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FACULDADE DE CIÊNCIAS

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THE T CELL SURFACE GLYCOPROTEIN CD6 AS AN INHIBITORY RECEPTOR AND AS TARGET FOR IMMUNOMODULATION

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular;
Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas de Abel Salazar e Faculdade de Ciências)

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3. Carmo, Alexandre & Henriques, Sónia. (2020). *Cell Activation and Signaling in Lymphocytes*. 10.1007/978-3-030-44436-5_5.
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Abstract

T lymphocytes are key elements of the immune system, constantly surveilling the human body for the presence of pathogens. When the T cell antigen receptor (TCR) encounters and binds its cognate antigen, presented by an antigen-presenting cell (APC), T lymphocytes are activated. This activation leads to cell proliferation, polarization and secretion of effector enzymes or cytokines, among other outcomes. TCR-mediated T cell activation is regulated by several proteins of both co-stimulatory and inhibitory nature, such as CD28 or CTLA4, for example, keeping it in check. One of such proteins is CD6, a transmembrane receptor without enzymatic activity that binds extracellular ligands CD166 and CD318. Initial reports have suggested CD6 to be a co-stimulator but, over the last decade, CD6 has been shown to effectually downmodulate T cell activation via its cytoplasmic tail. Despite the fact that the role of CD6 on T cell stimulation is still debated today, and that only recently its signalosome has begun to be uncovered, the use of itolizumab, a monoclonal antibody that binds CD6, has been approved for treating inflammatory disorders. The overall lack of information currently available regarding CD6 action mechanisms prompts the need to further investigate what signaling pathways are effectively modulated by this receptor and what effectors are involved in the process.

In the present work, we have demonstrated that, once phosphorylated, CD6 binds GTPase activating protein of Ras (RasGAP), an enzyme with GAP (*i.e.* inhibitory) activity over Ras, a GTPase that potentiates T cell activation. We have further devised a new Förster resonance energy transfer (FRET)-based biosensor to assess Ras GTPase activity in T cells in real time during APC-mediated activation and found that CD6 expression significantly decreases Ras activation in stimulated T lymphocytes. Having also found that CD6 expression additionally inhibits extracellular signal-regulated kinase (ERK)-1 and -2 activation, we conclude that CD6 downmodulates the full extension of the mitogen-activated protein kinase (MAPK) pathway via RasGAP recruitment. Here we have also shown that CD6 associates with the E3 ubiquitin-protein ligase Cbl proto-oncogene B (CBLB), a second inhibitory enzyme, via CD6 tyrosine residues 486, 489 and 629, and that the signalosome of CD6 is ubiquitinated in activated T cells, a post-translational modification with significant potential for signal modulation. Taking a step further into clarifying CD6-regulated signaling pathways, we have characterized the signaling potential of most tyrosine residues in CD6 intracellular domain and found that while some of them are inhibitory, others are co-stimulatory or neutral. Lastly, we have found that CD6 expression constitutively decreases RhoA activity, a GTPase crucial for cytoskeletal remodeling and regulated by described

interactors of CD6. This finding supports previous evidence that suggest that CD6 can, in a context-dependent manner, modulate cytoskeletal elements in T lymphocytes.

Altogether, our data are consistent with previous observations that CD6 inhibits T cell activation. Here, we have described two new interactors (RasGAP and CBLB) that can mediate its role and pinpointed which intracellular tyrosine residues of CD6 contribute to its net effect. Additionally, CD6-mediated regulation of RhoA activity hints at a new signaling axis through which CD6 can regulate cytoskeletal proteins. The present work represents a significant advancement in our understanding of CD6-mediated signaling and its overall role in the progression of T cell activation.

Resumo

Os linfócitos T são elementos-chave do sistema imune, constantemente em vigilância pelo corpo humano à procura de patógenos. Quando o recetor de antigénio da célula T (TCR) encontra e se liga ao seu antigénio cognato apresentado por uma célula apresentadora de antigénios (APC), o linfócito T é ativado. Esta ativação leva à proliferação e polarização celulares e à secreção de enzimas efetoras ou citocinas, entre outros efeitos. A ativação de linfócitos T mediada pelo TCR é regulada por várias proteínas de natureza tanto co-estimuladora como inibitória, como são exemplos CD28 ou CTLA4, mantendo-a controlada. Uma dessas proteínas é CD6, um recetor transmembranar sem atividade enzimática que se associa aos ligandos extracelulares CD166 e CD318. Observações iniciais sugeriram que CD6 fosse um co-estimulador mas, ao longo da última década, foi demonstrado que CD6 na realidade diminui a ativação de células T através da sua cauda citoplasmática. Apesar do papel de CD6 na estimulação de linfócitos T ainda ser objeto de debate, e de que apenas recentemente se começou a desvendar o seu signalosoma, a utilização de itolizumab, um anticorpo monoclonal que se liga a CD6, foi já aprovada para o tratamento de doenças inflamatórias. A falta de informação atualmente disponível sobre os mecanismos de ação de CD6 gera a necessidade de investigar quais as vias de sinalização efetivamente moduladas por este recetor e quais os efetores envolvidos nesse processo.

Neste trabalho, demonstramos que, quando fosforilado, CD6 se liga à proteína ativadora de GTPase da Ras (RasGAP), uma enzima com atividade GAP (*i.e.* inibitória) sobre Ras, uma GTPase que potencia a ativação de células T. Além disso, criámos um biossensor baseado na transferência de energia de ressonância de Förster (FRET) para avaliar a atividade GTPase de Ras em células T em tempo real enquanto estas são ativadas por APCs e descobrimos que CD6 diminui significativamente a ativação de Ras em linfócitos T estimulados. Tendo adicionalmente descoberto que a expressão de CD6 inibe a ativação da cinase regulada por sinais extracelulares (ERK)-1 e -2, concluímos que CD6 inibe a via de sinalização da proteína cinase ativada por mitogénio (MAPK) em toda a sua extensão através do recrutamento de RasGAP. Também mostramos aqui que CD6 se associa à proteína ligase-ubiquitina E3 Cbl proto-oncogene B (CBLB), uma segunda enzima inibitória, através dos resíduos de tirosina 486, 489 e 629 de CD6, e que o signalosoma de CD6 é ubiquitinado em células T ativadas, uma modificação pós-translacional com grande potencial para modulação de sinal. Dando mais um passo na clarificação das vias de sinalização reguladas por CD6, caracterizámos o potencial de sinalização da maioria dos resíduos de tirosina no domínio intracelular de CD6 e

descobrimos que apesar da maioria delas ser inibitória, outras são co-estimuladoras ou neutras. Por último, observámos que a expressão de CD6 diminui constitutivamente a atividade de RhoA, uma GTPase crucial para a remodelação do citoesqueleto e regulada por proteínas que se associam a CD6. Esta observação sustenta indícios prévios que sugerem que CD6 pode, dependendo do contexto, modular elementos do citoesqueleto em linfócitos T.

De uma forma geral, os nossos dados são consistentes com observações prévias de que CD6 inibe a ativação de linfócitos T. Aqui, descrevemos duas novas proteínas que se associam a CD6 (RasGAP e CBLB) e que podem mediar o seu papel e identificámos quais os resíduos de tirosina intracelulares de CD6 que contribuem para o seu efeito geral. Além disso, a regulação da atividade de RhoA por CD6 sugere um novo eixo de sinalização através do qual CD6 pode regular proteínas do citoesqueleto. O presente trabalho representa um avanço significativo na nossa compreensão da sinalização mediada por CD6 e o seu papel global na progressão da ativação de linfócitos T.

Abbreviations

aa	Amino acid(s)
ABP	Actin-binding protein
AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2
AKT	Protein kinase B
ALCAM	Activated leukocyte cell adhesion molecule
AP-1	Activator protein-1
APC	Antigen-presenting cell
AR	Abundance ratio
ARHGAP45	Rho GTPase-activating protein 45
BAT3	HLA-B-associated transcript 3
BCL10	B-cell lymphoma/leukemia 10
BCR	B-cell receptor
CARD11	Caspase recruitment domain family member 11
CBLB	Cbl proto-oncogene B
CD6Δd3	CD6Δdomain3
CIA	Collagen-induced arthritis
CK2	Casein kinase 2
CLP	Common lymphoid progenitor
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CUB	Complement C1r/C1s, Uegf, Bmp1
d1-3	Domain 1-3
DAG	Diacylglycerol
DC	Dendritic cell
DN	Double-negative
DP	Double-positive
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
ERM	ezrin, radixin and moesin
F-actin	Fillamentous actin
FP	Fluorescent protein
FRET	Förster resonance energy transfer
FYN	FGR/YES novel protein
G-actin	Glomerular actin
GADS	GRB2-related adaptor protein 2

GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRB2	Growth factor receptor bound protein 2
HSC	Hematopoietic stem cell
HVR	Hypervariable region
IFNɣ	Interferon ɣ
Ig	Immunoglobulin
IGLL1	Immunoglobulin lambda-like polypeptide 1
IKBKG	Inhibitor of nuclear factor-κB kinase regulatory subunit ɣ
IκBα	NF-κB inhibitor α
IKK	IκB kinase
IL	Interleukin
IP3	Inositol-1,4,5-tris-phosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITK	IL-2-inducible T-cell kinase
ITSM	Immunoreceptor tyrosine-based switch motif
KO	Knockout
LAG-3	Lymphocyte-activation gene 3
LAT	Linker for activation of T cells
LCK	Lymphocyte cell-specific protein-tyrosine kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MTD	Membrane-targeting domain
MTOC	Microtubule organizing center
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
PAMP	Pathogen-associated molecular pattern

PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDK1	Pyruvate dehydrogenase kinase isoform 1
PD-L1	Programmed cell death 1 ligand 1
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C
PLCG1	Phospholipase C ɣ1
PM	Plasma membrane
pMHC	Peptide-major histocompatibility complex
PNPLA2	Patatin-like phospholipase domain-containing 2
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
RA	Rheumatoid arthritis
Raichu	Ras and interacting protein chimeric unit
RAM-Ig	Rabbit anti-mouse immunoglobulin
Rap1	Ras-related protein 1
RasGAP	Ras GTPase-activating protein 1
RasGRP	RAS guanyl-releasing protein
RBD	Ras-binding domain
RBD-PKN	RhoA-binding domain of protein kinase N
RIP1	Receptor-interacting serine/threonine-protein kinase 1
rsCD6/CD166	Recombinant soluble CD6/CD166
sAg	Superantigen
SCD6	Soluble CD6
SEE	Staphylococcal enterotoxin E
SH2	SRC homology 2
SH3	SRC homology 3
SHIP-1	Inositol polyphosphate-5-phosphatase D
SHP-1	Protein tyrosine phosphatase non-receptor type 6
SHP-2	Protein tyrosine phosphatase non-receptor type 11
SLP76	Lymphocyte cytosolic protein 2

SNP	Single nucleotide polymorphisms
SOS	Son of sevenless homolog 1
SP	Single-positive
SPR	Surface plasmon resonance
SRCR	Scavenger receptor cysteine-rich
T_c cell	Cytotoxic T cell
TCR	T-cell receptor
TGFβ	Transforming growth factor β
T_h cell	Helper T cell
TIM-3	Hepatitis A virus cellular receptor 2
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
T_{reg} cell	Regulatory T cell
TSAD	T cell-specific adapter protein
UBASH3A	Ubiquitin associated and SH3 domain containing A
UBE2D3	Ubiquitin-conjugating enzyme E2 D3
WT	Wild-type
ZAP70	70 kDa zeta-chain associated protein

General introduction

T lymphocytes and the immune system

The immune system

The human body is comprised of several systems that co-exist and collaborate with the goal of ensuring survival and well-being. The immune system, specifically, is a complex network designed to protect the body from as many pathogens as possible, and it can be subdivided into two main categories: innate immunity and adaptive immunity. The particular characteristics that define each category ensure that they complement each other while effectively addressing the different strategies used by pathogens to invade an organism.

Innate immunity exists in plants, vertebrate and invertebrate organisms and relies on both physical barriers and cellular players. In metazoans, many different cell types are involved in this response, such as macrophages, dendritic cells, neutrophils, basophils and eosinophils. This first line of defense is characterized by a quick action and a lack of specificity towards the invading pathogen [1, 2].

Adaptive immunity, on the other hand, exists only in vertebrate organisms and can summarily be described as the opposite of its counterpart. The cell effectors at work in this system are lymphocytes: T lymphocytes (T for their generation in the thymus) and B lymphocytes (generated in the bone marrow). The lymphocyte-driven immune response is delayed, as compared with the innate one, although highly specific [3, 4].

None of these systems is more relevant than the other, and an efficient immune response is usually dependent not only on the intervention of both innate and adaptive effectors, but also on a well-organized communication between them (figure 1).

Innate immunity

Innate immunity is the front line of defense in the body against invading pathogens. Taking into consideration the rapid multiplication of some existing viruses or bacteria, the existence of a fast-acting system is indispensable [5].

Physical and chemical barriers constitute the first mechanism of protection in the innate response. Epithelial barriers, such as the skin, avert physical contact between pathogens and the internal environment. These pathogens are cleared from epithelial linings via secretions, namely mucus, tears or saliva. Other strategies, such as the acidic pH in the stomach, further hinder the survival of harmful agents [1, 2].

When pathogens overcome the aforementioned physical barriers, a second layer of responses comes into play. These threats to homeostasis (whether they may be viruses,

bacteria, fungi or other organisms) are identified by cells and recognized as pathogens via a number of conserved molecular motifs that are either present on their surface or secreted – and are therefore collectively termed pathogen-associated molecular patterns (PAMPs) [6, 7]. These molecules are often found across entire classes of microorganisms, and unable to mutate over time because of their crucial role to survival. Some examples of PAMPs are lipopolysaccharide (LPS) and lipoteichoic acid (LTA), found on the membrane of Gram-negative and -positive bacteria, respectively [8]. Other molecules belonging to this group are mannan and glucan, both present on the membrane of fungi [9, 10], double stranded RNA [11], commonly generated during viral replication, and formylated methionines positioned at the start of a protein sequence, a modification characteristic of bacterial protein synthesis [12].

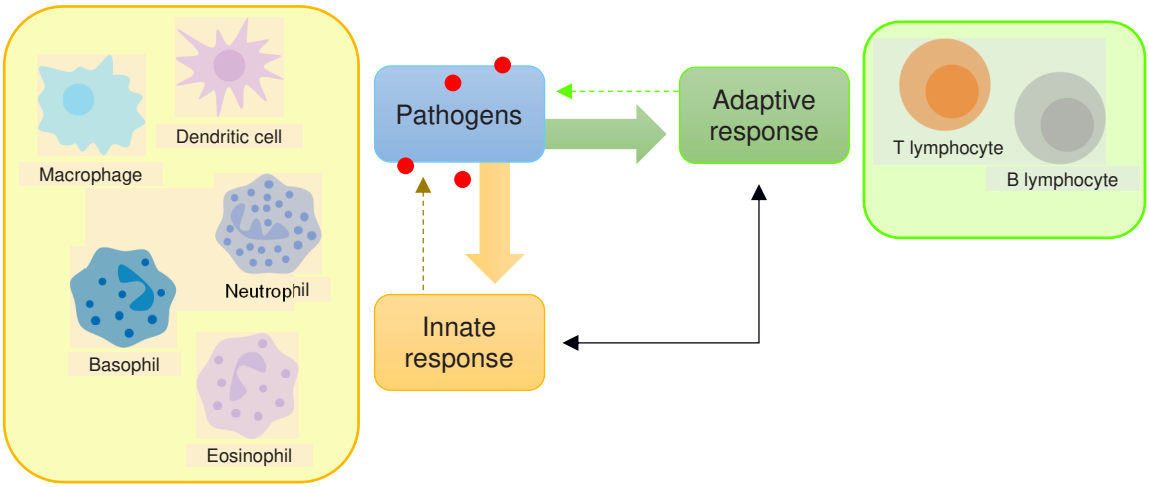

Figure 1. **The immune response to pathogens**. When pathogens (either viral, bacterial, or other) invade the human body, the two branches of the immune system are activated – innate and adaptive. The innate immune response is non-specific, quicker and involves different cell types, such as macrophages, dendritic cells or neutrophils (yellow rectangle). On the other hand, the response from the adaptive immune system is both pathogen-specific and of slower action. The cells involved in this response are T and B lymphocytes (green rectangle). When innate and adaptive responses in the immune system are activated by a pathogen, cells from both branches work to eliminate it while communicating with each other to reinforce activation and recruitment at the local level.

PAMPs are detected by a broad class of receptors called pattern recognition receptors (PRRs) – some exist in circulation, in soluble form, while others are membrane-bound or cytoplasmic [6, 13]. Among circulating PRRs, those belonging to the complement system are particularly relevant. These receptors are mainly produced by hepatocytes in the liver, but they can also be secreted by other cells such as macrophages and epithelial

cells. Although different pathways exist within the complement system, they all converge in the activation of the complement C3 protein. When C3 is activated and cleaved, two fragments are formed: C3a and C3b. While the former is a soluble messenger that attracts phagocytic cells, the latter remains bound to the infected cell and kick-starts a number of strategies to eliminate the pathogen: inducing the lysis of infected cells via pore formation, encapsulating them to promote their phagocytosis and recruiting inflammatory cells [14, 15]. Regarding cellular PRRs, the most notable members of this class are the transmembrane Toll-like receptors (TLRs), a family that includes ten different members in humans, each of them able to bind distinct PAMPs from distinct origins. Triggering of TLRs ultimately leads to translocation of transcription factors and increases transcription of inflammation-related genes [16, 17]. Many other classes of receptors exist [18], most notedly the C-type lectin receptors [19] and the NOD-like receptors [20], the latter being an example of intracellular PRRs.

Adaptive immunity

Besides the enumerated functions of the innate immune system, it is also responsible for recruiting effectors from the adaptive immune system – B and T lymphocytes [1]. Unlike innate immune cells, lymphocytes are highly specific in pathogen detection. Each lymphocyte in the human body is programmed since its generation to detect one specific antigen [21, 22] – so much so that small alterations in protein structure or single amino acid (aa) changes are sufficient to hinder antigen detection by these cells [23].

Antigens, which are broadly defined as any “substance capable of eliciting an adaptive immune response” [1], are detect by these cells via either their B- or T-cell receptor (BCR or TCR). Antigen-receptor binding is a distinct process in B and T cells. Each BCR complex contains two antigen binding sites and can directly bind its respective antigen upon encountering it in circulation [24, 25]. The TCR, however, has only one antigen binding site and cannot bind its antigen unless the latter is presented to it by a second cell – called an antigen-presenting cell (APC). An APC is any cell that, upon phagocytosis and intracellular cleavage of a pathogen, is able to display these processed molecules on the outside of its plasma membrane [26, 27]. Antigenic fragments, however, are not presented to lymphocytes directly on the surface of APCs – instead, they are presented in complex with the major histocompatibility complex (MHC) [28]. There are two main classes of MHC proteins - class I and class II - and each of them is responsible for presenting antigens to different subsets of T cells [29].

Upon the first encounter of a lymphocytic cell with the unique antigen it can recognize, an initial response is weak but triggers the proliferation of these cells and

formation of numerous clones with the same recognition capacity – these are termed memory cells [30, 31]. When the human body is exposed to the same antigen a second time, memory cells quickly proliferate and cooperate to eliminate the pathogen. This particular defense strategy is precisely the mechanism behind vaccination and is also the reason why an adaptive response is both quick and highly efficient upon encountering a given antigen for the second time [32, 33].

The response generated by B and T lymphocytes is distinct. B cells, once activated, secrete circulating antibodies that recognize the same antigen that triggered their production. Once in circulation, antibodies will have two main functions: first, they inhibit binding of the pathogen to host cells, preventing their infection; second, antibodies will bind pathogen-infected cells and mark them for phagocytosis [34]. T cells, on the other hand, are responsible for both directly destroying infected cells and for recruiting and activating other cell types to the site of infection. The different types of immune responses elicited by T cells are dependent on their subsets.

T cell subsets

T cells can be subdivided into two main categories: cytotoxic T cells (T_c), and helper T cells (T_h), and these can be further divided into subcategories as well (figure 2).

T_c cells, or killer T cells, are also frequently referred to as $CD8^+$ T cells once they can be identified based on the expression of the cell surface co-receptor CD8. T_c cells are activated by antigens presented in complex with MHC class I proteins, once these are specifically recognized by CD8 proteins [35, 36]. When T_c cells bind their cognate antigen, they become activated, and their main function is to directly trigger the apoptosis of infected cells using different mechanisms. One of these mechanisms, and the most relevant one, is the secretion of enzymes into the extracellular medium – specifically, perforins [37] and granzymes [38, 39]. In resting cells, these enzymes are stored in granules present in the cytoplasm and, upon lymphocyte activation, they are released from the cells and work in concert to trigger apoptosis: while perforins bind target cells, polymerizing and forming pores in the cell membrane, granzymes diffuse through these pores and activate a family of endoproteases called caspases (and the subsequent caspase pathway) to promote programmed cell death [39, 40]. The second mechanism is the ligation of the Fas ligand (Fas-L) on the surface of T_c cells to the Fas protein, expressed on several APCs, which also activates the caspase pathway leading to apoptosis [41]. Lastly, T_c cells can also act by secreting cytokines that recruit other immune cells and control viral replication [42, 43].

The second main subset of T cells, T_h cells, is characterized by the surface expression of the CD4 co-receptor. Unlike T_c cells, T_h cells are only activated by antigens

presented by class II MHC proteins [44, 45]. The general function of these cells is, as suggested by their name, to assist other cells and potentiate their effector role. They can be further divided into subcategories:T_h1, T_h2, T_h17 and regulatory T cells (T_{reg}).

