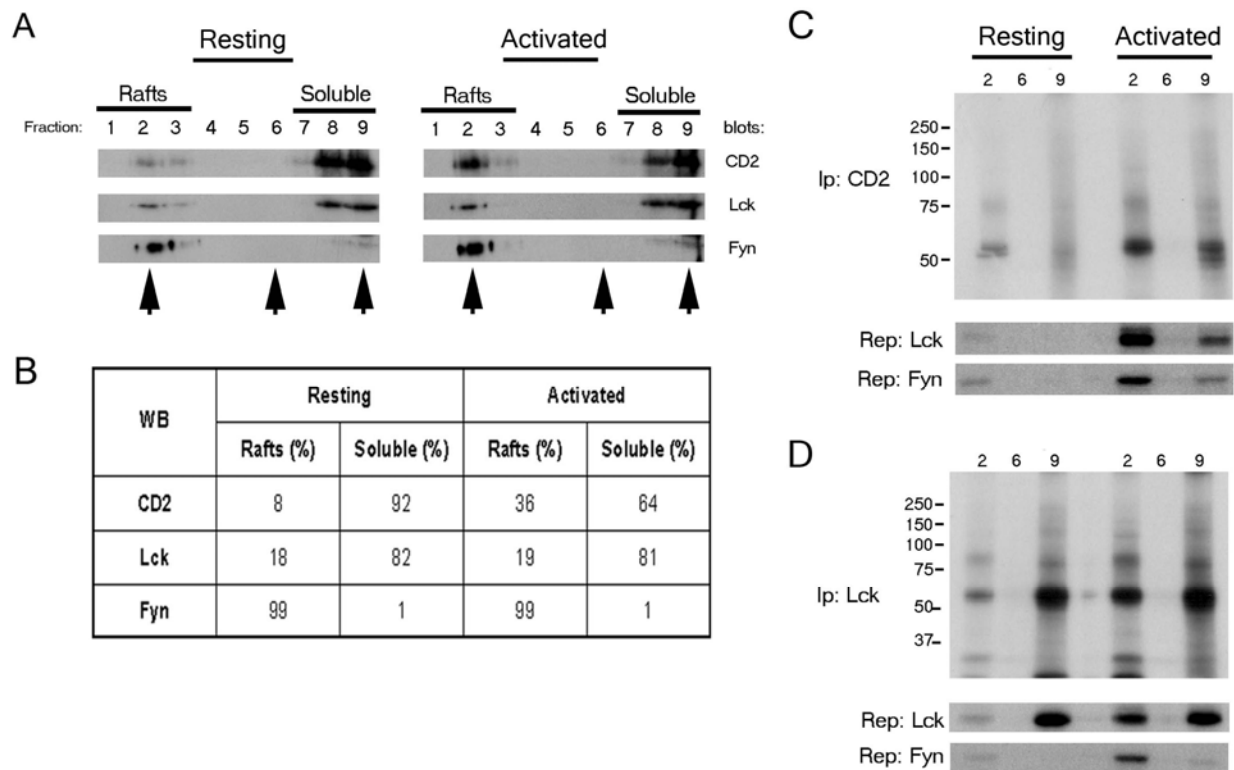


**Figure 6. Rat CD2 redistribution and molecular associations upon CD2 stimulation.** A) Rat splenocytes were stimulated through CD2 for 15 min at 37°C, or left unstimulated. Lysates were fractionated by sucrose gradient centrifugation and the recovered fractions were analyzed for the presence of CD2, Lck and Fyn. B) Densitometric analysis of the immunoblots illustrated in A. Table shows densitometry values of CD2, Lck and Fyn subcellular distributions, representing the proportion of the total amount of each molecule detected in the raft and soluble fractions. C-D) In vitro kinase assays were performed in immune complexes of CD2 (C), and Lck (D), prepared from the fractions indicated by arrows on A. Following SDS-PAGE, phosphorylated products were visualized by exposure of dried gels at -70°C to CL-Xposure films. Immune complexes were disrupted, and Lck and Fyn were reprecipitated, subjected to SDS-PAGE and exposed to autoradiography. Ip, immunoprecipitation; Rep, reprecipitation.

## DISCUSSION

It is increasingly clear that lipid rafts play an important role in T cell signaling, since the assembly of the TCR signaling machinery takes place within this membrane micro-environment enriched in glycosphingolipids, sphingomyelin and cholesterol [23, 42, 43]. Moreover, crosslinking the TCR with accessory proteins such as CD2, CD5, CD9 and CD28 readily increases the association of the TCR with lipid rafts [24-26]. The conventional view is that the TCR and other signaling molecules translocate to these platforms that facilitate signal transduction mechanisms. However, given that MHC-

engaging T cell receptors that originate activation and signaling, together with accessory molecules binding to the respective counter receptors, are polarized towards the sites of cell contact with antigen presenting cells, a fairer assessment is that lipid rafts coalesce into the interface of TCR-antigen recognition [44, 45].