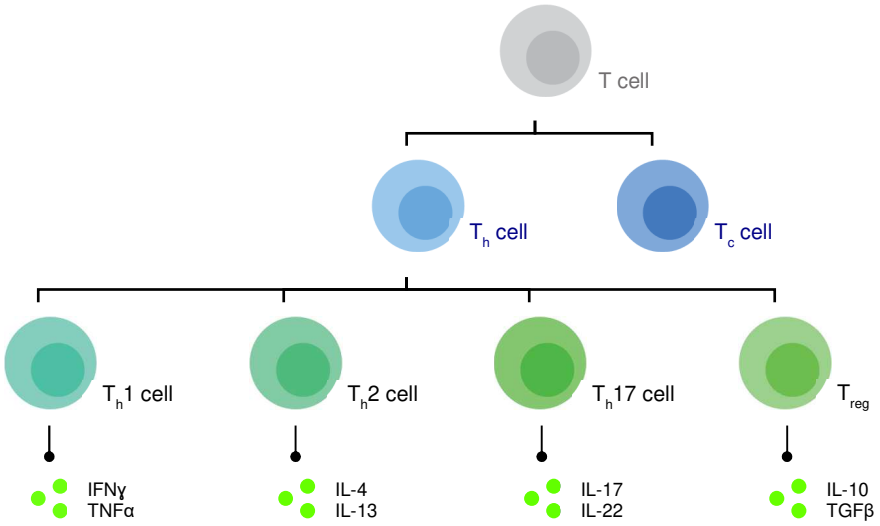

Figure 2. **The main subsets of mature T lymphocytes**. There are two main classes of lymphocytes: helper T cells (T_h), which mainly activate and recruit other cell types, and cytotoxic T cells (T_c), that directly kill infected cells. On their surface, these cells express CD4 or CD8, respectively. Naïve T_h cells can in turn differentiate into four main groups: T helper 1 (T_h1), T helper 2 (T_h2), T helper 17 (T_h17) and regulatory T cells (Treg). Differentiation into each category of T_h cells is induced by different compositions of extracellular milieu, and so are the cytokines they secrete, which are indicated below the representative image of each subset.

When naïve T_h cells (cells that have never encountered its cognate antigen) are activated, they can differentiate into any of these cell types, and lineage choice is heavily influenced by the extracellular milieu. Each T_h cell type will in turn produce specific cytokines that mediate its effector function [46-48].

Differentiation of T_h1 cells is induced by the presence of interferon ɣ (IFNɣ) and interleukin-12 (IL-12) [49-51] in the extracellular milieu, and fully differentiated T_h1 cells will in turn produce high levels of IFNɣ [52], tumor necrosis factor α (TNFα) [53], granulocyte-macrophage colony-stimulating factor (GM-CSF) [54] and IL-2 [55, 56], among others. T_h1 cells generally contend with intracellular pathogens, and their main roles are to activate macrophages [57] and T_c cells [58], although other cells, like B lymphocytes, are also targeted [59].

On the other hand, the polarization of T_h2 cells is usually associated with extracellular pathogens and occurs in the presence of IL-4 and IL-2 [60, 61], resulting mostly

in the production of IL-4 and IL-13, among others [62, 63]. T_h2 cells are crucial for antibody-mediated responses, triggering class switching in B cells (a process that results in antibody secretion) [64, 65], but also recruiting eosinophils [66] and basophils [67]. T_h1 and T_h2 cells were the first T_h subtypes to be described and, for some time, thought be the only existing ones [56].

T_h17 cells are a recently uncovered subtype [68, 69] that is generated from naïve T cells in the presence of IL-23, IL-6 or TGFβ [70-72]. T_h17 cells were named as such based on their production of high levels of IL-17 upon differentiation [69]. T_h17 cells have a proinflammatory role and have been linked to the genesis of autoimmune disorders [73, 74].

Lastly, T_{reg} cells [75] downmodulate the immune system and help preventing autoimmune diseases [76]. They are differentiated in the presence of IL-2 [77] and TGFβ [78] and produce mostly IL-10 [79] and TGFβ [80].

Thymopoiesis

The majority of mature T lymphocytes circulating in the body differentiate in the thymus from precursor cells termed common lymphoid progenitors (CLPs) that have migrated there from the bone marrow. CLPs derive from hematopoietic stem cells (HSCs) and are thought to originate both B and T lymphocytes [81, 82]. The gradual process of mature T cell generation in the thymus is called thymopoiesis and occurs continuously in the human body since before birth and into adulthood (figure 3) [83].

In their first stage of maturation, thymocytes do not express neither CD4 nor CD8, and are therefore designated double-negative (DN) cells. This stage can be further subdivided into four separate stages based on expression of the markers CD25 and CD44: DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$) and DN4 ($CD44^-$ $CD25^-$) [84, 85]. Although cells at DN1 and DN2 stages retain their ability to differentiate into other cell types, DN2 cells specifically are characterized by a higher proliferative profile and upregulation of T cell-specific genes, while repressing the expression of genes associated to other lineages [82, 86-88]. Cells at DN3, on the other hand, are fully committed to the generation of T lymphocytes and begin to express rearranged individual TCR chains – β, ɣ and δ chains [89]. How quickly and successfully these rearrangements occur will determine whether each lymphocyte expresses TCR αβ or TCR ɣδ dimers at its surface [90]. At this point, TCR αβ-expressing thymocytes come to their first checkpoint: β selection. This checkpoint selects for cells that have managed to successfully assemble a pre-TCR complex, which is formed upon the association of one functional TCR β chain, one pre-TCR α chain and six CD3 chains. Signaling through this complex will lead to CD25 downregulation – and mark entry into the DN4 stage. If the cell is unable to compose a pre-

TCR, it will die by apoptosis. In selected cells, the ensuing upregulation of both CD4 and CD8 marks the passage of thymocytes into the next developmental stage – double-positive (DP) cells [91, 92].

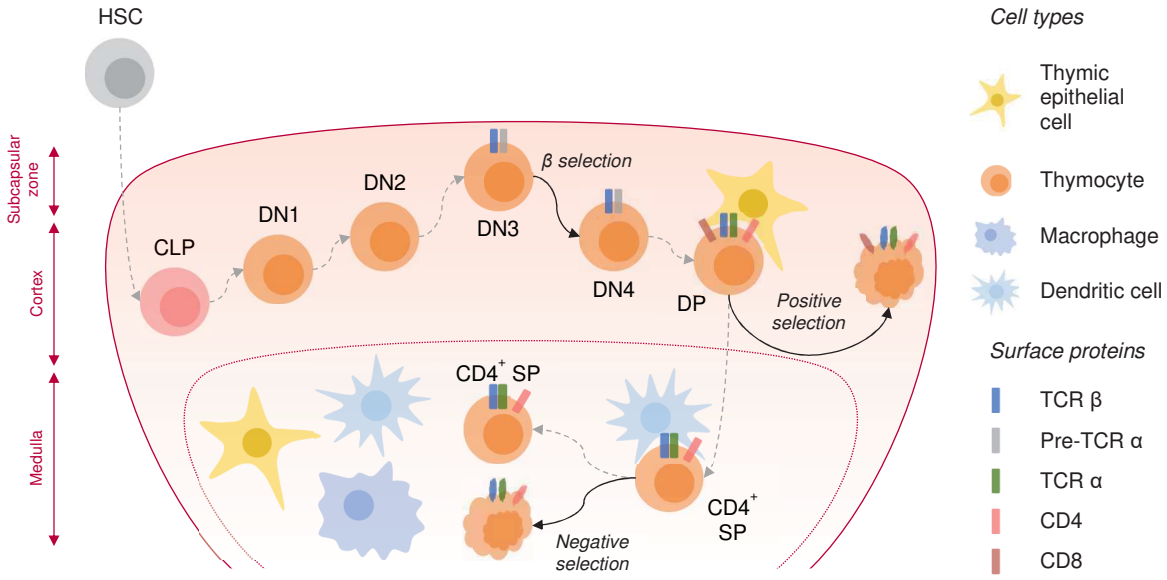

Figure 3. **Generation of mature T cells in the thymus (thymopoiesis)**. T cells develop in the thymus from common lymphoid precursors (CLP), that in turn arise from hematopoietic stem cells (HSC). According to the expression of the CD4 and CD8 co-receptors, T cells progress from double-negative (DN), to double-positive (DP) and to single-positive (SP) thymocytes – expressing either CD4 or CD8. DN cells can be further subdivided into four stages, determined by CD44 and CD25 expression (DN1-DN4). During thymopoiesis, there are three major checkpoints: 1) β selection, between DN3 and DN4 stages, which sorts thymocytes with a correctly arranged pre-TCR; 2) positive selection, between DP and SP stage, which sorts T cells with a TCR that recognizes self MHC complexes on antigen-presenting cells, and 3) negative selection, that sorts thymocytes that react too avidly to self MHC proteins. The small percentage of thymocytes that survive all checkpoints will then migrate out of the thymus to the periphery.

The next major checkpoint for developing thymocytes is positive selection and it is conditioned by proper rearrangement of the TCR α chain and the specificity of the resulting TCR αβ complex [93, 94]. As mentioned above, T cells are able to recognize foreign antigens when these are presented by MHC proteins. During positive selection, the newly formed TCR complexes in thymocytes are presented self-antigens (*i.e.*, antigens derived from the host) by self MHC proteins. Thymocytes expressing TCR complexes that bind self MHC are selected to live and further progress in maturation. T_c cells arise from cells where the TCR recognized class I MHC complexes, while T_h cells result from thymocytes that

recognized class II MHC complexes [95, 96]. On the contrary, if the TCR from a given thymocyte is not able to recognize the self MHC, that cell will die by neglect [97].

The recognition of MHC complexes, however, must not be too potent, at the possible cost of inducing autoimmunity. To ensure that circulating mature lymphocytes are tolerant to self-molecules, cells that react too strongly to the self MHC-self antigen complex in the thymus also die by apoptosis – this third sieve is called negative selection [98, 99]. Only around 5% of DP thymocytes survive both positive and negative selection [100]. Self-reactive T cells can also be posteriorly eliminated in the periphery, if they escape the thymus, in a process called peripheral tolerance [101]. There are many mechanisms involved, some of which are the induction of apoptosis in cells triggered by non-infected dendritic cells (DCs) or T_{reg} cell induction [102].

Thymocytes that have both been selected for survival and downregulated their expression of either CD4 or CD8 co-receptor (according to their MHC specificity) will at this point adjust their gene expression according to their effector function. After receiving survival signals and fully maturing, naïve T cells from both subsets will leave the thymus and circulate in the periphery [97].

The Immunological Synapse

The T cell receptor (TCR)-CD3 complex

The TCR is a protein heterodimer expressed on the membrane of T lymphocytes [103, 104]. In most T cells, the TCR is composed of α and β chains (αβ T lymphocytes) [105, 106] but, in around 0.5-5% of lymphocytes, it is alternatively composed of ɣ and δ chains (ɣδ T lymphocytes) [107]. Each of these chains has a constant and a variable domain, the latter of which binds peptide-MHC (pMHC) complexes [108]. The affinity of the TCR towards an antigen is determined by three complementarity-determining regions within the variable domains [109], and each individual T cell expresses a different TCR on their surface. This substantial diversity is obtained by rearrangement of the TCR variable (V), diversity (D) and joining (J) gene segments in a process called somatic V(D)J recombination [21].

The TCR heterodimer is non-covalently bound to CD3 – a complex that integrates four different chains (CD3 δ, ɣ, ε and ζ), grouped in three dimers: δε, ɣε, and ζζ. Together with the TCR dimer, they form the TCR-CD3 complex, henceforth referred to only as the TCR [110]. The cytoplasmic tails in the CD3 δ, ɣ and ε chains all encompass an immunoreceptor tyrosine-based activation motif (ITAM), while each of the ζ chains has three. These motifs ($YxxL/Ix_{(6-8)}YxxL/I$ [111]), although not unique to CD3 subunits, are crucial for signal transduction in lymphocytes, once they connect extracellular TCR-pMHC binding to intracellular lymphocyte activation [112].

How TCR-pMHC binding triggers cell activation is still a matter of debate, and different theories have been put forward to explain how extracellular engagement of the TCR translates into intracellular phosphorylation. Those models can be generally grouped in three classes: aggregation, conformational change and kinetic segregation (as reviewed in [113]).

The *aggregation-based models* stipulate that intracellular phosphorylation of the TCR takes place upon the accumulation of key molecules in the vicinity of the TCR. The co-receptor heterodimerization model, on one hand, suggests that, because the co-receptors CD4/CD8 also bind the pMHC complex along with the TCR, they recruit lymphocyte cell-specific protein-tyrosine kinase (LCK) to the TCR immediate vicinity. On the other hand, the pseudodimer model proposes that different TCR units in the same cell bind pMHC complexes that present a self-peptide, as well as their agonist peptide. This will ultimately lead to the formation of an aggregate when the co-receptor of a TCR-self-pMHC

complex binds instead the MHC associated to a TCR-agonist-pMHC complex, leading also to kinase accumulation [114, 115].

The *conformation-based models* state that TCR-pMHC binding induces a change in the conformation of the TCR (either the extracellular domains or the CD3 intracellular chains) that allows for the complex to be phosphorylated by kinases. Specifically, it has been shown that pMHC binding leads to extracellular conformational changes in the TCR α constant domain, possibly inducing receptor oligomerization [113, 116-118]. Other researchers have reported that TCR-triggered conformational changes take place mostly at the intracellular level of CD3 chains ε and ζ, potentially regulating the access and the effect of kinases [119-121].

Lastly, the *kinetic segregation models* affirm that TCR engagement results in its segregation from inhibitory phosphatases, increasing its phosphorylation levels. This model has been supported by observations that, upon T cell activation, the amount of active LCK is unchanged [122], which strongly suggests that TCR phosphorylation is modulated not by the activation of LCK but instead by its redistribution inside the cell. Phosphorylation of the TCR by LCK is counterbalanced by the activity of phosphatases, such as CD45 and CD148. One of these models postulates that TCR-pMHC engagement, followed by TCR clustering, results in the formation of proximity zones between the T cell and the APC, from which proteins with large extracellular domains, such as CD45 and CD148, are excluded [123]. The increase of the kinase/phosphatase ratio in these areas leads to TCR phosphorylation and triggering of downstream signaling, a theory that has been supported by numerous evidence over the last years [124, 125]. Other similar proposals suggest that pMHC binding prompts the association of the TCR to lipid rafts (lipid-enriched membrane microdomains [126]), where LCK is enriched, unlike CD45 and CD148 [127].

CD4 and CD8 co-receptors

TCR-pMHC engagement is further mediated by the co-receptors CD4 or CD8, two transmembrane glycoproteins that bind MHC complexes from class II or class I, respectively. While CD4 is a monomer that contains four extracellular immunoglobulin (Ig)-like domains, CD8 is a dimer that includes one α and one β chain, each of them comprising one extracellular Ig-like domain [128]. As aforementioned, expression of CD4 or CD8 on the surface of a T cell determines to which lymphocyte subset that T cell belongs: $CD4^+$ lymphocytes are T_h cells, while $CD8^+$ lymphocytes are T_c cells [129].

Both CD4 and CD8 interact with LCK. It is generally accepted that the main role of CD4/CD8 is to anchor LCK and recruit it to the vicinity of the TCR, in order to trigger ITAM phosphorylation [130-132]. Accordingly, CD4/CD8-pMHC binding lowers the threshold for

T cell triggering by antigenic peptides [133-135]. Furthermore, expression of either CD4 or CD8 has been shown to be required for optimal T cell activation, as assessed by quantification of IL-2 production [136-139].

Roles and regulation of SRC- and SYK-kinases

LCK and FGR/YES novel protein (FYN), as members of the same protein family SRC, share common structural features: one SRC homology 3 (SH3) domain, one SH2 domain, a kinase domain and an inhibitory C-terminal region [140]. Their distribution within the cell is determined by lipid modifications (myristoylation and palmitoylation) to their N-terminus, which allows them to bind the inner leaflet of the membrane [141]. The SH3 and SH2 domains in both LCK and FYN mediate binding to other proteins containing, respectively, proline-rich regions and phosphotyrosine residues. Despite an overlap in protein interactions, others remain kinase-specific [142]. Additionally, SRC-kinases encompass a unique region preceding their SH3 domain that, among other functions, mediates specific protein-interactions [143, 144]. Despite the many similarities, LCK and FYN do not completely co-localize in T cells, as FYN is also abundant in intracellular structures other than the membrane [145]. Within the plasma membrane, LCK and FYN have also been shown to concentrate their activity in different regions [146].

Activation of LCK and FYN is mostly determined by the conformational changes induced by phosphorylation of two tyrosine (Y) residues: LCK-Y394/FYN-Y420 (activating) and LCK-Y505/FYN-Y531 (inhibitory) [147]. Specifically, when their activating tyrosine residue is phosphorylated, enzymatic activity in these kinases is increased [148, 149]. On the other hand, when the C-terminal inhibitory tyrosine residue is phosphorylated [typically by C-Src kinase (CSK)], it binds the kinase's own SH2 domain, locking it in an autoinhibited conformation. Dephosphorylation of the same residue by CD45 releases the kinase from auto-inhibition, generating an activated conformation (figure 4) [150-156]. In LCK, CD45 also dephosphorylates the residue Y394 (although with lower affinity), and therefore plays a double part in the regulation of this kinase [157, 158]. Importantly, it has been demonstrated that LCK is constitutively active in T cells, suggesting that other factors, such as spatial regulation, are more relevant in the control of LCK activity [122].

Upon TCR-pMHC engagement, LCK is brought to close proximity of the TCR by CD4/CD8 [132, 159], leading to phosphorylation of the ITAMs on CD3 chains [131]. Each doubly phosphorylated ITAM becomes at this point a potential binding site for the SYK-family kinase 70 kDa zeta-chain associated protein (ZAP70), which rapidly accumulates at the membrane upon T cell activation. Unlike SRC-family kinases, ZAP70 does not have an SH3 domain but has, instead, two adjacent SH2 domains that mediate its binding to CD3

ITAMs [160, 161]. Next to its SH2 domains, ZAP70 contains a kinase domain. When bound to the TCR, this domain is phosphorylated by LCK, rendering ZAP70 active (figure 4) [162-164]. While FYN is also associated with the TCR and involved in T cell activation, its role is less clear than that of LCK [165, 166].

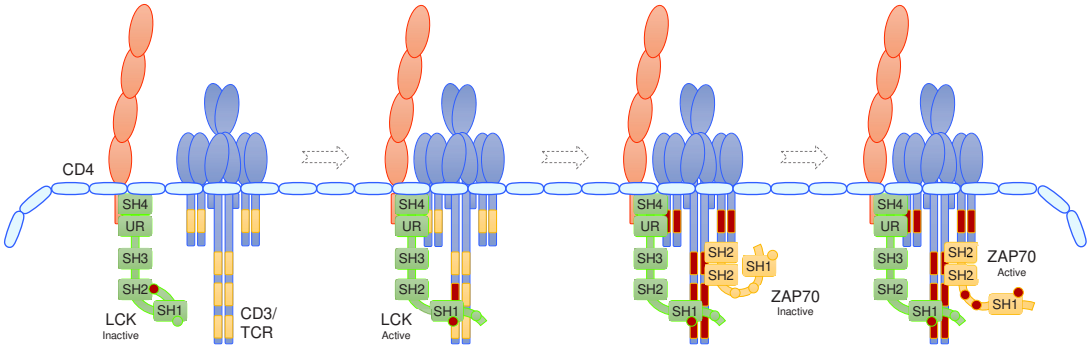

Figure 4. **Early steps of T cell receptor-mediated activation of T lymphocytes**. When a T cell is activated, the co-receptor CD4 (in T_h cells) or CD8 (in T_c cells) brings the SRC-family kinase LCK closer to the TCR. The activity of LCK is mostly controlled by two tyrosine residues – Y394 and Y505. When Y394 is phosphorylated and Y505 is dephosphorylated, LCK is released from its auto-inhibitory conformation and becomes active, phosphorylating in turn the ITAM motifs (in yellow) present in the cytoplasmic tail of CD3. When CD3 ITAMs are doubly phosphorylated, the SYK-family kinase ZAP70 is able to bind them via its SH2 domain pair and, at this point, ZAP70 is phosphorylated and activated by LCK. Active ZAP70 will then go on to phosphorylate proteins downstream of this aggregate, propagating activation.

When ZAP70 is active, it phosphorylates and recruits a number of substrates, the most critical of which is linker for activation of T cells (LAT) [167, 168]. Active LAT functions as a signaling platform that propagates TCR-originated signals and activates different pathways, promoting distinct outcomes of T cell activation [169]. The central role of LAT in amplifying T cell activation is illustrated by the fact that ZAP70-deficient Jurkat cells (a T cell line) are not able to propagate signals downstream of TCR engagement [170]. Moreover, patients with impaired ZAP70 function have severe combined immunodeficiency (SCID) [171].

Co-stimulation by CD28

Engagement of the TCR is not enough to trigger T cell activation to its full extent [172, 173]. For that, the action of a second signal – co-stimulation – is required, provided by the co-stimulatory receptor CD28 [174, 175]. CD28 is a 44 kDa transmembrane homodimer, and each monomer contains one extracellular Ig-like domain, in addition to

transmembrane and cytoplasmic domains [176]. CD28 has two ligands, CD80 and CD86, both of them expressed on the membrane of different APCs [177-179].

CD28 is phosphorylated upon T cell activation [180] and recruited to the immunological synapse upon its assembly [181]. “The immunological synapse (IS) is a specialized cell–cell junction between a thymus-derived lymphocyte (T cell) and an antigen-presenting cell (APC)” [182]. Much like CD3, CD28 has no intrinsic enzymatic activity. Therefore, it relies on intracellular associations to modulate T cell activation, mediated by either tyrosine-containing or proline-rich motifs. Two motifs within CD28 cytoplasmic tail have been shown to be the most important for these interactions, although their relative relevance is still a matter of debate: YMNM and PYAP [183, 184]. The first one – YMNM – mediates binding to phosphatidylinositol 3-kinase (PI3K) [185, 186], and has been suggested to be relevant for IL-2 production and lymphocyte proliferation [187]. CD28-mediated recruitment of PI3K is key to its co-stimulatory function, as PI3K stimulates the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3), which in turn recruits pyruvate dehydrogenase kinase isoform 1 (PDK1), leading to the phosphorylation of protein kinase B (PKB/AKT) and protein kinase C (PKC) θ [188].

The PYAP sequence, on the other hand, likely binds LCK [183, 189], and has also been associated with increased IL-2 secretion and proliferation, potentially in a more significant manner than YMNM [188, 190]. Additionally, CD28 also binds growth factor receptor bound protein 2 (GRB2) and GRB2-related adaptor protein 2 (GRAP2/GADS), while upregulating the anti-apoptotic protein BCL2-like 1 (BCL2L1) [191] and promoting stabilization of mRNA for IL-2, IFNɣ and TNFα [192].

CD28 plays a double role on T cell activation, both amplifying TCR-originated signals [193] and independently triggering diverse signaling pathways. Accordingly, a study focused on phosphorylation-dependent associations of CD28 showed that, while many are linked to TCR signaling, others are not [194]. CD28 has increasingly been shown to be a critical player in cytoskeletal dynamics, by directly inducing cytoskeletal remodeling [195], and by associating with diverse regulators of that process, namely capping protein regulator and myosin 1 linker 2 (CARMIL2) [196] and RCSD domain containing 1 (RCSD1) [194].

The linker for activation of T cells (LAT) signalosome

LAT was cloned in 1998 from Jurkat T cells, by the laboratory of Lawrence Samelson [167]. In that same year, the protein homologue in rat was also cloned [197]. LAT is a transmembrane protein that includes a long cytoplasmic tail that, while devoid of enzymatic activity, contains nine intracytoplasmic tyrosine residues conserved among human, mouse and rat [169]. While the molecular mass of the predicted protein was determined to be

around 25 kDa [167], observations reported this protein to migrate at 36-38 kDa, explaining its previous designation of pp36/38.

Upon T cell activation, LAT is quickly phosphorylated, a process mediated mostly by ZAP70 [167, 198], but also by IL-2-inducible T-cell kinase (ITK) [199] and LCK [200]. When phosphorylated, intracellular tyrosine residues on LAT become potential docking sites for SH2 domain-containing proteins. LAT has been reported to interact with a plethora of proteins, like phospholipase C ɣ1 (PLCG1) [201, 202], GRB2 [203], PI3K [204] and GADS [205] (figure 5). In line with the stimulatory nature of these proteins, Jurkat T cells deprived of LAT display a severe impairment in T cell activation. Specifically, LAT has been shown to be required for optimal PLCG1 and lymphocyte cytosolic protein 2 (LCP2/SLP76) phosphorylation, intracellular calcium increase, mitogen-activated protein kinase (MAPK) activation, Ras activation, CD69 upregulation and nuclear factor of activated T-cells (NFAT) and activator protein-1 (AP-1) transcriptional activity upon T cell triggering. These cells, however, display normal levels of TCR and ZAP70 phosphorylation, which demonstrates that LAT is the protein that connects immediate TCR triggering and interactors to downstream signaling [206, 207] (figure 5). Consistent with its role in TCR-mediated T cell activation, LAT-deficient mice lack peripheral mature T cells, once thymopoiesis is blocked at the DN stage [208].

Phospholipase C ɣ1 (PLCG1)

PLCG1 is an enzyme that binds LAT via Y132 upon phosphorylation of that aa, although LAT Y191 appears to have a role in this binding as well [209]. PLCG1 is key in T cell activation, and its main role consists in hydrolyzing phosphatidylinositol-4,5-bisphosphate (PIP2) into the second messengers diacylglycerol (DAG) and inositol-1,4,5-tris-phosphate (IP3) [210].

DAG is a membrane-bound lipid and, there, it recruits both Ras guanyl-releasing protein (RasGRP) and PKC [211, 212]. RasGRP is an activator of Ras and, consequently, of the MAPK pathway [213, 214]. Active PKC, on the other hand, will promote the assembly of a complex that includes the proteins caspase recruitment domain family member 11 (CARD11), B-cell lymphoma/leukemia 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), which will in turn activate the IκB kinase (IKK) complex. NF-κB inhibitor α (NFKBIA/IκBα) is bound to cytosolic nuclear factor-κB (NF-κB), masking its nuclear localization sequence. When IκBα is phosphorylated by IKK, it is degraded, which allows for NF-κB to translocate to the nucleus [210, 215, 216].

PLCG1-generated IP3, on the other hand, will bind specific receptors on the endoplasmic reticulum and promote calcium release into the cytoplasm (from the endoplasmic reticulum and subsequently from the extracellular milieu). Elevated calcium levels will in turn activate the phosphatase calcineurin and lead to NFAT dephosphorylation and subsequent translocation to the nucleus [217-220].

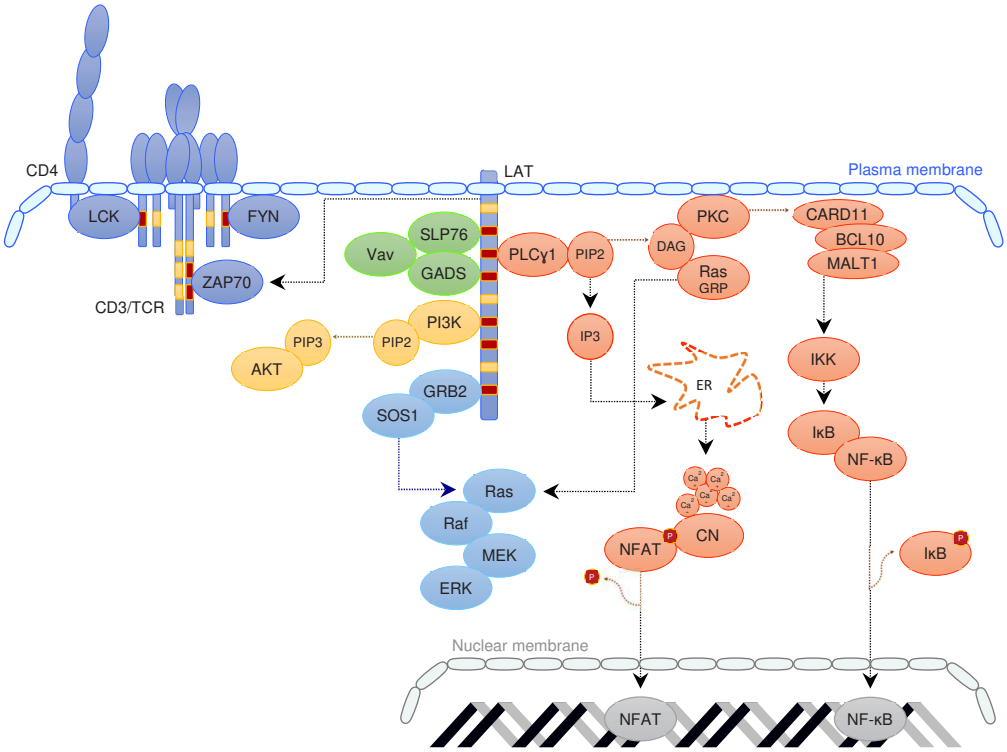

Figure 5. **Downstream propagation of T cell receptor-mediated T lymphocyte triggering**. Upon TCR engagement, CD4-recruited LCK is brought to the vicinity of the TCR, phosphorylating the ITAM motifs present on CD3 cytoplasmic tails, which then become binding sites for ZAP70. Once ZAP70 is activated by LCK, it phosphorylates LAT, leading to the formation of the LAT signalosome and consequent signal branching. Phosphorylated LAT binds: 1) both GADS and SLP76, the latter of which recruits Vav; 2) PI3K, which indirectly recruits AKT to the membrane by synthesizing PIP3; 3) GRB2, which is constitutively associated to SOS1, a GEF protein that activates Ras and the ensuing MAPK pathway and 4) PLCG1, an enzyme that hydrolyzes PIP2 into IP3 and DAG. While IP3 will trigger the release of calcium from intracellular storages, DAG will recruit both RasGRP (a second Ras activator) and PKC, leading to the formation of the CARD11/BCL10/MALT1 complex and activate IKK. The combination of the different signaling pathways activated by formation of the LAT signalosome upon TCR triggering will ultimately lead to translocation of different transcription factors (such as NFAT and NF-κB) to the nucleus and to the modulation of the expression of several activation-related genes.

Lymphocyte cytosolic protein 2 (LCP2/SLP76)

SLP76 is an adaptor protein that binds LAT directly and indirectly upon T cell activation. SLP76 is, as LAT, a crucial adaptor for the progression of signaling, as shown by the absence of mature peripheral T cells in knockout (KO) mouse models for either protein [208, 221]. Moreover, similarly to LAT, SLP76 is also key to the progression of T cell activation, and its absence hinders a number of distinct signaling pathways [208, 222].

Structurally, in addition to a proline-rich domain and an SH2 domain in its structure, this adaptor further includes tyrosine residues that are phosphorylated by ZAP70 upon TCR triggering [223, 224]. It is generally accepted that SLP76 binds LAT by associating with GADS SH3 domain, which will in turn bind phosphorylated LAT [205], a complex that is further stabilized by PLCG1 [209]. SLP76 recruits, at this point, a number of signaling proteins, such as the guanine nucleotide exchange factor (GEF) Vav [225, 226], ITK [227, 228], ArfGAP with dual PH domains (ADAP) [229, 230] and PI3K [231].

Phosphatidylinositol 3-kinase (PI3K)

PI3K refers to a group of lipid kinases involved in many cell processes [232]. PI3K can be activated by different membrane receptors (the TCR among them), and many proteins have been described to link TCR triggering to PI3K activations and recruitment to the membrane, namely SLP76 [231], LAT [167], FYN [233, 234] and Ras [235, 236].

PI3K is a heterodimer composed of two separate units: a smaller regulatory unit and a larger catalytic one [237]. As briefly mentioned before, PI3K mediates the synthesis of PIP3 from PIP2, its leading substrate, and therefore its central function is to regulate levels of this lipid inside the cells. PIP3 can bind proteins containing a pleckstrin homology (PH) domain, such as AKT [238], recruiting them to the membrane. Once AKT binds PIP3, it is phosphorylated by PDK1 at threonine 308 and activated [239]. Activated AKT can then phosphorylate downstream targets, such as mechanistic target of rapamycin kinase (mTOR), forkhead box O (FOXO) transcription factors, glycogen synthase kinase 3 (GSK3) and MDM2 proto-oncogene (MDM2), promoting cell survival and proliferation [240].

Growth factor receptor bound protein 2 (GRB2)

GRB2 is an adaptor cytosolic protein that includes one SH2 and two SH3 domains. GRB2 binds LAT-phosphorylated tyrosine residues via its SH2 domain, and recruits proteins containing proline-rich sequences via the SH3 domains [210].

In T cells, GRB2 is constitutively associated to son of sevenless homolog 1 (SOS1), an activator of Ras [241-243]. Therefore, when T cells are activated, GRB2 recruits SOS1 to the LAT signalosome. The GRB2-SOS1 and PLCG1-RasGRP1 (previously detailed) axes constitute the two main pathways through which Ras is activated in T lymphocytes [244-246], although their relative importance in the process has been subject of discussion [245, 247].

Inhibitory mechanisms of T lymphocytes

Activation of the adaptive immune system is, as mentioned before, a fundamental process to identify and destroy foreign threats to homeostasis. T cell activation, specifically, is an intracellular multimolecular response that, starting with TCR engagement, incorporates diverse co-stimulatory signaling pathways to promote proliferation and cytokine secretion, among other outcomes. Thus far, we have outlined the most significant co-stimulatory pathways involved in lymphocyte activation.

However, unchecked activation of T cells can lead to an exaggerated response to antigen or even autoimmunity. For that reason, along with the TCR and co-stimulatory proteins, T cells also express proteins that repress activation – these regulators are called immune checkpoints. The development of checkpoint inhibitors, *i.e.*, molecules that prevent binding of checkpoint proteins to their ligands, therefore increasing the anti-tumor effector activity of T lymphocytes, led to the attribution of the Nobel Prize in Physiology or Medicine 2018 to James Allison and Tasuku Honjo [248]. The outstanding work of these two researchers was focused on checkpoint inhibitors targeting the proteins programmed cell death protein 1 (PDCD1/PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). Many immune checkpoints have been described so far, among them PD-1, CTLA-4, Hepatitis A virus cellular receptor 2 (HAVCR2/TIM-3) and lymphocyte activating 3 (LAG-3) [249].

Programmed cell death protein 1 (PD-1)

PD-1 was first described in 1992 [250]. It has two ligands: programmed cell death 1 ligand 1 (PD-L1/CD274, [251]) and programmed cell death 1 ligand 2 (PD-L2/CD273, [252]). PD-1 expression is upregulated upon activation in both thymocytes and T cells [253]. The inhibitory potential of this protein was first uncovered when model animals lacking PD-1 expression developed autoimmune pathologies, such as lupus-like glomerulonephritis and arthritis, while also increasing the incidence of diabetes in nonobese diabetic (NOD) mice

[254, 255]. Furthermore, engagement of PD-1 by PD-L1.Ig was found to decrease both proliferation and cytokine production in human $CD4^+$ T cells activated via CD3 [251].

PD-1 is composed of one extracellular Ig-like V-type domain, a transmembrane domain, and a cytoplasmic tail [256]. Within the cytoplasmic tail, PD-1 contains two tyrosine residues, each embedded in either an immunoreceptor tyrosine-based inhibitory motif (ITIM, V/L/I/XpYXX/L/V) or a immunoreceptor tyrosine-based switch motif (ITSM, TXpYXXV/I). The ITSM recruits both protein tyrosine phosphatase non-receptor type 6 (PTPN6/SHP-1) and protein tyrosine phosphatase non-receptor type 11 (PTPN11/SHP-2) phosphatases and has been reported to the be indispensable for PD-1 inhibitory activity [257]. The ITIM binds SHP-2 as well and, although not indispensable, it is required for full PD-1 inhibitory action [258].

Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)

A second transmembrane protein that restrains T cell activation is CTLA-4 [259]. CTLA-4 is a glycoprotein from the immunoglobulin superfamily that incorporates a cytoplasmic tail, a transmembrane domain and one extracellular Ig-like V-type domain, through which CTLA-4 directly binds the same ligands as CD28: CD80 and CD86 [177, 179]. CTLA-4 main inhibitory mechanism is to compete with the co-stimulator CD28 for the same ligands [260-262]. Although previously hinted, the role of CTLA-4 in downplaying T cell activation was first confirmed by the generation of CTLA-4-KO mice. These animals were shown to be short-lived, presenting elevated levels of lymphoproliferation, as well as severe tissue infiltration and damage [263-265].

In addition to preventing CD28 extracellular engagement, CTLA-4 further constrains T cell activation by recruiting inhibitory proteins via its cytoplasmic tail, namely SHP-2 [266] and protein phosphatase 2A (PP2A) [267]. Although CTLA-4 has no classical ITIM or ITSM, its cytoplasmic portion has been shown to be indispensable for its inhibitory role [268].

Hepatitis A virus cellular receptor 2 (HAVCR2/TIM-3)

Aside from PD-1 and CTLA-4, other immune checkpoints are also crucial for the downmodulation of T cell responses. One example is HAVCR2, most commonly known as T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3). TIM-3 was first cloned in 2002 and initially identified as a T_h1-specific immunomodulatory protein [269], although later reports demonstrated this protein is also present in other cell types, like T_{reg} or NK cells [270, 271]. The protein includes one extracellular Ig-like V-type domain, a

membrane-adjacent mucin domain, an extracellular domain and a cytoplasmic tail, containing five conserved tyrosine residues [272].

Galectin-9 was the first described ligand for TIM-3, and binding has been shown to downmodulate T_h1 responses (such as IFNγ production), promote Th1 cell death and induce peripheral tolerance [273, 274]. Other TIM-3 ligands have been described since then, namely phosphatidylserine, high mobility group protein B1 (HMGB1) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), all directly binding to its Ig-like extracellular domain [275]. These interactions modulate other cell processes like antigen presentation and phagocytosis of apoptotic cells [276]. Although the molecular action mechanism of TIM-3 is still a matter of debate, there has been mounting evidence pointing to an HLA-B-associated transcript 3 (BAT3)-mediated effect [277]. In the basal state, TIM-3 is bound to BAT3 but, when ligand-bound, intracellular tyrosine residues are phosphorylated, releasing BAT3 from TIM-3 and allowing for the recruitment of inhibitory phosphatases [275].

Lymphocyte-activation gene 3 (LAG-3)

LAG-3 was first identified in 1990 [278]. This transmembrane protein contains four Ig-like domains (one V-type and three C-type), one transmembrane domain and a cytoplasmic tail. The principal LAG-3 ligand is MHC II [279], but other proteins, such as galectin-3, hepatic sinusoid endothelial cell lectin (LSECtin), fibrinogen-like protein1 (FGL1) and α-synuclein have also been described as LAG-3 ligands as well [280].

Mechanistically, unlike CTLA-4, LAG-3 does not compete with CD4 for ligand binding – instead, it uses CD4-MHC II stable contacts as a selection mechanism for inhibition [281]. While some reports have stated that the conserved “KIEELE” sequence within the cytoplasmic tail is indispensable for LAG-3 inhibitory function [282], others have disputed this finding and instead identified two other intracellular motifs (FXXL and a C-terminal EX repeat) as the main responsible for LAG-3-mediated inhibition, the latter of which mediates association to LAG-3-associated protein (LAP) [283, 284]. In a broader context, LAG-3 has been shown to inhibit antigen-activated $CD4^+$ T cells, decreasing proliferation, CD25 expression and cytokine production [285].

CD6 characterization and role in T cell activation

Structural organization

CD6 is a transmembrane glycoprotein with a molecular mass varying between 105 and 130 kDa, depending on alternative splicing events and post-translational modifications [286-290]. The full-length form of human CD6 was first cloned in 1995 [289]. Authors predicted a protein with 668 aa, including a cytoplasmic tail of 244 aa that is 71.5% homologous to that of mouse CD6. Important signaling features were found to be conserved between human and mouse CD6, including nine tyrosine residues, two proline-rich motifs and 22 serine residues. Additionally, human CD6 further includes ten casein kinase 2 (CK2) and three PKC phosphorylation motifs. CD6 homologue in rat was later cloned in 2003. Comparison of the cytoplasmic domains of rat, mouse and human showed that all tyrosine residues, all PKC phosphorylation sites, five CK2 phosphorylation sites and two proline-rich regions are conserved among them [291], underlining the potential relevance of these motifs for CD6-mediated signaling.

Both serine and threonine residues in CD6 are phosphorylated in a resting state. Tyrosine residues, on the other hand, only become phosphorylated upon stimulation, while serine residues become hyperphosphorylated [292]. Two particular clusters of serine residues, located within CK2 phosphorylation motifs, have been identified to be constitutively phosphorylated (S480/S482/S484 and S560/S562/S565/S567/S568), a process likely mediated by CK2, considering their localization [293].

While no full structure has yet been obtained experimentally for CD6, the structure of its extracellular domains has been resolved in 2015, showing that CD6 three extracellular scavenger receptor cysteine-rich (SRCR) domains are arranged in an angular fashion [294]. The same study also observed that two particular CD6 single nucleotide polymorphisms (SNPs) that had been previously associated to susceptibility to multiple sclerosis (R225W and A257V) [295] are located in SRCR domain 2 (d2) and have the potential to disrupt protein stability which, authors suggest, may explain the link between reduced CD6 expression and multiple sclerosis [294]. Another CD6 SNP – S351N – introduces a glycosylation site in SRCR d3 and decreases its affinity for CD166 (CD6 SRCR d3-binding ligand) by 10-fold, potentially by physically hindering the association.

Expression

CD6 is mostly expressed on the surface of peripheral T cells and thymocytes, but expression has also been detected on specific subsets of natural killer cells, B cells, hematopoietic precursor cells and cells from the brain parenchyma [296-299].

Expression of CD6 varies from birth to adulthood, increasing as cells develop from double-negative thymocytes to double-positive thymocytes and to single positive thymocytes. Within single-positive thymocytes, CD6 expression is slightly higher in $CD4^+$ T cells [300]. These variations in expression are consistent with CD6 expression being particularly relevant in the last stages of thymopoiesis, which is supported by the fact that CD6-KO mice display a decreased percentage of $CD4^+$ and mature $CD8^+$ T cells, while not having significant differences in other stages of development. This result indicates that, in the absence of CD6, negative selection is enhanced [297].

The *CD6* gene is located in chromosome 11 (11q13), in close proximity to the *CD5* gene, and spans 13 exons [286]. The physical proximity of these genes suggests that CD5 and CD6, two proteins similar in many aspects, resulted from gene duplication [301]. While the first six *CD6* exons code for the extracellular portion of the protein, the seventh codes for the transmembrane region, and the remaining six for the intracellular tail. Each SRCR domain (1-3) is coded by a separate exon (3-5, respectively).