Therefore, rather than considering individual TCRs or other signaling molecules being cumulatively trapped by the raft, depending on certain characteristics such as intrinsic affinity for the raft lipids or interactions with raft-peripheral proteins, a TCR-centered perspective should ponder instead how the whole of the raft is captured at the sites of antigen recognition. There are currently many open theoretical possibilities, but a simple one advances that lipid rafts need not be fairly large or complex structures: homotypic FRET studies have shown that some individual rafts can form high density clusters of nanometer size (4–5 nm), containing as few as 4 GPI-anchored molecules [46]. Moreover, Douglass and Vale showed that the raft associated molecule LAT, as well as Lck, had a much larger diffusion coefficient than non-raft CD2, suggesting that lipid rafts can be highly mobile and diffuse to the TCR-activation spots [47]. They also suggest that, as Lck and LAT change from a highly mobile to a motionless state when they encounter a signaling cluster, it is possible that protein-protein interactions, rather than any sort of raft-addressing labels, can play a major role in establishing specific interactions between raft and non-raft proteins. In this paper we demonstrate that the association of rat CD2 with membrane lipid rafts is in fact mostly determined by the physical interaction established with the protein tyrosine kinase Lck.

CD2 has been shown to associate to both Lck and Fyn protein tyrosine kinases [14, 15], and deletion of the cytoplasmic tail eliminates these interactions and abolishes CD2-mediated signaling [12, 48]. However, given that our results, using several mutant molecules of CD2 and Lck and different cells lines, show an absolute correlation between the capacity of rat CD2 to interact with raft-resident Lck and to be found in lipid rafts, we can conclude that it is Lck, and not Fyn, that retains CD2 at the rafts. It is however unlikely that Lck is responsible for the actual co-transport of CD2 to the rafts. There is little evidence that in non-activated cells CD2 and Lck interact outside lipid rafts, both in experiments where mutated Lck was excluded from the rafts (Fig. 5B), as well as from the analysis of associations of the different fractions of physiological cells (Fig. 6). Moreover, whereas there is an increase of 4.5 fold of CD2 into the rafts upon CD2-induced activation, there is no net shift of Lck to the rafts (Fig. 6 A and B). Furthermore, Yang and Reinherz [28] have shown that the recruitment of activated CD2 to the rafts does not require the cytoplasmic tail, and hence, any association with Src-like kinases. Therefore, it is plausible that following TCR-antigen recognition and CD2 engagement to its ligand and upon an encounter with a lipid raft, transitory interactions mediated through the CD2

ectodomain with a raft resident receptor may allow for a transient overlap of the raft with CD2 complexes and induce raft-resident Lck to dock to the cytoplasmic domain of CD2.

Interestingly, when in the rafts, CD2 seems to bind equally well to Fyn and to Lck, and upon activation, more phosphorylated Lck and Fyn can be recovered from CD2 immune complexes (Fig. 6C). This may simply reflect the increment of CD2 in rafts with the consequent increased association of CD2 with the kinases, although an enhancement in the activity of the kinases may have to be considered. Regardless of that, CD2 stimulation clearly induces a functional association between Lck and Fyn (Fig. 6 D). Lck and Fyn bind to non-overlapping sequences of CD2 [14, 29], but other CD2 partners compete for these sequences. The CD2 proline-rich motif PPPPGHR is complexed with the GYF domain of CD2BP2, but only outside lipid rafts, as upon translocation, the SH3 domain of Fyn displaces CD2BP2 [29]. The penultimate CD2 proline motif PPLPRPR has been reported to associate to the SH3 domains of both Lck and CD2BP1 [14, 49], and so it is possible that the association between CD2 and Lck may be similarly regulated and confined to lipid rafts as well. As the amounts of Lck and Fyn, in and outside the rafts, remain unchanged following activation, the increase of the phospho-Fyn signal co-precipitated with Lck (Fig. 6 D – bottom panel) has to be due to an increase in the amount of Fyn associating with Lck, or to the phosphorylation of Fyn induced by Lck [22]. Conversely, the augmented Lck signal in the raft fraction of activated cells can be caused by an increase in the phosphorylation of Lck, possibly catalyzed by Fyn (Fig. 6 D – middle panel). Therefore, a central role of CD2 in the reorganization of lipid rafts upon cellular activation could be the facilitation of the association between Lck and Fyn.