CD6 can be alternatively spliced in different regions of the sequence. At least five different *CD6* mRNAs have been identified that arise from alternative splicing of the exons encoding the intracellular region (CD6a-e) in human cells. Analysis of the signaling potential of these isoforms showed that the last two tyrosine residues (Y629 and Y662) of CD6 are critical for phosphorylation of the protein upon CD6/CD3-mediated T stimulation, suggesting a critical role for them in CD6-originated signaling cascades [286, 302].

Another CD6 isoform has also been described where, upon splicing of exon 5, CD6 lacks SRCR d3 – the domain that mediates CD6 interaction with CD166 [287, 303]. This isoform, termed CD6Δdomain3 (CD6Δd3), is generally either co-expressed with full length CD6 in resting lymphocytes, or not expressed at all. Still, in some cases it may be the only extracellular isoform expressed. Strikingly, expression of CD6Δd3 increases in T cells activated via the TCR, and this is often accompanied by a parallel decrease in the expression of full length CD6 [287].

Besides the different isoforms of CD6 found in T cells, CD6 can also occur in a circulating soluble form (sCD6). Its levels are increased in patients with Sjögren's syndrome, and even further increased in the presence of additional immunological challenges [304]. Another study demonstrated that, in patients with systemic inflammatory response syndrome, elevated levels of sCD6 were associated with a higher mortality risk [305].

Secretion of sCD6 likely results, at least partially, from activation-induced proteolytic cleavage of full-length CD6. This hypothesis is supported by western blot analysis of T cell supernatants once these show that, upon cell activation, one band is detected at the same molecular weight of recombinant soluble CD6 (rsCD6, corresponding to its ectodomains) [306]

Ligand interactions

To this date, two different ligands have been identified for CD6 (figure 6). The first one, activated leukocyte cell adhesion molecule (ALCAM/CD166), was identified and cloned in 1995 [307]. CD166 is a 100 kDa glycoprotein [308] belonging to the immunoglobulin superfamily, and it contains five extracellular Ig-like domains. CD166 is expressed on the surface of different cell types, such as monocytes, lymphocytes, mesenchymal stem cells, phytohemagglutinin (PHA)-activated peripheral blood mononuclear cell (PBMCs), epithelial cells from different tissues (like breast, skin and thymus), hepatocytes and fibroblasts [307-309]. Interestingly, CD166 was also detected, via immunoreactivity assays, in neurons [308]. The presence of both CD6 and CD166 in the brain suggested very early on a potential role for this interaction in the nervous system. This observation is particularly curious when reconciling the near-exclusive expression of CD6 on lymphocytes, the immune-privileged status of the brain, and the established association between CD6 SNPs and multiple sclerosis.

CD166 binds CD6 via CD6 SRCR d3 [303, 310]. A detailed structural analysis revealed that, within CD6 SRCR d3, the residues critical for binding CD166 are D291, S292, E293, Y295, E298, R314, Y327, S329, N346, N348, L349, Q352 and S353. These residues were used to outline the precise region interfacing CD166, which was found to be highly conserved between human, mouse and rat [294].

CD6-CD166 binding partially mediates T cell-thymic epithelial cell adhesion [307]. Blocking this binding using either rsCD6 or recombinant soluble CD166 protein (rsCD166) decreases IL-2 secretion by activated T cells [311]. Moreover, addition of either recombinant soluble CD6-Fc or ALCAM-Fc reduces the proliferation of peripheral blood lymphocytes (PBLs) activated by dendritic cells [312].

The second ligand for CD6 was only recently identified: CUB domain-containing protein 1 (CDCP1), also known as CD318. This 130-kDa glycoprotein has three extracellular complement C1r/C1s, Uegf, Bmp1 (CUB) domains and, unlike CD166, CD318 binds CD6 via CD6 d1 [299]. This protein is also expressed on the membrane of different cell types, like epithelial cells (from lung, thymic and breast origin), chondrocytes and fibroblasts.

CD318 is involved in the adhesion of T cells to stimulated synovial cells, and elevated levels of soluble CD318 were also detected in synovial fluids from patients with rheumatoid arthritis (RA) and juvenile inflammatory arthritis [299, 313]. In concentration gradients similar to those observed in RA (serum/synovial fluid), CD318 was shown to be a chemoattractant to T cells [299].

Although most studies so far on the role of CD318 are focused on cancer progression [314], the same study that identified the molecule as a CD6 ligand demonstrated that CD318-KO mice, much like CD6-KO mice, were protected in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, evidenced by lower clinical scores and lower IL-17 and IFNɣ secretion by splenocytes in recall assays [299]

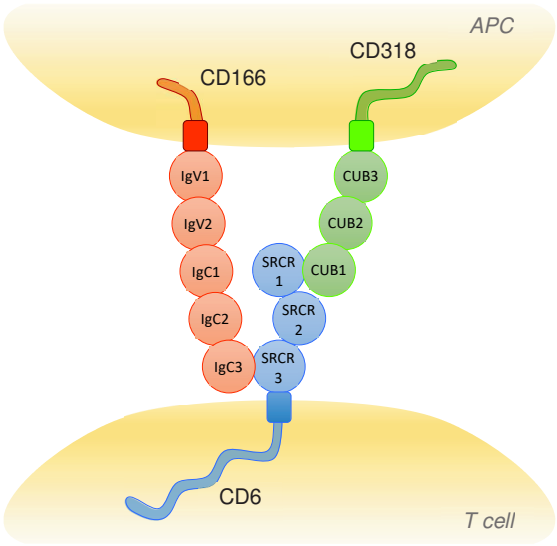

Figure 6. **CD6 and its ligands**. CD6 is expressed mostly on the membrane of T cells, and it encompasses three extracellular scavenger receptor cysteine-rich (SRCR) domains, a transmembrane (TM) domain, and a long intracellular domain. To this day, two CD6 ligands have been identified: CD166 and CD318. CD166 is a transmembrane protein expressed by different cell types. Besides a TM domain and a cytoplasmic tail, CD166 has five extracellular immunoglobulin-like domains – two membrane-proximal V-type domains and three C-type domains, the most distal of which binds CD6 SRCR d3. CD318, on the other hand, binds CD6 via SRCR d1. It includes a short cytoplasmic tail, a TM domain and three extracellular CUB domains.

In addition to CD166 and CD318, CD6 also binds bacteria: rsCD6 is able to aggregate both Gram-positive and -negative bacteria via the recognition of LTA and LPS, respectively. This interaction is also relevant when mediated by full-length CD6, once full length CD6 expression in 2G5 Jurkats cells increases LPS-triggered phosphorylation of

MAPKs [315, 316]. The physiological role of the interaction between CD6 and bacteria has also been shown in animal models. Specifically, rsCD6 administration increases survival in a mouse model of sepsis induced by *Staphylococcus aureus*, while decreasing secretion of pro-inflammatory cytokines (like IL-1β) in mouse splenocytes treated with LTA and peptidoglycan (PGN) [315]. Accordingly, CD6-KO mice presented decreased survival rates when subject to two different models of bacterial sepsis induced by either cecal ligation and puncture or *Escherichia coli* infection [317]. Taken together, these data support a protective role for CD6 against bacterial infections.

CD6 is additionally able to bind to some fungal species, namely *Schizosaccharomyces pombe* [318].

Function

The first studies on the role of CD6 using recombinant proteins pointed to a stimulatory function. Antigen-mediated stimulation of human PBLs in the presence of chimeric CD6 protein (or CD6 SRCR d3) resulted in decreased cell proliferation and IL-2 production [311], which suggested that CD6 potentiates T cells activation (via SRCR d3-mediated interactions). Similar results were obtained by another group, who showed that pre-treatment of PBLs with rsCD6 diminished both CD3- and PHA-induced proliferation [319]. Moreover, pre-incubation of Raji B cells with rsCD6 diminished the number of mature synapses formed with Jurkat T cells [319].

Concomitantly, other studies used anti-CD6 antibodies to understand its role on the progression of activation. Proliferation and phosphorylation of intracellular substrates of purified human T cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin were both increased when cells were simultaneously treated with IOR-T1 (a CD6 antibody) and rabbit anti-mouse (RAM)-Ig. Furthermore, IOR-T1+RAM-Ig alone trigger the release of intracellular calcium from storage and induce IL-2 mRNA expression. Importantly, all of these readouts were inhibited by herbimycin, a SRC-family protein tyrosine kinase inhibitor [320]. A different study further showed that, in human PBLs, CD6 crosslinking enhanced both proliferation and NFAT activation induced by CD3 antibodies to a similar extent as CD3 and CD28 co-crosslinking [312]. Additionally, cell proliferation was inhibited when human PBLs and DCs were co-cultured with antibodies against CD6 or ALCAM, or the recombinant soluble form of those proteins [312]. Altogether, these data seemed to generally position CD6 as a co-stimulatory-protein.

This notion was first challenged in 2012 by Oliveira *et al.* in a report that clearly demonstrated the inhibitory nature of CD6-mediated signaling in activated T cells [321]. In that report, CD6 expression was knocked down in primary human T cells using specific

morpholinos. Posteriorly, $CD6^+$ and $CD6^-$ T cells were activated with anti-CD3 antibodies (OKT3 clone), and the levels of intracellular calcium released were measured. This experiment demonstrated that calcium levels in $CD6^-$ cells were much higher than in $CD6^+$ cells. In a parallel experiment, using a human T cell line with residual CD6 expression (E6.1 Jurkat T cells), authors further showed that this effect was entirely dependent on CD6 cytoplasmic tail, as T cells expressing a truncated version of CD6 were activated to the same extent as untransfected cells [321]. The inhibitory effect of CD6 expression was also observed in later readouts of T cell activation. Specifically, $CD6^+$ cells produced significantly lower levels of IL-2, compared with untransfected cells after 24 h activation with anti-CD3 antibodies. Accordingly, when the interaction between human PBLs and APCs (Raji B cells) was blocked with an anti-ALCAM antibody, proliferation was increased. Blocking this interaction with anti-CD6 antibodies had the opposite effect, which authors attribute to a different effect than ligand binding, activating CD6 restraining properties instead [321].

The emergence of CD6-KO mouse models came to confirm these observations. The first model was generated in 2016 by the laboratory of Francisco Lozano. Cells from different developmental stages were isolated from normal and CD6-KO mice, and triggering using anti-CD3 monoclonal antibodies (mAbs) resulted in increased intracellular calcium levels in DP thymocytes from CD6-KO mice, compared with wild-type mice [297].

A second KO model for the CD6 protein came shortly after, generated to study the involvement of CD6 in the progression of multiple sclerosis. This pathology was mimicked in mice by immunization with MOG_{79-98} (myelin oligodendrocyte glycoprotein) peptide and CFA (Complete Freund's Adjuvant). Antigen-specific recall assays performed in splenocytes from immunized animals showed that $CD6^+$ T cells secreted lower amounts of both IFNɣ and IL-17. Additionally, mature $CD4^+$ T cells were isolated and stimulated for 5 h with anti-CD3 and anti-CD28 antibodies, and flow cytometry analysis was used to show that the percentage of either $CD25^+$ or $CD69^+$ T cells was lower in activated normal cells, when comparing to cells from CD6 KO mice [322].

Despite growing evidence in the last decade that CD6 conveys inhibitory signaling during T cell activation, this is not yet an established precept. In fact, CD6 is still broadly described as a co-stimulatory receptor and, even within the scientific community studying CD6 signaling mechanisms, there is no consensus regarding its function during lymphocyte triggering.

Signaling partners and signaling axes

CD6 has no intrinsic enzymatic activity. Still, as previously detailed, its cytoplasmic portion entails a number of aa and motifs that constitute potential anchoring sites for effector

proteins. Specifically, besides CK2 and PKC phosphorylation sites, proline-rich regions can bind SH3-domain containing proteins, while phosphorylated tyrosine residues can bind SH2 domain-containing proteins. CD6 becomes phosphorylated on its tyrosine residues in T cells stimulated with anti-CD3 antibodies, an effect augmented by anti-CD4 or anti-CD2 antibody co-crosslinking [292]. While both serine and threonine residues are phosphorylated in a resting state, tyrosine residues only become phosphorylated upon stimulation [292]. The observations showing that CD6 is post-translationally modified upon activation suggest that those modifications might mediate activation-dependent interactions.

Therefore, uncovering its intracellular interactions is key to understanding which signaling pathways underlie the effect of CD6 expression on the progression of T cell activation. The signalosome of CD6 began to be uncovered in 2003 (figure 7), when Castro and colleagues demonstrated that CD6 co-precipitated CD5, LCK, FYN, ZAP70 and ITK in a rat T cell line [291]. The association between CD6 and LCK/FYN emerged as a potential mechanistic explanation as to why herbimycin inhibited the co-stimulatory effects of CD6 mAbs treatment on TPA-treated T cells, namely on proliferation, tyrosine phosphorylation and IL-2 mRNA production [320]. Additionally, the highly phosphorylated fraction of CD5 associated to CD6, along with the aforementioned kinases, further suggesting a role for CD6 in CD5 [291]. Likewise, CD5 was demonstrated to associate with CD6 in HUT-78 T cells and primary thymocytes, showing this interaction is also important in human T cells [323]

The idea that CD6 could play a role in the early stages of T cell triggering was reinforced by the observation that CD6 accumulates at the synaptic region formed between APCs and T lymphocytes (primary or immortalized), co-localizing with CD3 at the central supramolecular activation cluster (cSMAC) in Jurkat-Raji conjugates [319, 323]. Accordingly, CD6 was shown to co-precipitate CD3 in both HUT-78 T cells and primary human cells, strengthening the concept that CD6 (directly or indirectly) interacts with the TCR/CD3, taking part in the membrane-proximal signalosome formed upon synapse formation. While earlier studies indicated that CD6 translocation to the IS was dependent on CD166 engagement [321], recent data from our laboratory indicate that it is instead the cytoplasmic region of CD6 that mediates this re-distribution (unpublished data).

CD6 has also been shown to be involved in the regulation of the MAPK pathway, as CD6 crosslinking with monoclonal antibodies is enough to increase MAPK phosphorylation in both T cell lines and PBMCs. Moreover, in PBMCs this effect is mimicked by cell incubation with Fc-ALCAM, and synergistically augmented by CD3 co-crosslinking. The impact of CD6 crosslinking in MAPK phosphorylation is dependent on CD6 cytoplasmic tail, as well as SRC kinases [324]. This particular signaling cascade has been demonstrated to

be regulated by two constitutively phosphorylated serine clusters (likely by CK2) in CD6 cytoplasmic tail (S480/482/484 and S560/562/565/567/568) [293]. Still, the effector protein connecting CD6 to the MAPK canonical pathway remains undescribed to date.

Importantly, a potential link between CD6 and the cytoskeleton has also been suggested when CD6 was shown to co-precipitate the PDZ domain-containing protein syntenin-1 that, among other functions, works as an adaptor for cytoskeletal proteins [325].

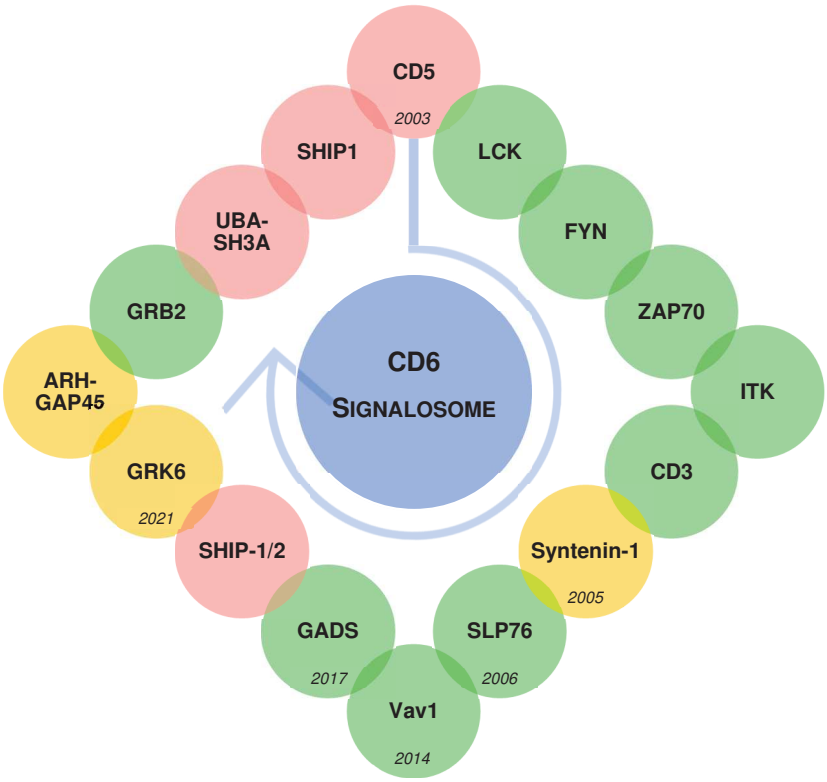

Figure 7. **CD6 intracellular signalosome**. Composition of CD6 intracellular network, ordered by date of discovery. Green circles enclose proteins that stimulate the progression of T cell activation, red circles are for inhibitory proteins, and yellow circles imply proteins with a less straightforward role. Although the first proteins know to associate to CD6 were mostly of stimulatory nature, subsequent description of CD6 signalosome has uncovered an assembly of proteins with diverse functions, hinting at a complex and highly regulated role for CD6.

The first CD6 inducible association described was with SLP76, an adaptor protein that binds CD6 Y662, the most C-terminal CD6 tyrosine residue, when it is phosphorylated [326]. Afterward, GADS was also shown to bind phosphorylated CD6 Y629 [327]. A joint binding model has been proposed suggesting that GADS SH3 domain, bound to SLP76 proline-rich region, is recruited to CD6 Y629, at the same time that SLP76 is recruited to Y662. This implies that, while there is tyrosine-enzyme specificity, both tyrosine residues

are equally relevant for the association of SLP76 and GADS, once they are recruited to CD6 cytoplasmic tail as a single unit [327].

There are nine tyrosine residues on the cytoplasmic tail of CD6, along with other potential docking sites and signaling motifs. There is no study to date on how many or what specific CD6 tyrosine residues are phosphorylated upon lymphocyte activation, and it is also still unknown whether different kinases phosphorylate different tyrosine residues. In fact, SLP76 and GADS remain the only SH2-mediated CD6 associations described so far, and the only interactions mapped to a specific residue.

While only a few CD6 protein interactions were described in the first decade of 2000, many insights were put forward in the ensuing years, providing an increasingly focused outlook on the role of CD6 in T cell activation [328]. The positioning of CD6 as a LAT-equivalent adaptor came in 2014, when Bernard Malissen's laboratory generated genetically modified mice expressing tagged forms of LAT, ZAP70 and SLP76. This experimental approach, followed by the use of affinity-purification coupled with mass spectrometry (AP-MS), allowed them to identify high-confidence interactions for each of these proteins and assemble their respective signalosomes. After initially determining that CD6 belonged to the signalosomes of both SLP76 and ZAP70, authors demonstrated that ZAP70, unlike LAT, was necessary for both CD6 phosphorylation and CD6-SLP76 association upon T cell activation. Moreover, in the absence of LAT, CD6 crosslinking was enough to induce SLP76 and MAPK phosphorylation, although TCR-mediated transcriptional changes in LAT-deprived T cells were dramatically reduced. They also observe that blocking CD6-ALCAM interaction in the absence of LAT precludes formation of T cell-B cell conjugates. All these data prove that there is a number of signaling events mediated by CD6 taking place in T cells in a LAT-independent manner [328].

This view was furthered by a separate study from the same group where the importance of high confidence interactions established downstream of TCR-triggering was determined and compared based on its prevalence and stoichiometry. This analysis demonstrated that the interactions CD6-Vav1 or CD6-SLP76 were quantitatively comparable to those between LAT and SLP76, inositol polyphosphate-5-phosphatase D (INPP5D/SHIP-1) or thymocyte selection associated (THEMIS). This observation strengthens the position of CD6 as a transmembrane adaptor that, similarly to LAT, is crucial for signal branching downstream of the TCR [329].

A similar approach was again put to use in 2021, to uncover the signalosome of CD5 and CD6 [330]. These two proteins have been considered in the past to be similar in many ways: they belong to the same superfamily, are structurally identical and share a number of interactors. Interestingly, both CD5 and CD6 were initially regarded as co-

stimulatory proteins, and only through the generation of KO animals have researchers demonstrated that their expression, *per se*, was inhibitory.

However, when analyzing the clusters of proteins assembled around CD5 and CD6, proteins belonging to the high confidence interaction network of CD5 were shown to be generally of inhibitory nature, like Cbl proto-oncogene B (CBLB) and ubiquitin associated and SH3 domain containing A (UBASH3A) [331, 332]. On the other hand, the signalosome of CD6 includes proteins with both stimulatory and inhibitory functions. Upon T cell activation, the CD6 interactome includes the enzymes ZAP70 and Vav1, as well as the adaptors SLP76, GRB2 and GADS. Nevertheless, UBASH3A and SHIP-1 are also associated (directly or indirectly) with CD6 upon T cell triggering [330]. This divergence between CD5 and CD6 signalosome appears to indicate that, while CD5 can be generally classified as an inhibitory protein, CD6 likely has a more nuanced role, which potentially explains why, in different physiological or pathological contexts, CD6 plays a different role depending on the activation status, the nature of the trigger or even the timepoint.

Role in disease

The relevance of CD6-mediated signaling has been underlined by the surge of CD6-KO mice. These animals were shown to be more susceptible to collagen-induced arthritis (CIA), an established model of RA, as evidenced by their higher disease incidence and clinical score [297]. A separate report, however, has described the opposite phenotype, showing CD6-KO animals have less exacerbated clinical scores in a CIA setting [333]. CD6-KO mice have further been described to be more susceptible to models of bacterial sepsis, as they display increased immunological responses and bacteria loads and decreased survival [317], and of lupus-like disease, where these animals display higher levels of both nucleosome and dsDNA autoantibodies [334]. On the other hand, absence of CD6 expression was shown to be protective in the EAE mouse model of multiple sclerosis, with CD6-KO animals having lower clinical scores, T_h1- and T_h17-specific cytokine production and T cell infiltration in the spinal cord [322]. Interestingly, CD318-KO mice presented a similar phenotype, being protected from EAE development as assessed by the same parameters [299], while CD166-KO mice have been reported to have a more severe disease phenotype, compared with their healthy counterparts [335]. Lack of CD6 expression was shown to be protective also in experimental autoimmune uveitis, an animal model for autoimmune uveitis [336].

Although these recent reports highlight the role of CD6 in the progression of inflammatory diseases, the protein has been a therapeutic target for many years, with demonstrated positive results. Itolizumab, a CD6 monoclonal antibody that binds CD6

SRCR d1, has been approved since 2013 for treatment of severe plaque psoriasis in India [337, 338], where it has further been re-purposed and approved to treat cytokine release syndrome in patients with COVID-19 [339]. These overall consistent results are in line with the ability of Itolizumab to decrease cytokine production, T cell differentiation, activation and proliferation [340, 341]. Treatment with itolizumab has also been shown to result in clinical improvements for patients with RA [342], and clinical trials are ongoing for use in graft versus host disease (GVHD), uncontrolled asthma and lupus nephritis [343]. The predominant role of CD6 in both physiological and pathological settings underlines the importance of uncovering its signaling properties and action mechanisms.

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Goals

CD6 is an inhibitory transmembrane receptor expressed mostly by T lymphocytes and thymocytes. Although initial reports on its function pointed to a co-stimulatory role, in the last decade CD6 has been shown to downmodulate T cell activation upon TCR triggering. Nevertheless, the nature of CD6-mediated signaling, *i.e.* the net effect of its expression and engagement on stimulated T cells, is still controversial. This controversy is furthered by the complex nature of CD6 signalosome (encompassing both co-stimulatory and inhibitory proteins) and our sparse knowledge of its mechanism of action. Identifying and dissecting the pathways downstream of CD6 is particularly important in the present moment taking into consideration the growing utilization of itolizumab, a monoclonal antibody that binds CD6, to treat inflammatory diseases.

Bearing in mind this information, the goals of the present thesis are:

- Identify new interactions established by CD6 upon T cell activation with inhibitory enzymes;
- Dissect the signaling pathways involved in CD6-mediated inhibition of T cell activation downstream of newly identified interactors;
- Assess the signaling potential of individual CD6 intracellular tyrosine residues;
- Based on our findings and on previous observations, explore the potential role of CD6 in the regulation of other signaling axes than those directly triggered by its intracellular effectors.

Results

Chapter 1: CD6-mediated inhibition of T cell activation via modulation of Ras

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CD6-mediated inhibition of T cell activation via modulation of Ras

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Abstract

Background: CD6 is one of many cell surface receptors known to regulate signal transduction upon T cell activation. However, whether CD6 mediates costimulatory or inhibitory signals is controversial. When T cells engage with antigen presenting cells (APCs), CD6 interacts with its ligand CD166 at the cell–cell interface while the cytosolic tail assembles a complex signalosome composed of adaptors and effector enzymes, that may either trigger activating signaling cascades, or instead modulate the intensity of signaling. Except for a few cytosolic adaptors that connect different components of the CD6 signalosome, very little is known about the mechanistic effects of the cytosolic effectors that bind CD6.

Methods: Jurkat model T cells were transfected to express wild-type (WT) CD6, or a cytoplasmic truncation, signaling-disabled mutant, CD6Δcyt. The two resulting cell lines were directly activated by superantigen (sAg)-loaded Raji cells, used as APCs, to assess the net signaling function of CD6. The Jurkat cell lines were further adapted to express a FRET-based unimolecular HRas biosensor that reported the activity of this crucial GTPase at the immunological synapse.

Results: We show that deletion of the cytosolic tail of CD6 enhances T-cell responses, indicating that CD6 restrains T-cell activation. One component of the CD6-associated inhibitory apparatus was found to be the GTPase activating protein of Ras (RasGAP), that we show to associate with CD6 in a phosphorylation-dependent manner. The FRET HRas biosensor that we developed was demonstrated to be functional and reporting the activation of the T cell lines. This allowed to determine that the presence of the cytosolic tail of CD6 results in the down-regulation of HRas activity at the immunological synapse, implicating this fundamental GTPase as one of the targets inhibited by CD6.

Conclusions: This study provides the first description of a mechanistic sequence of events underlying the CD6-mediated inhibition of T-cell activation, involving the modulation of the MAPK pathway at several steps, starting with the coupling of RasGAP to the CD6 signalosome, the repression of the activity of Ras, and culminating in the reduction of ERK1/2 phosphorylation and of the expression of the T-cell activation markers CD69 and IL-2R α chain.

Keywords: Cell surface molecules, Inhibitory receptors, Signaling transduction, Immunological synapse, GTPases, FRET

Introduction

CD6 is a type I transmembrane glycoprotein expressed mainly by peripheral T lymphocytes, medullary thymocytes and B1a lymphocytes [1–3]. It remains controversial whether CD6 is a co-stimulatory or inhibitory receptor as, depending on the particular cellular [4–8] or animal [9–11] model, CD6 has been reported to either activate or repress T-cell responses. It may well be that,

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contingent on the cell subset where it is expressed and the context or phase of the immunological response, CD6 would behave as a dual-function molecule [12].

Upon T cell receptor (TCR) triggering, tyrosine residues of the cytosolic tail of CD6 are rapidly phosphorylated and become putative docking sites for specific SH2 domain-containing enzymes and adaptors [13]. These reactions allow CD6 to assemble a large signalosome that may include both positive as well as negative [14–18] regulators of T-cell activation, and also intracellular adaptors such as SLP-76 and TSAD [19, 20]. Although the combination of all these mediators has the potential to balance signaling strength and fine-tune T cell activation [21], it has been shown that in the absence of the CD6 cytosolic tail, T cells or T cell lines respond more vigorously to stimulation [8] suggesting that, mechanistically, the overall effects of CD6 correspond to those of an inhibitory membrane scaffold protein.

CD6 shares with the related T-cell antigen CD5 many genetic, structural, and functional characteristics [21]. Long established as an inhibitory receptor [22], CD5 was suggested to constrain T-cell signaling due to its association with the SH2 domain-containing activation-repressive effectors E3 ubiquitin-protein ligase CBL, Ras GTPase-activating protein 1 (RasGAP), and protein-tyrosine phosphatase SHP-1 [23–25]. Recently, also CD6 was shown to be coupled by SHP-1 upon direct extracellular ligation with its conventional ligand, CD166 [17]. However, the contribution of these effectors towards CD5- or CD6-mediated signaling has only been inferred and not formally demonstrated. Indeed, the actual participation of SHP-1 in CD5 function has recently been challenged, since no differences in CD5-based signaling between thymocytes from SHP-1-deficient mice and thymocytes from SHP-1-positive control mice were observed [26].

A key event in TCR-induced signaling is the activation of the family of small GTPases collectively known as Ras proteins [27, 28]. The most important Ras members are encoded by the genes *HRAS*, *NRAS* and *KRAS* [29, 30], and the fundamental biological role of this family of GTPases is highlighted by the fact that mutations in RAS genes are found in over 25% of all human cancers [31, 32]. Ras proteins share 90% identity in the first 165 aa, which are followed by a hypervariable region (HVR) of 20 aa (19 aa in KRas2B, the predominant of two alternative-splicing generated isoforms of the *KRAS* gene) and end with a conserved C-terminal CAAX box (in which C = cysteine, A = aliphatic, and X = any but commonly serine or methionine). Although the different Ras proteins and isoforms display subtle differences in their subcellular localization [33] and traffic between different endomembrane compartments [34, 35], they can all respond to mAb-mediated TCR triggering, albeit with different sensitivities, and seem capable of colocalizing with the TCR/CD3 complex at the plasma membrane (PM) of T cells or T cell lines [36, 37]. It remains disputed, however, whether Ras proteins are preferentially activated at the PM or on the Golgi apparatus [36–39].

Ras proteins cycle between GTP-bound active and GDP-bound inactive forms, and are activated upon TCR triggering through the coincident action of specific guanine nucleotide exchange factors (GEFs), such as SOS-1 and RasGRP1 [40, 41], and the repression or exclusion of GTPase-activating proteins (GAPs), such as RasGAP and neurofibromin which accelerate the hydrolysis of Ras-bound GTP [27, 42]. The best characterized Ras effector is the serine/threonine-protein kinase Raf-1 [43, 44], but activation of Ras feeds into multiple signaling pathways that promote the activation of transcription factors such as AP-1 and NFAT [45–47], and the upregulation of genes encoding critical factors for T-cell proliferation and effector functions, including the T cell-growth factor IL-2, the α chain (CD25) of the high-affinity IL-2 receptor, and the early activation marker CD69 [48–51].

We investigated the interaction between CD6 and putative downstream signaling effectors and show in this study that CD6 interacts at the cytosolic level with RasGAP in a phosphorylation-dependent manner and, importantly, that deletion of the cytosolic tail of CD6 results in an increase in the GTPase activity of HRas at the immunological synapse (IS), as assessed using a novel HRas biosensor based on Förster resonance energy transfer (FRET). This study thus constitutes the first clear evidence for a molecular mechanism underlying the inhibitory function of CD6, highlighting the critical role of this receptor in modulating the strength of signals originating at the immediate steps after TCR triggering, and in regulating T cell activation overall.

Material and methods

Cell lines and cell culture

Jurkat E6.1 and Raji cells were cultured in RPMI-1640 (Cytiva) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin–streptomycin and 1% (v/v) sodium pyruvate (all from Thermo Fisher Scientific). HEK293T cells were cultured in DMEM (Cytiva), supplemented as RPMI. Cell cultures were kept in a controlled incubator (BINDER) at 37 °C and 5% CO_2.

CD6 constructs and expression in E6.1 cells

CD6-encoding sequences were amplified by PCR from pEGFP-N1/CD6FL [52] and corrected to coincide with the GenBank accession no. U66142.1 by point mutating the appropriate single residues. The sequence encoding the cytosolic deletion mutant CD6Δcyt includes a

stop codon to substitute for K429, and still maintains 5 aa of the cytosolic tail-sequence for proper membrane attachment and stability. cDNA sequences were cloned via *Bam*HI/*Mlu*I and *Not*I restriction sites into the lentiviral expression vector pHR, which was then transfected into HEK293T cells along with p8.91 and pMDG for lentiviral production, as described elsewhere [53]. After 72 h, the medium was collected, filtered and used to stably transduce E6.1 cells. All transfected cell lines were checked for CD6 expression level homogeneity by flow cytometry and, when needed, cell sorting was performed based on CD6 and CD3 expression.

For flow cytometry analyses, 0.5×10^6 parental or transfected E6.1 cells were collected and washed twice in cold flow cytometry buffer [0.2% (w/v) BSA (NZYTech), 0.1% (v/v) sodium azide (Merck) in PBS (Thermo Fisher Scientific)]. Cells were then incubated with FITC-conjugated CD6 mAb (BL-CD6, BioLegend) for 30 min at 4 °C. After washes, cells were filtered with 100 µm nylon filters (Corning Life Sciences) into FACS tubes. Acquisition was performed on a BD Accuri™ C6 Flow Cytometer (BD Biosciences) and analyzed using the FlowJo™ v10 Software (BD Biosciences).

Activation of E6.1 cells

Superantigen-mediated T cell activation was performed as previously described [54], using 5×10^4 Raji cells preloaded with 1 µg/ml staphylococcal enterotoxin E (SEE, Toxin Technology) co-cultured with 5×10^4 CD6 wild-type (WT)- or CD6Δcyt-expressing E6.1 cells, for 24 h at 37 °C. In parallel, E6.1 cells were also co-cultured with unloaded Raji cells, or RPMI alone. Cells were then stained with APC-conjugated CD69 (FN-50) or CD25 (BC96) mAbs (all from BioLegend), and cell activation was measured by flow cytometry. Raji cells were gated out using PeCy7-conjugated CD19 mAb (HIB19, Thermo Fisher Scientific).

E6.1 cell activation using pervanadate was performed as described [55], using 50×10^6 cells. Cells were activated for 5 min at 37 °C, pelleted for 30 s, the supernatants discarded, and then cells were lysed for 30 min on ice in 500 µl of lysis buffer [1% (v/v) Triton X-100, 10 mM Tris·Cl pH 7.4 (NZYTech), 150 mM NaCl (NZYTech), 1 mM EDTA (Merck), 1 mM PMSF (Merck), 1 mM orthovanadate (Sigma)]. Lysates were centrifuged for 10 min at 11,500 × *g* at 4 °C and the supernatants were recovered for further analysis. 50 µl of lysate were mixed with 50 µl 2 × Laemmli buffer (Bio-Rad), with or without β-mercaptoethanol, and denatured for 5 min at 95 °C, to be run in SDS-PAGE. The remaining 450 µl of lysate were pre-cleared with 50 µl of Protein A Sepharose® CL-4B reconstituted beads (Cytiva) for 45 min at 4 °C by end-over-end rotation. Beads were pulled down by centrifugation at 500 × *g* for 1 min, the lysates recovered and then used for immunoprecipitation with 100 µl of Protein A Sepharose beads pre-packed with 1 µg of CD6 mAb (MEM98, EXBIO). Immunoprecipitates were washed three times with 500 µl lysis buffer. Beads were then resuspended in 50 µl of 2 × Laemmli buffer and denatured for 5 min at 95 °C for use in SDS-PAGE.

For E6.1 cell activation using mAbs, 5×10^6 E6.1 cells were harvested, washed with cold PBS, and kept on ice until activation. Cells were simultaneously activated for 5 min at 37 °C with CD3 (UCHT1) and CD28 (CD28.2) mAbs (all from BioLegend), at 3 and 5 µg/ml, respectively. Cells and lysates were then processed as above.

SDS-PAGE and western blotting

Protein samples were separated in polyacrylamide gels (7 to 12%, depending on the target protein) and transferred to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Western blotting (WB) was performed as described previously [56]. Primary antibodies used were anti-CD6 mAb (MEM98, EXBIO), anti-RasGAP mAb (B4F8, Santa Cruz Biotechnology), anti-p21 Ras mAb (Cytoskeleton, Inc), anti-α-tubulin mAb (B-5-1-2, Merck), anti-ERK 2 rabbit polyclonal (C14, Santa Cruz Biotechnology), and Phospho-p44/42 MAPK rabbit polyclonal (Cell Signaling Technology); secondary antibodies used were HRP-conjugated goat anti-mouse IgG (BioLegend), rat anti-mouse IgG (Abcam) for detection of CD6 in MEM98 immunoprecipitates, and mouse anti-rabbit IgG (Santa Cruz Biotechnology). Membranes were developed with Amersham ECL Select/Prime Western Blotting Detection Reagent (Cytiva). Data were acquired using the ChemiDoc XRS+ System (Bio-Rad) and analyzed by Fiji [57].

Construction of the unimolecular HRas/Raf-1 FRET biosensor MRS2

MRS2 was generated by assembling elements from GTPase FRET biosensors previously created and validated. Sequences for HRas (aa 1–172) and Raf-1 (aa 51–131) were retrieved from the described Raichu-Ras sensor (GenBank accession no. AB046925) [58]. The sequence for Raf-1 was modified in three nucleotides to match the GenBank accession no NM_002880.4. The split sequences from HRas [N-terminal, aa 1–172; C-terminal (CT), aa 173–189] included in the construct are according with the GenBank accession no NM_001130442.3. Sequences for Clover, mRuby2, linker 1 (L1), L3 and L4 were retrieved from the pCAGGS-Raichu-RhoA-CR plasmid [59]. pCAGGS-Raichu-RhoA-CR was a gift from Michael Lin (Addgene plasmid #40258; http://n2t.net/addgene:40258; RRID:Addgene_40258). L2 was amplified

by PCR from an optimized version of the Raichu-RhoA probe [60], kindly given by João Relvas (i3S, Porto).

For assembly, two sections of MRS2 were synthesized: from Clover to HRas, and from Raf-1 to HRas-CT. Both fragments and the L2 were assembled using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs Inc.). MRS2-S17N was obtained via site-directed mutagenesis of MRS2 by PCR. Constructs were cloned into the pHR vector via the restriction site *Mlu*I, as described [53]. All plasmids were verified by sequencing.

Cellular distribution of MRS2

1×10^5 MRS2- and MRS2-S17N-expressing E6.1 cells were plated on glass coverslips previously coated with 0.01% poly-L-lysine (Merck) in PBS, for 30 min at 37 °C. After adhesion, the medium was removed and cells were fixed with paraformaldehyde 2% (Electron Microscopy Sciences) in PBS for 10 min, and thoroughly washed. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min and washed. Nuclei were stained with DAPI. Samples were washed and mounted on coverslips using Vectashield mounting medium (Vector Laboratories). Images were acquired in a Zeiss Axio Imager Z1 microscope (Carl Zeiss, Germany) equipped with an Axiocam MR ver3.0 camera (Carl Zeiss) and a Plan-Apochromat 63x/1.40 Oil DIC objective, and controlled through the Axiovision 4.9 software (Carl Zeiss, Germany).

Live cell imaging of immunological synapses

5×10^4 E6.1-MRS2 and E6.1-MRS2-S17N cells were washed twice with RPMI without phenol red (Cytiva), resuspended in the same medium, and plated on poly-L-lysine-coated µ-slides (ibidi) for 30 min at 37 °C. Meanwhile, 1×10^5 Raji cells were incubated with SEE (1 µg/ml) in RPMI for 45 min at 37 °C, and posteriorly washed. Slides containing the biosensor-expressing E6.1 cells were placed under the microscope for imaging and initially filmed for 5 min, at which point acquisition was paused and sAg-loaded Raji cells were carefully added to the plate. Acquisition was resumed and proceeded for an additional 30 min.

Sequential frames were acquired every minute using an inverted motorized Nikon Ti widefield microscope (Nikon) equipped with an Andor iXon888 EMCCD camera (Andor), a PL APO LAMBDA 60x/1,4 Oil DIC objective, a Lumencor SpectraX light source, and controlled through the NIS-Elements AR software (version 5.02). All acquisitions were performed in a controlled environment at 37 °C and 5% CO_2 using an Okolab microscope incubator system (Okolab). To image the Clover fluorophore emission, the lumencor Spectra X cyan filter 470/24 was used for excitation with a GFP/DsRed—bs 493/574, and a 514/30 emission filter. For mRuby2, the lumencor Spectra X green filter 550/15 was used for excitation with a GFP/DsRed—bs 493/574, and a 617/73 emission filter. For the FRET channel, the lumencor Spectra X Cyan filter 470/24 was used for excitation, a GFP/DsRed—bs 493/574, and a 617/73 emission filter. All LEDs were used at 10%. All images from fluorescence channels were acquired with no binning and 100 ms exposure time. Additionally, a brightfield image was acquired with the lamp at 6.5 V and an exposure time of 10 ms.

Results

CD6 modulates CD69 and CD25 upregulation following T cell activation

We set-up an in vitro cellular system in which T-cell activation could be measured as the upregulation of the T-cell responsive antigens CD69 and CD25 in Jurkat E6.1 cells interacting with superantigen (sAg)-loaded Raji cells, here used as antigen presenting cells (APCs). Jurkat cells express only residual levels of endogenous CD6 (Fig. 1a), so they constitute an excellent experimental model in which to express CD6 variants and mutants to assess the function of the different components of the molecule. Expression plasmids encoding the wild-type (WT) CD6 molecule and a cytosolic-truncation mutant (CD6Δcyt) were transduced into E6.1 cells, which then expressed these CD6 forms at equivalent levels (Fig. 1a), and each of these cell lines was subjected to interaction with Raji cells previously incubated, or not, with sAg. As seen in Fig. 1b, c and Additional file 1: Fig. S1, E6.1-CD6WT and E6.1-CD6Δcyt cells were strongly activated only in the presence of Raji cells pre-incubated with sAg. Moreover, E6.1-CD6Δcyt cells were more strongly activated than E6.1-CD6WT cells, as assessed by the increased fraction of cells expressing CD69 and CD25. This indicates that the cytosolic tail of CD6 is responsible for reducing the strength of activation, confirming our previous findings [8].