What can then be the role of the two membrane proximal cysteine residues? The physical segregation in different membrane phases could be easily overcome if proteins, such as rat CD2, should have a lipid raft insertion motif. For example, CD8 can exist as a CD8 $\alpha\beta$  heterodimer, with the  $\beta$  subunit facilitating raft-association and the  $\alpha$  unit providing an extracellular binding site for MHC class I and an intracellular motif that binds to Lck [50]. Since CD8 $\alpha\beta$ , and not the CD8 $\alpha\alpha$  homodimer, partitions into rafts, it can induce the association with lipid rafts of a significant fraction of the T cell receptor. It is possible that rat CD2 may have a similar role, such as linking cytoskeleton orientation with lipid raft organization. However, we were not able to prove that the membrane proximal cysteine residues are indeed palmitoylated, although the level of association of mutant CD2 with the rafts tends to be lower than the wild type molecule. It is also possible that CD2 may establish protein-protein interaction with yet other raft-resident molecules to strengthen raft localization. The determination of the function of these two amino acid residues will no doubt require further investigation.

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### **III. Conclusions**

An immune response requires numerous receptor-mediated signals to be delivered to T cells and this coordinated activation in response to foreign antigen will guarantee antigen-specific T cell clonal development and differentiation. The specificity of the response lies on the recognition of the foreign peptide in the context of an MHC molecule by the T cell receptor, however, the modulation and fine-tuning of the T cell receptor signals, hence, the specific developmental program and cellular behaviour, is achieved by the integration of signals provided by accessory receptor-ligand interactions. The intricate assimilation of all these signals still remains a daunting challenge that needs to be clarified before any possible medical benefits from predicting T cell behaviour can be anticipated. Progress in the clarification of this area of research has been accomplished by studies on accessory receptors behaviour and on the internal molecular interactions triggered by these numerous receptors engaged during T cell activation, such as the ones described in this thesis.

CD6, a 105-130 kDa type I cell surface glycoprotein, is one of these accessory receptors, whose expression is largely restricted to thymocytes and mature T lymphocytes [1]. Although several studies point towards a role for CD6 as a costimulatory molecule in T cell activation, given that antibody-mediated CD6 engagement can potentiate proliferative T cell responses [2-4], its exact function has not yet been elucidated. In this thesis we present data showing that CD6 can exert its function via association with CD5, inducing CD5 tyrosine phosphorylation, also implying CD6 in the regulation of CD5 function. The long cytoplasmic domain of CD6 is well fitted for signal transduction as, apart from numerous tyrosine residues, it contains two well-conserved proline-rich sequences, potential binding sites for SH3-containing proteins [5, 6].

We have demonstrated that the rat homologue of CD6 is able to associate with protein tyrosine kinases of different families, namely Lck and Fyn from the Src-family, ZAP-70 of the Syk-family and the Tec-family kinase Itk, and it is therefore reasonable to envisage that the ability of CD6 to induce CD5 phosphorylation derives from the CD6-associated kinases. We have shown that Lck together with Itk is the most efficient combination for the phosphorylation of an exogenous CD5 peptide containing the ITAM-like motif. Since we have found that Itk alone was unable to phosphorylate a CD5 peptide and that Lck was able to phosphorylate Itk, we propose that rather than having a synergistic effect, the kinases regulate each others activities. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity by inducing autophosphorylation events [7] which probably renders Itk capable of regulating the activity of Lck. These suggestion is in conformity with a more downstream role of Itk observed in T cell activation [8]. However, until now, there are no established functional roles for these interactions.

The immune system has always been an attractive model to study the complexity of biological regulation, as it is one system that has intricate developmental pathways and demands well fine-tuned responses. In such a system where cellular functions are regulated in a tissue, temporal or stimuli-dependent manner the generation of alternative mRNA transcripts and proteins encoded from a single gene may have an extreme importance. In fact, a mechanism that contributes to one further level of regulation of immune responses is alternative splicing. This is a crucial and ubiquitous mechanism for generating protein diversity and regulating protein expression which is widespread and has particular prevalence in the immune system. Several immunologically relevant genes have been found to undergo alternative splicing; however, understanding how this large number of proteins and protein isoforms regulate the function of the immune system is still a crucial challenge.

One of the molecules that has long been known to be alternatively spliced is CD6; different CD6 isoforms, both from human and mouse, have been described arising from alternative splicing of exons coding for sequences within the cytoplasmic domain [5, 9-11], but until now no specific physiological function has been attributed to any of these isoforms. Cloning of the rat homologue of CD6 [12] allowed us to notice that an additional isoform, CD6 $\Delta$ d3, resulting from alternative splicing of an exon coding for the third SRCR domain, the CD166-binding domain, was expressed on different tissues of lymphoid origin (Castro *et al.* 2007, unpublished results). Subsequently, we have identified in human T lineage cells four novel CD6 extracellular isoforms that arise from alternative splicing. The work performed with these isoforms, and presented in this thesis, led us to the demonstration of a rare example of a functional role of an alternatively spliced isoform, the CD6 $\Delta$ d3 isoform.