CD6 interacts with RasGAP in a phosphorylation-dependent manner

Among other potential effector candidates that we tested in preliminary assays, we found that RasGAP interacted with CD6 in a tyrosine-phosphorylation dependent manner (Fig. 2). E6.1-CD6WT cells were activated with pervanadate, a potent and selective inhibitor of phosphotyrosyl-protein phosphatases which allows for the extensive tyrosine phosphorylation of intracellular proteins and is particularly effective in inducing overt T-cell activation [55, 61]. RasGAP was co-precipitated with CD6WT only upon cell activation (Fig. 2, top left). We further established that the interaction between CD6 and

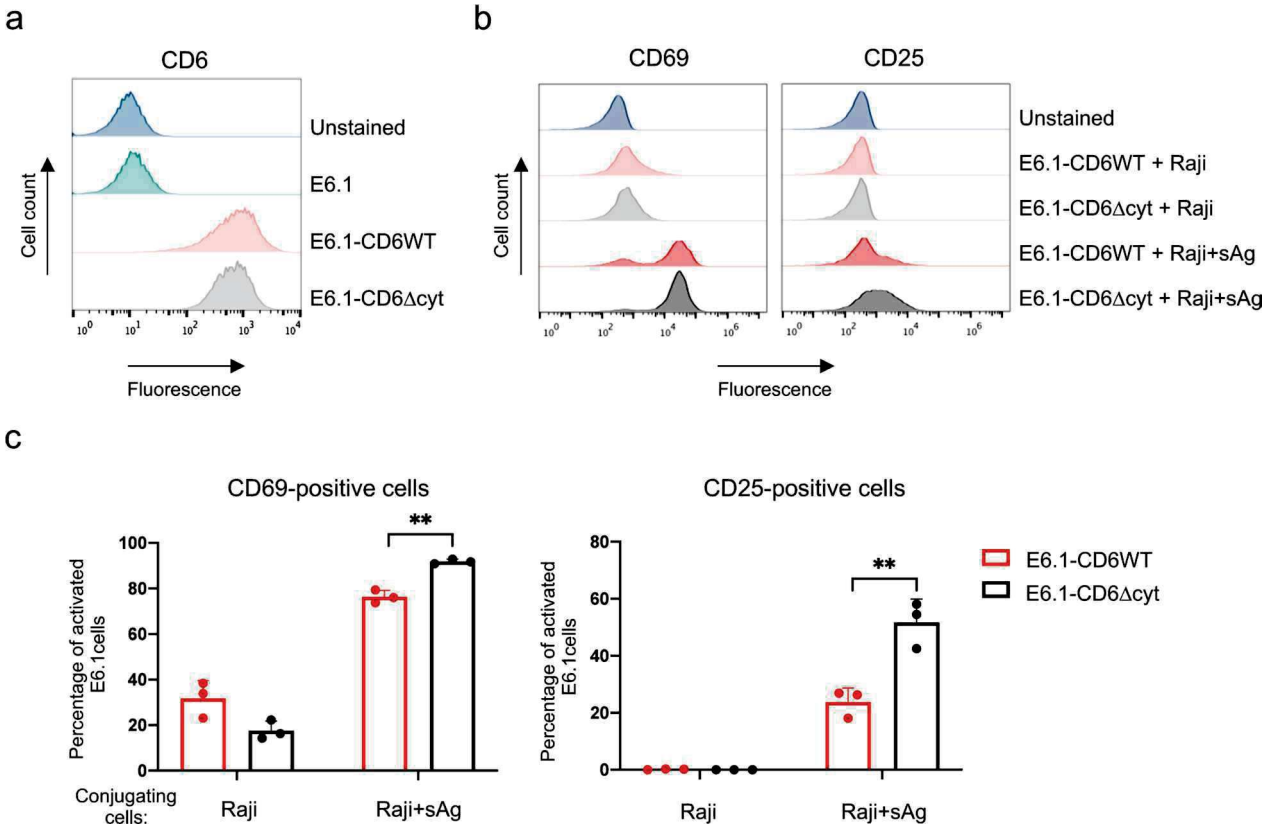

Fig. 1 CD6 inhibits CD69 and CD25 upregulation following T cell activation. **a** E6.1 cells were stably transduced with vectors encoding CD6WT or CD6Δcyt. Cells were stained with fluorochrome-conjugated CD6 mAbs, and protein expression was assessed by flow cytometry. Histograms show the expression of the corresponding CD6 forms in each cell line. **b** E6.1-CD6WT and E6.1-CD6Δcyt cells were cultured for 24 h in the presence of unloaded or sAg-loaded Raji cells, and expression of CD69 and CD25 was then assessed by flow cytometry. Shown are representative histograms of CD69 and CD25 expression of one of three independent experiments. **c** Percentage of CD69-positive (left panels) and CD25-positive (right panels) E6.1-CD6WT or E6.1-CD6ΔDcyt cells cultured with Raji cells without or with sAg. Each dot represents one independent experiment. Bars represent mean and SD for each condition, that were compared using a paired *t* test. **, $p < 0.01$

RasGAP is mediated by the cytosolic tail of CD6 since in E6.1 cells expressing CD6Δcyt, RasGAP was not co-precipitated with the mutant CD6 molecule before or following activation.

Design, expression and activity of the FRET sensor MRS2, which reports on HRas activation upon T-cell stimulation by sAg-loaded APCs

Having established that CD6 recruits the signaling-inhibitory mediator RasGAP upon T-cell activation, and created a cellular system wherein CD6 clearly suppresses T-cell signaling, we next assessed whether Ras could be influenced by CD6 function. We thus created and optimized a biosensor to track Ras and measure changes in its activity in real time using FRET microscopy. The unimolecular FRET reporter we developed, MRS2, was modified from the original "Ras and interacting protein chimeric unit" (Raichu) probes [58] by: (1) replacing the cyan and yellow fluorescent proteins (FP) for the much brighter Clover (green) and mRuby2 (red) FPs [59], which confer greater photostability to FRET reporters, have a wider FRET dynamic range and higher sensitivity to detect ratio changes, and may be less phototoxic to live imaged cells [59, 62, 63]; (2) inserting, based on the studies of Komatsu et al. [64], an extended flexible linker between the sequence of HRas and that of the Ras-binding domain (RBD) of Raf-1, to reduce the basal FRET signal and thereby increase the gain of FRET biosensors; and (3) adding the C-terminal sequence of HRas to target the reporter to the proper subcellular compartments. MRS2 thus comprises, in the following order, the FP Clover, HRas amino acids M1-N172, a 129 aa-long linker, the RBD of Raf-1, the FP mRuby2, and the C-terminal aa P173-S189 of HRas (Fig. 3a). To control for variations in MRS2 output arising from experimental artifacts, namely a predicted accumulation of the probe at the IS resulting in increased FRET that did not result from the activation

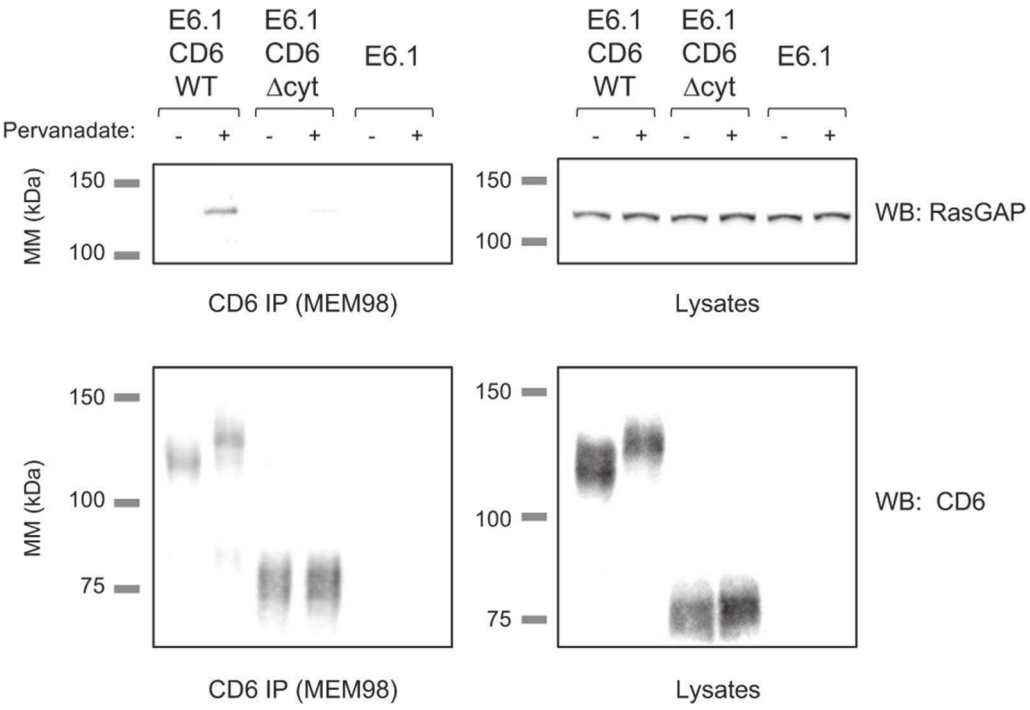

Fig. 2 RasGAP associates with the cytoplasmic tail of CD6 depending on tyrosine phosphorylation. E6.1-CD6WT, E6.1-CD6Δcyt, as well as untransfected E6.1 cells were incubated with pervanadate for 5 min at 37 °C, lysed in Triton X-100 lysis buffer, and CD6 was immunoprecipitated. RasGAP was detected by WB in CD6WT but not in CD6Δcyt immunoprecipitates in activated cells (left panels). WB of cell lysates (right panels) confirms the presence of the indicated molecules at equivalent levels. Images are of one of two independent experiments. MM, molecular mass; IP, immunoprecipitation; WB, western blotting

of Ras (*i.e.*, bystander FRET), we generated a second, inactive, sensor (MRS2-S17N), wherein a point mutation of Ser17 of HRas precludes its activation [65].

E6.1 cells were stably transduced with MRS2- or MRS2-S17N-expressing plasmids and the expression of the full length biosensors was confirmed by WB (Fig. 3b). In addition to the full-length proteins, having a molecular weight (MW) of ~ 120 kDa, other species that were approximately half the size of the probe (58 and 60 kDa) could be detected in the blots, likely resulting from proteolytic cleavage. Transduced cells were imaged by fluorescence microscopy to confirm the proper cellular distribution of the probes, and whereas mRuby2 could only be detected at the plasma membrane, Clover was distributed between the membrane and the cytosol (Fig. 3c). This suggested that a fraction of the proteins can indeed be cleaved and while the N-terminal half may diffuse within the cytoplasm, the C-terminal hemi-probe, because it contains the membrane-associating C-terminal domain of HRas, remains localized to the membrane.

The probes that remain intact should nonetheless be bound to the membrane. To confirm that the integral probe is functional and able to report on the activity of HRas over time, we plated the transduced cells on poly-L-lysine-coated coverslips and filmed them for 35 min under a fluorescence microscope, in the absence of any type of stimulation. The activity of the biosensors was only detected at the cell surface (Additional file 2: Movie S1) and is presented as the ratio between FRET and Clover emissions, quantified at the single cell-level. This ratio at steady-state (basal level) increased slightly over time but remained stably and consistently higher than that of MRS2-S17N (Additional file 1: Fig. S2a). Normalization of data from MRS2 cells to that of the control MRS2-S17N cells demonstrated that the sensor remained stable over the course of the experiment in the unstimulated cells (Fig. 3d).

We next assessed whether MRS2 is able to report on HRas activation and localization during cell-mediated antigen presentation. Biosensor-transduced E6.1 cells were plated and filmed as they interacted with either unloaded or with superantigen (sAg)-loaded Raji cells (Additional file 3: Movie S2). Whereas in the occasional contacts between E6.1 and unloaded Raji cells there was no clear accumulation of either the MRS2 or MRS2-S17N biosensors at the cellular interfaces, in the presence of sAg, Raji cells were stably adhered to by E6.1 cells, and both of the probes accumulated at the IS during the

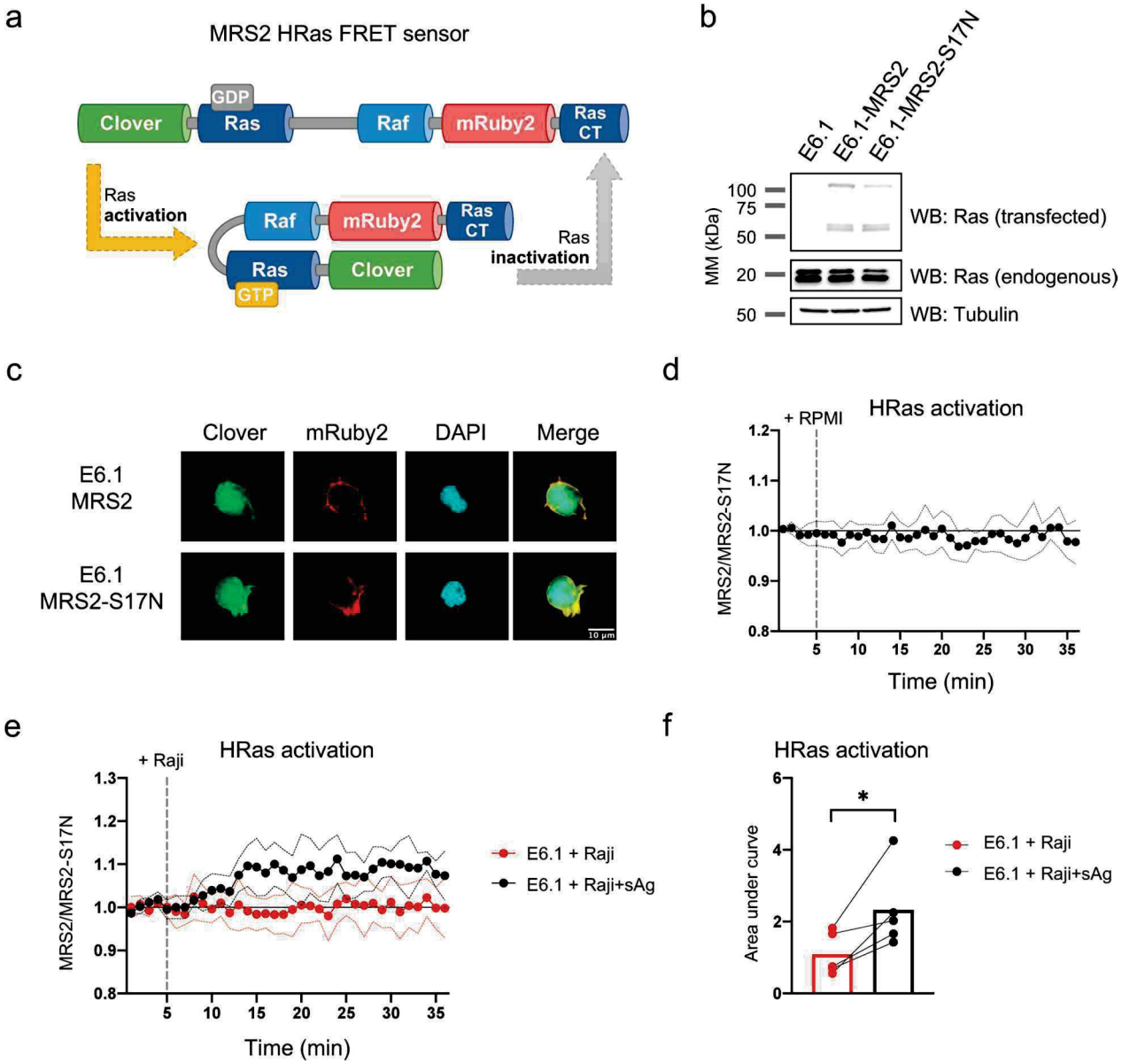

Fig. 3 The FRET biosensor MRS2 reports on HRas activation. **a** Schematic representation of the MRS2 fusion protein and its conformation when HRas is active or inactive. **b** MRS2- and MRS2-S17N-encoding plasmids were stably transduced into E6.1 cells, and the expression of the fusion proteins was assessed by WB, displaying a size of ~ 120 kDa. Protein species of 58 and 60 kDa presumably represent proteolytic cleavage by-products of the full probe. **c** Distribution of MRS2 and MRS2-S17N in E6.1 cells assessed by fluorescence microscopy. **d** MRS2- and MRS2-S17N-expressing E6.1 cells were filmed for 5 min at 37 °C (1 min frames), medium was then added, and cells were filmed for a further 30 min (see Additional file 2: Movie S1). FRET/Clover ratio was assessed for each time-point, and the ratio from MRS2-expressing E6.1 cells was normalized to that of MRS2-S17N-expressing cells. **e** E6.1 cells expressing MRS2 and MRS2-S17N formed conjugates with Raji cells, unloaded or pre-loaded with sAg (see Additional file 3: Movie S2). Raji cells were added at the 5 min mark. FRET/Clover ratios from MRS2-expressing E6.1 cells were divided by those of MRS2-S17N-expressing E6.1 cells for each condition, and normalized for the average of the first three time points to establish the basal activity before the addition of Raji cells. **d**, **e** Dots and lines represent mean ± SD of 5 independent experiments. In each experiment, 10 individual cells (in **d**) or 9–10 cell pairs (in **e**) were assessed for both MRS2 and MRS2-S17N biosensors. **f** Area under the curve of graph in (**e**), with the baseline starting at 1. Each dot represents one of 5 independent experiments, and lines represent paired experiments. 9–10 cells were assessed in all conditions of each individual experiment. *, $p < 0.05$, paired *t* test

period of the interactions. The FRET signals reported by MRS2 (Additional file 1: Fig. S2b) were normalized to the corresponding data from MRS2-S17N cells, revealing that while E6.1 cells interacting with unprimed Raji cells did not exhibit HRas activation, the formation of a synapse between E6.1 cells and sAg-loaded Raji resulted in a sustained elevation of HRas activity (Fig. 3e, f). Therefore, the MRS2 FRET sensor is capable of reporting on the activation and localization of HRas upon T-cell recognition of APC-presented antigen.

The cytosolic tail of CD6 constrains HRas activation and downstream ERK1/2 phosphorylation

The potential regulation of Ras activation by CD6 was then investigated in our cellular system, with E6.1 cells expressing either the MRS2 or the MRS2-S17N biosensors being further transduced with plasmids encoding CD6WT or the cytosolic truncation mutant CD6Δcyt. E6.1-CD6WT-MRS2 and E6.1-CD6Δcyt-MRS2 cells were placed in culture with Raji cells pre-incubated with sAg and imaged during the formation of synaptic contacts for 30 min (Fig. 4a and Additional file 4: Movie S3). As cellular contacts were established and T-cell activation progressed, the MRS2 biosensor concentrated at the synapses for the duration of the assays. Strikingly, the level of HRas activation in E6.1-CD6Δcyt-MRS2 cells was higher than that of the corresponding CD6WT-expressing cells. By normalizing the FRET/Clover ratios by those of the corresponding CD6WT or CD6Δcyt cells expressing the silent MRS2-S17N biosensor (Additional file 1: Fig. S2c), we could calculate that CD6WT-expressing cells exhibited 50% lower activation of HRas upon IS assembly than CD6Δcyt-expressing cells over the course of the 30 min assay (Fig. 4b, c).

We next assessed in E6.1-CD6WT, E6.1-CD6Δcyt and also parental E6.1 cells the levels of phosphorylation of the extracellular signal-related kinases (ERK)-1/2, downstream of Ras in the MAPK pathway. Cells were activated with anti-CD3 and anti-CD28 mAbs, and phosphorylated ERK1/2 was detected by WB (Fig. 5a). As can be seen, the ratio of phosphorylated to total ERK1/2 was significantly lower in E6.1-CD6WT cells than in E6.1 or E6.1-CD6Δcyt cells after activation (Fig. 5b). This indicates that CD6, and more particularly its cytosolic tail, is responsible for restraining the whole of the MAPK pathway, consistent with the observed effects of the repression of HRas at the initiation of the signaling cascade.

Discussion

For the past decade the role of CD6 on T-cell activation has been vigorously debated. Previous reports indicated that CD6 might function as a co-stimulatory protein that amplifies TCR-mediated activation [5–7, 66, 67]. Most of the studies utilized CD6 antibodies to address CD6 function but, as now seems clear, the effects of antibodies are not always clear cut and may produce experimental artifacts. Firstly, CD6 antibodies were routinely used in multicellular assays in order to block the CD6-CD166 interaction, whereas the antibodies were subsequently found to be non-blockers (reviewed in [68]). Secondly, the use of antibodies to cross-link CD6 may in fact result in the extensive aggregation of the CD6 cytosolic tail-associated signalosome, overriding any subtle regulatory effects that physiologically engaged CD6 might otherwise have.

The functional role of CD6 now has translational implications. In recent years, CD6 has emerged as a potential target for therapy in several autoimmune indications such as multiple sclerosis, Sjögren's syndrome and asthma [69–71], and the humanized anti-CD6 monoclonal antibody Itolizumab is already in clinical use to treat chronic plaque psoriasis, rheumatoid arthritis and COVID-19 [72–74]. It had been conjectured that Itolizumab modulates T-cell activation by disrupting the interaction between CD6 and its APC-expressed ligand CD166 [17]. However, using functional assays and biophysical approaches we have shown that Itolizumab does not physically block the CD6-CD166 interaction [54]. It therefore remains undetermined whether the antibody represses T-cell activation by interfering with the physiological function of a "co-stimulatory" CD6 molecule or by eliciting the "inhibitory" potential of CD6.

Notwithstanding the beneficial effects of CD6 mAbs and the investigation of the mechanisms underlying CD6-targeted therapy in the clinic, there is still much to be learned from basic, molecular studies of CD6. Using an experimental system constituted by APCs presenting sAg to primary T-cells or T-cell lines in which we manipulated the expression of CD6, we previously suggested that CD6 constrains T-cell activation on the basis that it decreases calcium signaling in cells expressing full-length CD6, comparing with cells expressing a CD6 cytosolic deletion mutant [8]. In the present study using FRET microscopy in Jurkat T cells, we now show that the cytosolic tail of CD6 is responsible for the repression of HRas activation at the synapse established with sAg-loaded Raji cells, reinforcing the view that CD6 is fundamentally inhibitory.