The concentration of CD6 at the immunological synapse depends on the alternative splicing regulated expression of SRCR domain 3, the CD166-binding domain. Furthermore, we have shown that upon human T lymphocyte stimulation, the expression of full-length CD6 is partially down-regulated and there is a switch to the expression of the shorter CD6 $\Delta$ d3 isoform which without doubt represents a product of activation. Another molecule of the immune system that has a similar type of regulation by alternative splicing is CD45 [13, 14], however the CD6 $\Delta$ d3 isoform described in this thesis illustrates a more evident function as it controls the interaction with the ligand. Upon TCR stimulation, instructions for the production of CD6 $\Delta$ d3 are generated and as a consequence the activity of CD6 at the immunological synapse is reduced along with a possible change of behaviour.

The preliminary results presented here on  $\text{Ca}^{2+}$  signals and tyrosine phosphorylation at the synapse after blocking CD6-CD166 binding or crosslinking studies

together with our previous unpublished results from rat CD6, where we show that the expression of CD6 but not its localization in terms of the synapse can down-modulate  $\text{Ca}^{2+}$  signals, led us to hypothesize that although CD6 expression may result in a general inhibitory outcome, the engagement of CD6 and consequent accumulation at the synapse does not preclude the possibility of a positive co-stimulatory role for CD6.

The role of CD6 as a modulator of antigen-induced signals still waits clarification as it may be dependent on interactions with other surface molecules, like has been observed for CD2. This surface glycoprotein belongs to the immunoglobulin superfamily and is expressed on all T lineage and natural killer cells [15]. Stimulation of CD2, either alone, or in synergistic combination with the T cell receptor, has long been known to transduce mitogenic stimulation in T cells [16-18]. However, as CD2 also interacts with CD5 [19], positive signaling of CD2 can potentiate the CD5 inhibitory effect in thymocyte selection and T cell activation, as demonstrated in vitro [20, 21] or in vivo, using the CD2/CD5 double-knockout mouse [21]. The stimulatory properties of CD2 can be attributable to its cytoplasmic domain as clearly demonstrated by structural and functional studies on human and rat CD2 [17, 22-24] and consequent association with the tyrosine kinases Lck and Fyn, [25-27]. Through these associations it is connected to downstream signaling pathways.

The work presented in this thesis further establishes another role for the association of CD2 with Lck, since we have found, using several CD2 and Lck mutants and different cell lines, that the interaction of CD2 with Lck is required for the localization of rat CD2 in lipid rafts. There are still many speculative models for the function and behavior of lipid rafts, but current knowledge supports a TCR-centered perspective, where the small and highly dynamic rafts and raft-associated proteins [28, 29] are continuously moving until they encounter a signaling cluster and the proper protein-protein interactions are created. Our observations also strengthens this view, as we demonstrate that the localization of rat CD2 within lipid rafts is mostly dictated by the physical interaction of CD2 with Lck, rather than by any sort of raft-targeting signal such as the two membrane-proximal cysteines. Moreover, our results indicate that Lck and CD2 do not interact outside of lipid rafts in non-activated cells, this together with the fact that human CD2 does not require the cytoplasmic tail to be recruited to rafts upon activation [30], led us to the postulation of the following model: upon TCR and CD2 engagement, interaction of CD2 extracellular domain with a raft-resident receptor may allow CD2 cytoplasmic tail to transiently interact with raft-resident Lck and therefore become captured to the rafts. When in the rafts, CD2 associates equally well with Lck and Fyn. Additionally, we observed for the first time a functional interaction between Lck and Fyn in lipid rafts upon

CD2 crosslinking, suggesting that this association may be mediated by CD2. Therefore, the interactions of CD2 with Lck and Fyn and consequently the interaction between both kinases may be favored in lipid rafts. Consistently, CD2-associated molecules, such as CD2BP2, compete with the SH3 domain of Fyn but only outside rafts [31], and it is possible that another partner, CD2BP1, may also compete with Lck [26, 32] within rafts. Moreover, as activation of Fyn in lipid rafts has been shown to be achieved by Lck [33] and that phosphorylation of Lck may possibly be catalyzed by Fyn, CD2-mediated interaction of both kinases may act to boost kinase activity in lipid rafts.

Further studies should aim at questions that remain regarding the role of the two membrane proximal cysteines, that despite the fact that we were not able to detect palmitoylation of amino acid residues, when mutated induced a slightly lowered level of association of the mutant CD2 with the rafts. Progress towards the understanding of the basic mechanisms that control the integration of complex processes such as TCR signaling, immunological synapse formation and rafts dynamics will only be achieved through the use of appropriate tools to characterize the behaviour of as many molecules as possible.

## **Conclusions references**

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