Major advantages of FRET microscopy include the continuous monitoring of the enzymatic activity over time and, in the case of T-cell activation promoted by APCs, the ability to track the sensors and determine the site of activation. Little information is available regarding the spatio-temporal regulation of the different Ras members in T cells or T cell lines, except that KRas2B is expressed predominantly on the plasma membrane (PM), whereas

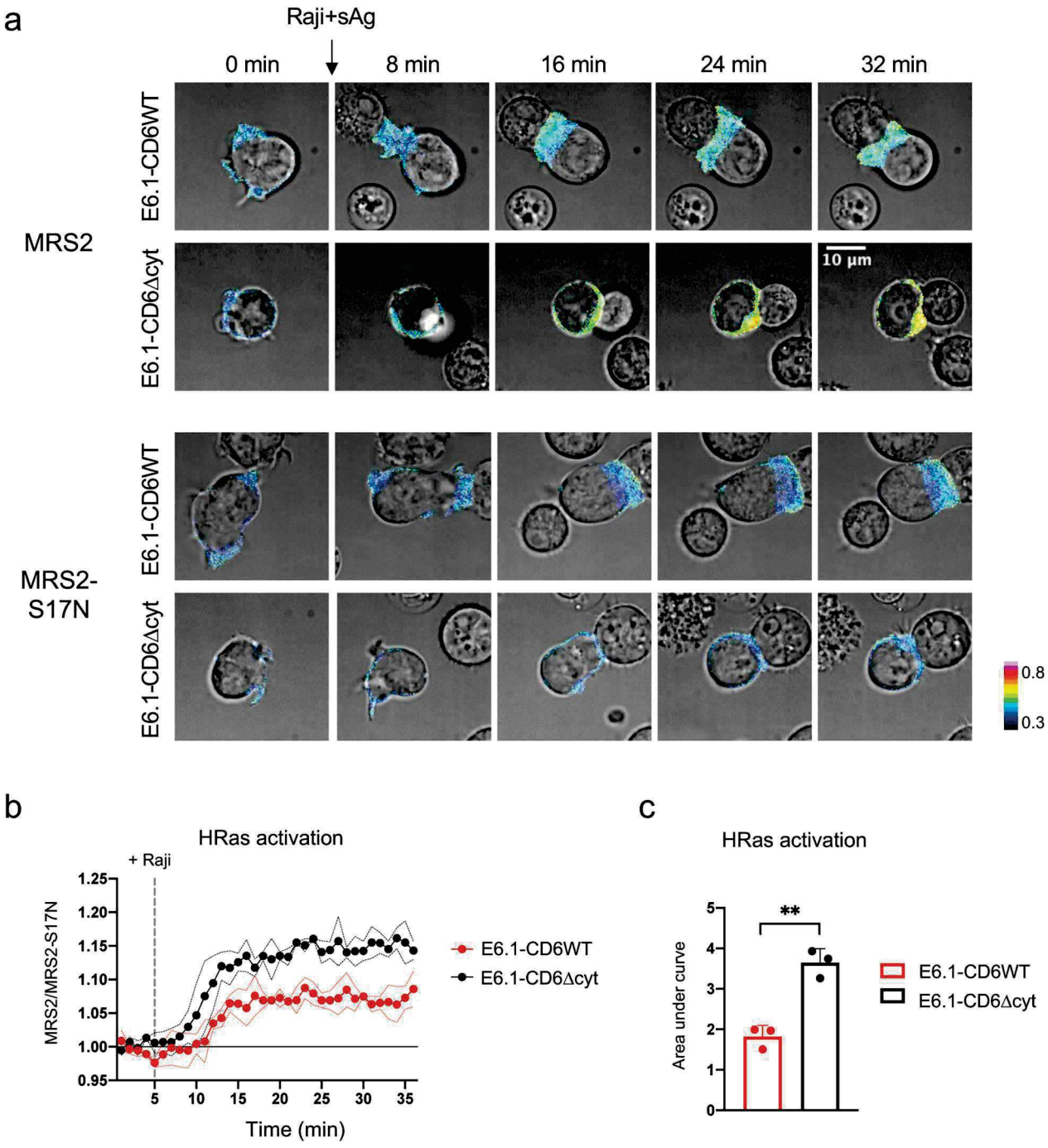

Fig. 4 The cytosolic tail of CD6 constrains HRas activation in E6.1 cells upon APC-mediated activation. **a** Representative images of E6.1-CD6WT and E6.1-CD6Δcyt cells, expressing either MRS2 or MRS2-S17N, during the formation of conjugates with sAg-loaded Raji cells (full movies at Additional file 4: Movie S3). FRET/Clover ratio is presented as a thermal range (scale in bottom right). **b** FRET/Clover ratios from MRS2-expressing cells (see Additional file 1: Fig. S2c) were divided by those of MRS2-S17N-bearing cells and normalized to the average of the first three points before the addition of Raji cells. Dots and lines represent mean ± SD of 3 independent experiments. **c** Area under the curve of graph in B, where baseline = 1. Bars represent mean and SD, and each dot represents an independent experiment. 9–10 cells were assessed in all conditions of each individual experiment. Comparisons were performed using a paired *t* test. **, $p < 0.01$

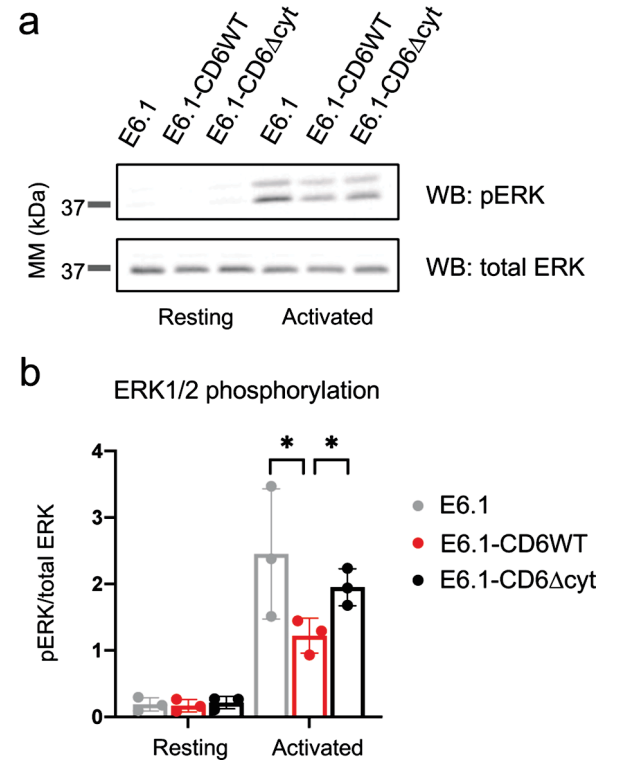

Fig. 5 CD6 downmodulates ERK1/2 phosphorylation in activated cells. **a** Parental E6.1 cells, E6.1-CD6WT and E6.1-CD6Δcyt cells were left undisturbed or were activated with CD3 (3 µg/ml) and CD28 (5 µg/ml) mAbs for 5 min at 37 °C, and lysed. Total and phosphorylated ERK1/2 levels were detected in lysates by WB, and quantified using Fiji. **b** Quantification of ERK1/2 phosphorylation from three independent experiments. Bars represent mean and SD. The probability that the phosphorylation of ERK was similar between E6.1-CD6WT and parental E6.1 or E6.1-CD6Δcyt cells was assessed using a paired *t* test. *, $p < 0.05$

NRas and HRas traffic between the PM and Golgi. Ras sub-cellular localization is mostly determined by the membrane-targeting domain (MTD, last 10–15 aa of Ras), which contains part of the HVR and the C-terminal CAAX box. Prenylation of the CAAX cysteine, followed by AAX tripeptide proteolysis and cysteine carboxymethylation enables Ras to loosely associate with membranes and also determines its addressing to the endoplasmic reticulum and Golgi [35, 75, 76]. Transport to the PM, however, requires a further posttranslational modification, the palmitoylation of yet other cysteine residue(s) within the MTD [35, 77]. HRas contains two cysteines that can be modified by palmitic acid, NRas and KRas2A contain one, and KRas2B has none [78]. Instead, a polybasic domain of six lysine residues in KRas2B can substitute for the palmitoylated cysteines as an alternative PM-targeting signal. Palmitoylation is reversible, and given that Ras acyltransferases are located in the Golgi while deacylating enzymes co-fractionate with the PM, Ras proteins can continually cycle between the different endomembrane compartments, except for KRas2B that resides mainly at the PM [79]. The FRET sensor for HRas that we developed, MRS2, was therefore modified to specifically replace the C-terminal sequence of KRas2B by that of HRas and thus faithfully report on the spatiotemporal mode of activation of the endogenous HRas.

Although it is undisputed that Ras responds to TCR triggering, there is controversy on whether HRas is activated on the Golgi apparatus [36, 39] or predominantly at the PM [37, 38]. In this study we have only observed the presence and activation of the MRS2 sensor at the PM during the formation of the T cell:APC synapse and never in the Golgi apparatus, supporting the view that the PM is the predominant site of Ras activation in Jurkat cells. However, caution should be taken to establish definitive conclusions, given that the mentioned studies used different methodologies and, to our knowledge, FRET-based live imaging of Ras has not been previously performed in T cells or T cell lines to be able to draw comparisons. Having maintained the same architecture of the extensively studied and validated Raichu FRET probes [58], in MRS2 we split the HRas sequence between the N- and the C-terminal ends within the HVR linker domain that connects the conserved N-terminal region and the MTD. It is therefore possible that the N-terminal 7 aa of the HVR that were separated from the MTD may have some additional roles, such as the interaction of Ras with lipid rafts, as previously suggested [80]. Although the topic of lipid rafts is also still very controversial regarding not only the biological function of these membrane microdomains but even their existence [81, 82], a detailed analysis of interaction of the sub-components of the MRS2 biosensor or variant probes with lipid rafts should deserve future consideration and help to unravel without any ambiguities the precise subcellular location of Ras activation in T lymphocytes.

One other limitation that FRET-based Ras/Raf-1 unimolecular biosensors may have is the permanent proximity imposed on the RBD to its Ras binding site via the linker, possibly artificially prolonging the measured Ras activation kinetics. The dislocation of FP modules may not be synchronous with the physiological Ras-RBD dissociation because the structure of the biosensor in an activated state may hinder the access of regulatory proteins (reviewed in [83]). In fact, using bimolecular or single-molecule FRET or biochemical methods in a number of different model systems, it has been shown that the activity of Ras peaks well before 5 min of stimulation, and decaying subsequently [84–86]. This contrasts with the sustained activation of HRas that we observe (at least 30 min after initial sAg recognition) using the

unimolecular MRS2 biosensor. However, there are plenty of examples in the literature documenting that in T cell activation models the activity of Ras is sustained for long periods [87, 88], even exceeding 3 h after CD3+CD28 mAb stimulation, which is fully in line with our observations. Moreover, in contrast with mAb activation triggered at the start of the conventional assays, our cellular system allows for T cell stimulation to be continuous as long as the T cell:APC pairs are engaged. Thus, although not excluding a minor contribution of the biosensor design to a prolonged recorded Ras activation, it is fully plausible that the MRS2 probe faithfully reproduces the behavior of Ras during physiological T cell activation.

The finding that RasGAP interacts with CD6 implied that the regulation of Ras could be a key part of the inhibitory function of CD6. Several other GAPs for Ras have been described in T cells [89] and, besides Raf-1, active Ras binds to a number of other effectors, including the lipid kinase PI3K, and RalGDS, a GEF for Ral GTPases [90, 91], each of these enzymes possibly leading to the activation of a variety of transcription factors. However, only RasGAP is equipped with an SH2 domain capable of binding to phosphorylated tyrosine residues of inhibitory receptors, acting immediately downstream of the TCR to restrain T-cell activation [23, 27]. As the absence of the cytosolic tail of CD6 seems to override the repression caused by the intact CD6 molecule in all steps of the canonical Ras-initiated MAPK signaling cascade, it seems more likely than ever that CD6 is an active repressor of signal transduction initiated by TCR triggering.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-022-00998-x.

Additional file 1: Figure S1. Gating strategy to detect $CD69^+$ or $CD25^+$ cells following sAg-mediated activation. E6.1-CD6WT and E6.1-CD6Dcyt cells were cultured for 24 h in the presence of unloaded or sAg-loaded Raji cells, and expression of CD69 and CD25 was assessed by flow cytometry. (a) The gate was defined based on unstained E6.1 cells, and positivity is represented in the right half of dot plots of FSC *vs.* marker expression. (b) Representative dot plots for each condition analyzing CD69 (left panels) or CD25 (right panels) expression. FSC: Forward scatter. **Figure S2.** Activity of the HRas biosensor MRS2 in unstimulated and activated E6.1 cells. (a) MRS2- and MRS2-S17N-expressing E6.1 single cells were filmed for 5 min at 37 ºC (1 min frames). At the 5 min mark, medium was added and cells were filmed for another 30 min. FRET/Clover ratios were assessed for each time-point. (b) Biosensor-expressing cells were initially filmed for 5 min alone. Then, unloaded or sAg-loaded Raji cells were added and allowed to form conjugates with the E6.1 cells for 30 min. FRET/Clover ratios were calculated for cells expressing MRS2 that interacted with Raji or Raji + sAg, and the same was performed for E6.1-MRS2-S17N:Raji pairs. (c) E6.1-CD6WT or E6.1-CD6Δcyt cells expressing either MRS2 or MRS2-S17N were filmed during conjugate formation with sAg-primed Raji cells. Biosensor-expressing E6.1 cells were filmed alone for 5 min, and for 30 min after sAg-Raji cells were added to the preparation. FRET/Clover ratios were calculated for each of the four experimental conditions. (a-c) Dots and lines represent mean ± SD of 5 independent experiments, with 10 individual cells (in a) or 9–10 cell pairs (in b and c) assessed for each condition in each experiment.

Additional file 2: Movie S1. Stability of the HRas FRET biosensor MRS2 in the absence of cell stimulation. (a) The activity of MRS2 was assessed using single cell live FRET microscopy. E6.1 cells expressing MRS2 were plated on poly-L-lysine-coated coverslips at 37 ºC and filmed for 5 min (1 min frames). At that point, medium was added and filming was resumed for another 30 min. FRET/Clover ratio is presented in a thermal scale (bottom right, range from 0.3 to 0.8). Images (two examples) are representative of 50 analyzed cells from 5 independent experiments. Full data are shown in Fig. S2a. (b) Activity of the inactive HRas biosensor MRS2-S17N, measured in the same conditions as in (a).

Additional file 3: Movie S2. MRS2 responsiveness to cell triggering. MRS2- or MRS2-S17N-expressing E6.1 cells were imaged using FRET live microscopy during conjugate formation with Raji cells. E6.1 cells were initially imaged alone and, after 5 min, filming was paused and Raji cells (unloaded or pre-loaded with sAg) were added to the preparation. Filming was resumed for an additional 30 min. FRET/Clover ratios are presented in a thermal scale (from 0.3 to 0.8). Two pairs of cells are shown for each condition, representative of 48–50 analyses for each E6.1:Raji combination, from 5 independent experiments. Full data are shown in Fig. S2b.

Additional file 4: Movie S3. CD6 downmodulates HRas activation. E6.1 cells expressing MRS2 or MRS2-S17N were further transduced with CD6WT or CD6Δcyt. Cells were imaged using live FRET microscopy as they formed synaptic conjugates with sAg-loaded Raji cells. E6.1 cells were initially imaged alone for 5 min, at which point imaging was paused and sAg-loaded Raji cells were added to the preparation. Filming was resumed for 30 additional min as synaptic contacts established. FRET/Clover ratios are presented in a thermal scale (from 0.3 to 0.8). Two pairs of cells are shown for each condition, representative of 48–50 analyses for each E6.1:Raji combination, from 5 independent experiments. Full data are shown in Fig. S2c.

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Author contributions
SNH and AMC designed the study. SNH performed most experiments. SNH, RFS and LO designed and developed expression vectors and established cell lines, and cell activation protocols. All authors discussed the results and defined the conclusions. SNH generated the figures. SNH and AMC wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Expression vectors and established cell lines are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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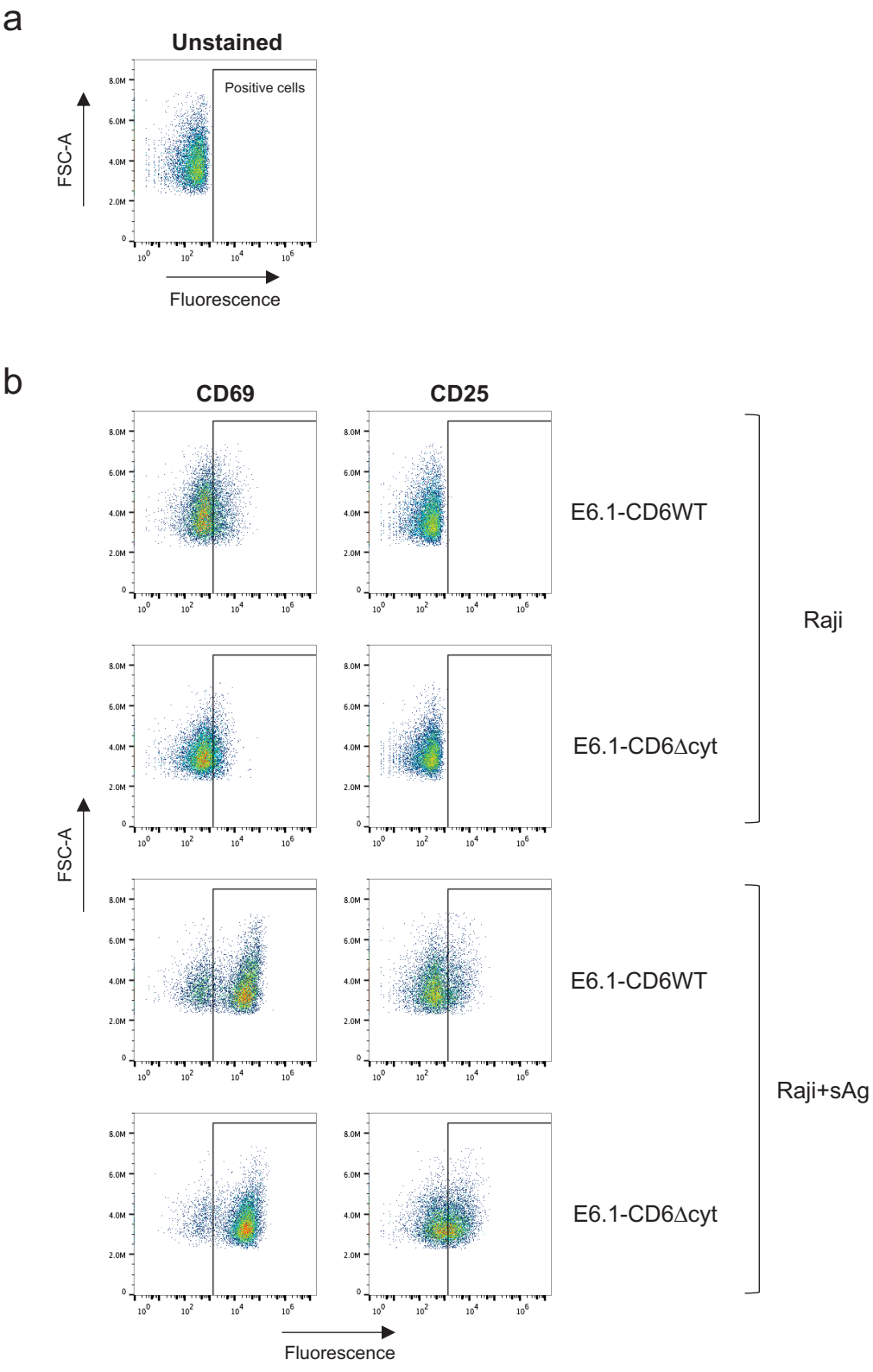

Figure S1. Gating strategy to detect CD69⁺ or CD25⁺ cells following sAg-mediated activation. E6.1-CD6WT and E6.1-CD6Δcyt cells were cultured for 24 h in the presence of unloaded or sAg-loaded Raji cells, and expression of CD69 and CD25 was assessed by flow cytometry. (a) The gate was defined based on unstained E6.1 cells, and positivity is represented in the right half of dot plots of FSC *vs.* marker expression. (b) Representative dot plots for each condition analyzing CD69 (left panels) or CD25 (right panels) expression. FSC: Forward scatter.

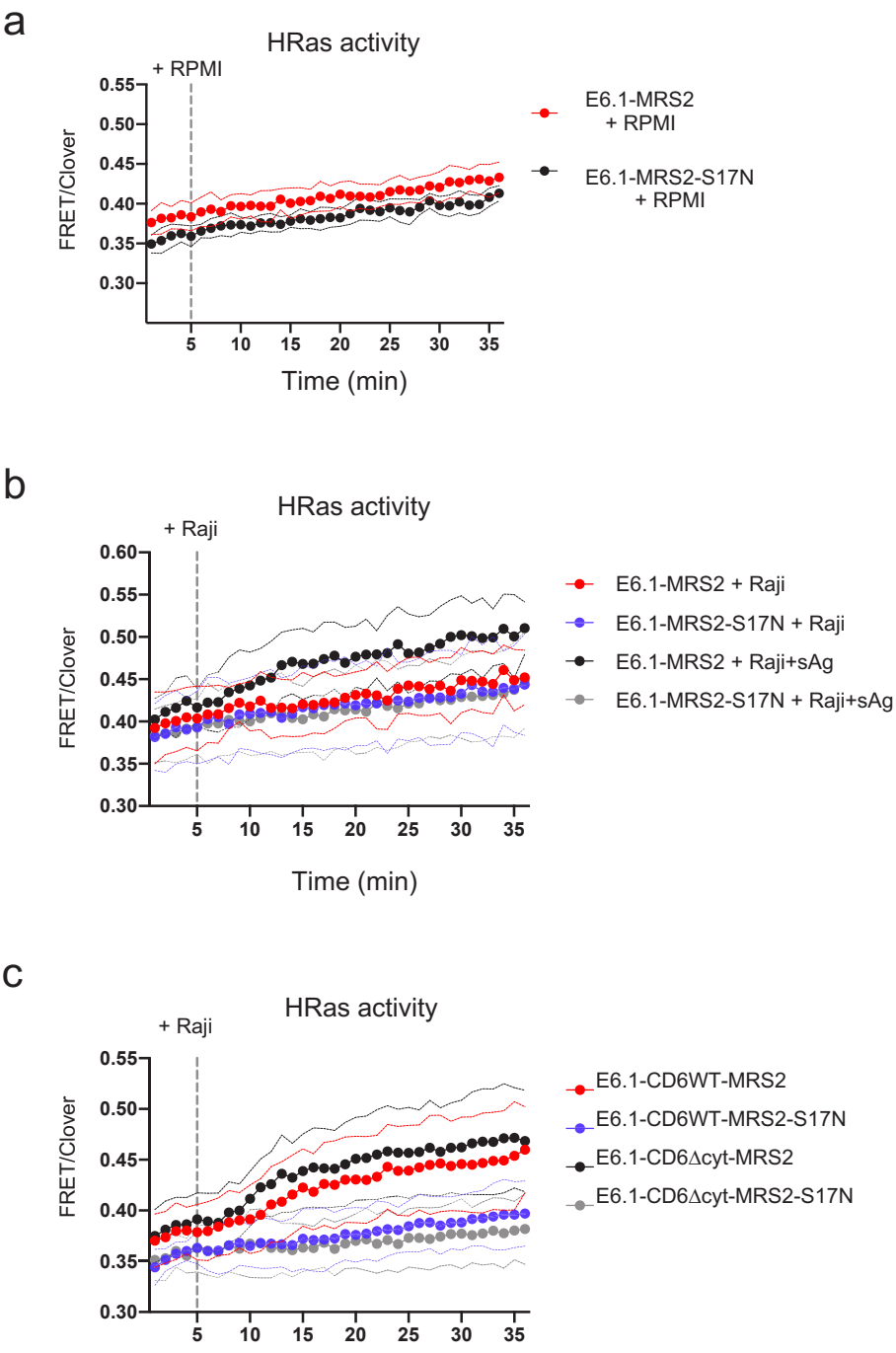

Figure S2. Activity of the HRas biosensor MRS2 in unstimulated and activated E6.1 cells. (a) MRS2- and MRS2-S17N-expressing E6.1 single cells were filmed for 5 min at 37 ºC (1 min frames). At the 5 min mark, medium was added and cells were filmed for another 30 min. FRET/Clover ratios were assessed for each time-point. (b) Biosensor-expressing cells were initially filmed for 5 min alone. Then, unloaded or sAg-loaded Raji cells were added and allowed to form conjugates with the E6.1 cells for 30 min. FRET/Clover ratios were calculated for cells expressing MRS2 that interacted with Raji or Raji+sAg, and the same was performed for E6.1-MRS2-S17N:Raji pairs. (c) E6.1-CD6WT or E6.1-CD6Δcyt cells expressing either MRS2 or MRS2-S17N were filmed during conjugate formation with sAg-primed Raji cells. Biosensor-expressing E6.1 cells were filmed alone for 5 min, and for 30 min after sAg-Raji cells were added to the preparation. FRET/Clover ratios were calculated for each of the four experimental conditions. (a-c) Dots and lines represent mean ± SD of 5 independent experiments, with 10 individual cells (in a) or 9-10 cell pairs (in b and c) assessed for each condition in each experiment.

Results

Chapter 2: Signaling properties of CD6 intracytoplasmic tyrosine residues and their association with CBLB

Introduction

CD6 is a transmembrane protein expressed mostly by thymocytes and T cells [1-3]. Initial studies on the role of CD6, mainly performed using blocking antibodies or recombinant proteins, suggested a co-stimulatory function for this protein during T cell activation [4-7]. However, our laboratory and others have shown that CD6 expression *per se* downmodulates both early and late outputs of TCR triggering [2, 8, 9].

Structurally, CD6 encompasses three extracellular scavenger receptor cysteine-rich (SRCR) domains, a transmembrane domain, and a 245 aa-long cytoplasmic tail [10, 11]. CD6 cytoplasmic tail is complex, including several motifs that can potentially mediate inter-protein interactions, such as proline-rich motifs, CK2 phosphorylation motifs, PKC phosphorylation motifs and nine conserved tyrosine residues [10]. Upon T cell activation, CD6 tyrosine residues become phosphorylated, potentially creating different anchorage points for SH2 domain-containing proteins [12]. Because CD6 has no intrinsic enzymatic activity, its function is likely dependent on intracellular protein interactions. Some of those interactions, like with LCK, ZAP70, SLP76, GADS and SHP-1, have been identified in recent years [13-16]. In accordance with the diversity of CD6 interactors, Mori and colleagues have found, upon outlining the signalosome of CD6 in activated T lymphocytes, that CD6 binds proteins with both inhibitory and co-stimulatory functions [17]. These results suggest that CD6 can play a more nuanced in T cell triggering than initially anticipated.

In collaboration with Simon Davis at the University of Oxford, we performed recently a comprehensive characterization of the interactions established between the phosphorylated tyrosine residues of CD6 and the SH2 domains of several proteins expressed in T lymphocytes. We performed surface plasmon resonance (SPR)-based assays where we determined the dissociation constant (K_d) of each interaction between the six C-terminal tyrosines of the cytoplasmic tail of CD6 and different effector-specific SH2 domains. The observation of high affinity interactions that have previously been described, namely between CD6 and SLP76 [14] or GADS [15], reinforced the potential of this technique in the identification of new interactions, to be demonstrated in further assays. Results from this characterization showed that CBLB can bind CD6 with high affinity, particularly via CD6 tyrosine (Y) 556. CBLB has been previously shown to associate upon T cell activation with CD5 [18], an inhibitory protein structurally and functionally related to CD6, suggesting that a similar interaction might be established by CD6 to mediate its net inhibitory effects.

CBLB is an E3 ubiquitin-protein ligase, a class of proteins involved in ubiquitin-mediated degradation of proteins by the proteasome – the ubiquitin-proteasome system [19-22]. This system is reliant on three separate classes of enzymes: ubiquitin-activating

enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3) [23-26]. These enzymes sequentially activate and transfer ubiquitin molecules to target proteins, a process called protein ubiquitination. This post-translational modification (PTM) allows for these proteins to be recognized by the ubiquitin-binding domains of the proteasome, a selective machinery inside of which proteins are hydrolyzed [27-29]. E3 proteins, such as CBLB [30-32], confer specificity to this system, identifying the proteins to be degraded. CBLB encompasses a tyrosine kinase binding (TKB) region, conserved among the Cbl family of proteins, a RING finger (RF) domain and a ubiquitin-associated (UBA) domain [33].

CBLB has been described to be a critical downmodulator of T cell activation [34-37]. This is illustrated by the fact that CBLB-deficient mice develop autoimmunity, with multiple T cell infiltrates, and are more susceptible to collagen-induced arthritis [35, 36]. Additionally, T cells from these mice are hyperresponsive to TCR stimulation [36]. On a signaling level, while CD28 engagement is required for full TCR-mediated activation of normal T cells (which would otherwise result in anergy), the absence of CBLB uncouples T cell activation from CD28 engagement [36, 38, 39]. Authors have suggested that CBLB-mediated restrain of T cell activation is due to regulation of Vav activation and PLCG1 phosphorylation [35, 39].

In the present study, we show that CD6 binds CBLB via the intracellular residues Y486, Y489 and Y629 when these become phosphorylated. Furthermore, we have assessed the functional role of several individual CD6 intracellular tyrosines by replacing them with phenylalanine residues, and have verified that, while Y486 has an activating role, Y503, Y556, Y629 and Y662 mediate inhibitory signaling. These findings support the notion that CD6 is a complex adaptor protein that associates with different effectors and, because of that, can mediate different signaling cascades, possibly depending on the activation context. Lastly, we have also demonstrated that the CD6 signalosome undergoes ubiquitination in activated lymphocytes, a process partially dependent on the Y486 residue, and that this PTM in CD6 is positively correlated with its association to CBLB.

Materials and methods

Cell lines and culture

T and B lymphocyte-derived cell lines (Jurkat E6.1 and Raji, respectively) were grown in RPMI medium (Cytiva) with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin–streptomycin and 1% (v/v) sodium pyruvate (Thermo Fisher Scientific). Virus-packaging

cells (HEK293T cells) were grown in DMEM (Cytiva), with the same additives as RPMI. All cells were maintained in a controlled incubator (BINDER) at 37 °C and 5% CO2.

Constructs

The sequence for wild-type CD6 (CD6WT) has been previously described [40]. Individual tyrosine mutants and the triple tyrosine mutant (Y486/489/629F) of CD6 were generated via site-directed mutagenesis of the CD6WT construct, and the CD6Y-F construct was synthesized (Integrated DNA Technologies, Inc.). The CBLB-encoding sequence was amplified by PCR from CBLB cDNA ORF Clone, Human, C-DYKDDDDK (Flag®) tag (SinoBiological). The HA tag (TACCCATACGATGTTCCAGATTACGCT) was added at the end of the CD6WT, CD6 mutant or CBLB sequences by PCR, preceded by a small linker. All sequences were cloned into the lentiviral expression vector pHR using the BamHI, MluI or NotI restriction sites and posteriorly sequenced. The gene encoding CD5 was knocked out of the Jurkat E6.1 cell line using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 technology, as described [41, 42]. The single guide RNA (sgRNA) was constructed by annealing of the oligo pair CACCGATGGGGTCTCTGCAACCGC (oligo 1) and AAACGCGGTTGCAGAGACCCCATC (oligo 2).

Transduction of Jurkat E6.1 cells

Jurkat E6.1 cells were transduced as detailed elsewhere [43]. In summary, pHR, p8.91 and pMDG plasmids were simultaneously transfected using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific) into HEK293T cells. Virus-containing cell medium was collected 72 h later, centrifuged and filtered, prior to being added to Jurkat T cells. New medium was added 24 h later and replaced after another 24 h. Protein expression was confirmed by either flow cytometry or SDS-PAGE and, if necessary, cells were sorted for CD3 or CD6 expression.

T cell activation assay

Activation of T cells was assessed as previously detailed [40]. Briefly, 5×10^4 parent or CD6(WT or mutant)-expressing Jurkat cells were co-cultured for 24 h with 5×10^4 staphylococcal enterotoxin E (SEE, Toxin Technology)-loaded Raji cells in complete RPMI. Raji cells had been previously loaded with 1 µg/ml SEE and washed prior to addition to

Jurkat cells. After 24 h, cultures were collected for flow cytometry analysis. As controls, Jurkat cells were further co-cultured with unloaded Raji cells or medium alone.

Flow cytometry

0.5 x 10^6 Jurkat cells were collected and washed twice with ice cold FACS buffer [0.2% (w/v) BSA (NZYTech), 0.1% (v/v) sodium azide (Merck) in phosphate-buffered saline (PBS, Thermo Fisher Scientific)]. Cells were then incubated for 30 min at 4 °C with specific antibodies, washed twice with cold FACS buffer and filtered to FACS tubes using 100 µm nylon filters (Corning Life Sciences). Data were acquired on a BD Accuri™ C6 Flow Cytometer (BD Biosciences) and analyzed on the FlowJo™ v10 Software (BD Biosciences). Antibodies used were: PerCP/Cyanine5.5-conjugated CD3 mAb (OKT3, BioLegend, San Diego, CA USA), FITC-conjugated CD6 mAb (BL-CD6, BioLegend), PE/Cyanine7-conjugated CD5 mAb (Y2/178, Santa Cruz Biotechnology), APC-conjugated CD69 (FN50) or CD25 (BC96) mAbs (BioLegend). Raji cells were gated out of samples from T cell activation assays using PeCy7-conjugated CD19 mAb (HIB19, Thermo Fisher Scientific).

Sodium pervanadate activation and immunoprecipitation

50 x 10^6 E6.1 cells were used for each experimental condition. Cells were activated with sodium pervanadate as previously detailed [44] for 5 min at 37 °C, and pelleted by centrifugation. The supernatant was discarded and cells were lysed for 30 min in 500 µl of lysis buffer [1% (v/v) Triton X-100, 10 mM Tris·Cl pH 7.4 (NZYTech), 150 mM NaCl (NZYTech), 1 mM EDTA (Merck), 1 mM PMSF (Merck), 1 mM orthovanadate (Sigma)], while on ice. Samples were then centrifuged for 10 min at 11,500 × *g* at 4 °C to discard cell debris, while the supernatant was recovered and incubated with 50 µl of Protein A Sepharose® CL-4B beads suspension (Cytiva) for 45 min at 4 °C in end-over-end rotation, for pre-clearing of unspecific protein-bead binding. Beads were pelleted by centrifugation at 500 × *g* for 1 min and the supernatant was recovered. At this point, pre-cleared lysates were further incubated for 1 h with 100 µl of Protein A Sepharose beads and 1 µg of precipitation antibody [HA mAb (16B12, BioLegend) or anti-ubiquitin mAb (UBCJ2, Enzo Life Sciences)] at 4 °C, in rotation. Beads were then cleaned by centrifugation, discarding the supernatant, adding 500 µl of lysis buffer and vortexing – three times. Proteins (from either lysates or immunoprecipitates, after removing the lysis buffer) were denatured for 5 min at 95 °C with 50 µl of 2 × Laemmli buffer (Bio-Rad), with or without β-mercaptoethanol, depending on the specifications of the detection antibody to be used in western blotting.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Proteins were loaded onto 10% polyacrylamide gels, run for approximately 1 h at 200 V and transferred into nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 h at room temperature (RT) in agitation using 5% (w/v) skimmed milk powder (Nestlé S.A.) in Tris-buffered saline-Tween 20 [TBS-T; 20 mM Tris-HCl pH 7.6 (NZYTech), 137 mM NaCl (NZYTech), 0.1% (v/v) Tween 20 (Bio-Rad)], and incubated with primary antibody overnight at 4 °C with agitation, and washed three times with TBS-T, prior to incubation with secondary antibody 1 h at RT in agitation. After new washes, membranes were developed using Amersham ECL Select/Prime Western Blotting Detection Reagent (Cytiva), and images were acquired in the ChemiDoc XRS+ System (Bio-Rad), for posterior analysis in Fiji [57]. Primary antibodies used were: anti-HA mAb (16B12, BioLegend), anti-CBLB mAb (246C5a, Abcam) or anti-ubiquitin mAb (UBCJ2, Enzo Life Sciences), and the secondary antibody used was HRP-conjugated goat anti-mouse IgG (BioLegend).

Mass spectrometry

Mass spectrometry-based proteomics data acquisition has been described [45]. The data analysis procedure was published [45] with the following optimized parameters: i) for protein identification three databases were considered: *Homo sapiens* Proteome (75,069 entries, 2020_05), a common contaminants database, and a FASTA file containing the aa sequence of the CD6 mutated protein; ii) for determination of differentially expressed proteins, the CD6/Control ratio was set to ≥ 2.0 for the selection of upregulated proteins and to ≤ 0.5 for downregulated proteins combined with a “high” confidence in at least two out of three analyzed samples; iii) for precursor quantification, the minimum replicate features parameter was set to 0%.; iv) the Tune software version was 2.11 (Thermo Scientific, Bremen, Germany).

Immunocytochemistry

Glass coverslips were coated with 0.01% poly-L-lysine (Merck) in PBS for 30 min at 37 °C, and thoroughly washed with PBS three times. 5×10^4 Raji cells were incubated with SEE (1 µg/ml) in RPMI for 30 min at 37 °C and posteriorly transferred to the coverslips, where incubation continued for 15 min. 5×10^4 Jurkat cells were then added and allowed to interact with Raji cells for 30 min. After that time, excess volume was aspired and cells

were fixed for 10 min with 2% paraformaldehyde (Electron Microscopy Sciences) in PBS at RT, followed by thorough washing. Cells were then permeabilized with 0.1% Triton X-100 (v/v) in PBS for 30 min, and incubated with blocking solution (0.5% (w/v) BSA) for 1 h at RT. At this point, samples were sequentially incubated with antibodies for 1 h at 4 °C, washed three times between incubations, then incubated with DAPI (Thermo Fisher Scientific) for 5 min for nuclear staining, washed again and mounted on coverslips with Vectashield mounting medium (Vector Laboratories). Data were collected using Zeiss Axio Imager Z1 or Zeiss Axio Imager Z1 Apotome microscopes (Carl Zeiss, Germany) with an Axiocam MR ver3.0 camera (Carl Zeiss) and a Plan-Apochromat 63x/1.40 Oil DIC objective, operated via the software Axiovision 4.9 or 4.8 (Carl Zeiss, Germany), respectively. Antibodies used were: anti-HA mAb (16B12, BioLegend), biotin-conjugated anti-CD6 mAb (OX-124, Absolute Antibody), Alexa Fluor® 488-conjugated anti-CD3 mAb (UCHT1, BioLegend), Alexa Fluor™ 568-conjugated goat anti-mouse IgG, and Alexa Fluor™ 647-conjugated streptavidin (Thermo Fisher Scientific).

Statistical analyses

Statistical analyses were performed on GraphPad Prism version 9.5.1 for (GraphPad Software, Inc.; www.graphpad.com).

Results

Expression of HA-tagged CD6 mutants in E6.1 Jurkat cells

To study the potential role of the seven C-terminal tyrosine residues of the CD6 cytoplasmic tail in their interaction with CBLB, we created seven different CD6 constructs where each tyrosine (Y) was replaced by a phenylalanine (F), preventing residue phosphorylation and subsequent association of SH2-domain containing effectors. We further created an eighth construct where all nine CD6 intracellular tyrosines were replaced by phenylalanine residues. Additionally, we added an HA tag to all constructs at the C-terminus of the CD6 sequence, for specific immunoprecipitation of CD6 molecules.

All constructs were stably transduced using lentivirus in E6.1 Jurkat cells. After transduction, we confirmed equivalent expression levels of total CD6, CD3 and CD5 among different cell lines by flow cytometry (fig. 1). When necessary, cells were sorted to obtain comparable levels of protein expression.

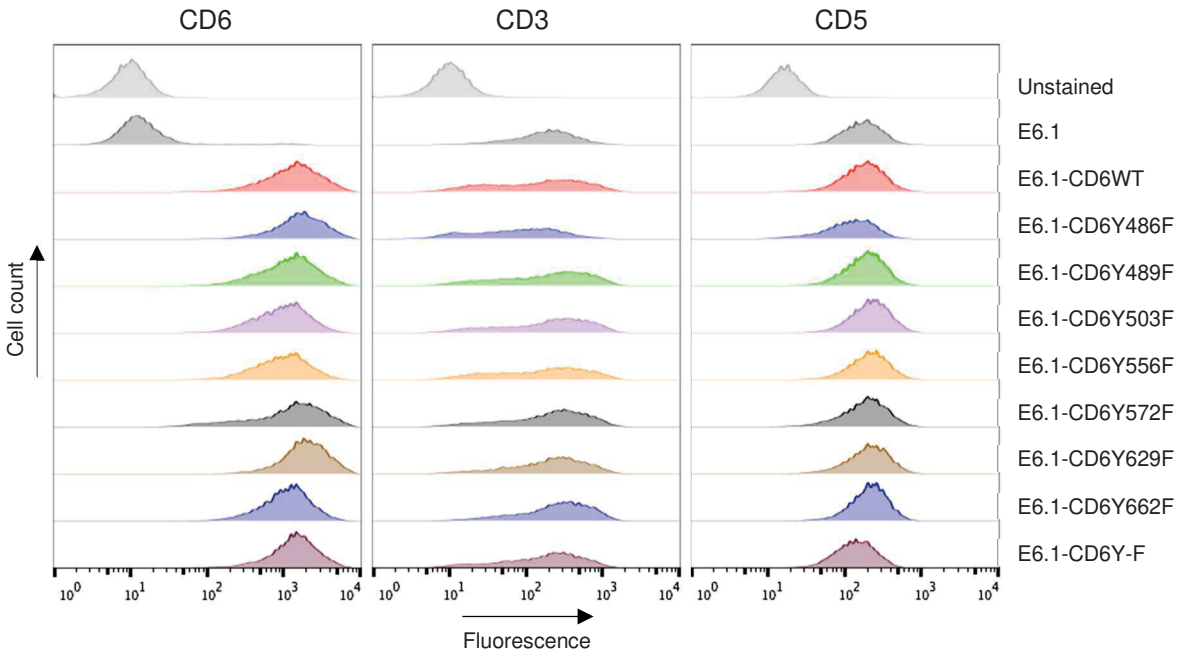

Figure 1. **Characterization of CD6-expressing E6.1 cell lines**. Parental E6.1 Jurkat cells were stably transfected using lentiviral plasmids with CD6WT, CD6 tyrosine point mutants (CD6YxF), or CD6 having all intracellular tyrosine residues mutated to phenylalanines (CD6Y-F). Approximately one week after transfection, all cell lines were stained with FITC-conjugated anti-CD6, PerCP/Cyanine5.5-conjugated anti-CD3 or PE/Cyanine7-conjugated anti-CD5 mAbs for flow cytometry analysis. Histograms are representative of the expression profiles of CD6, CD3 and CD5 in all cell lines.

CBLB associates with CD6 and promotes ubiquitination of the CD6 interactome

To determine whether CBLB physically associates with CD6 and to map this potential association at the aa level, the newly established E6.1 cell lines expressing different CD6 mutants were activated using sodium pervanadate, an inhibitor of protein phosphotyrosyl phosphatases. CD6 was then precipitated from these cells, and co-precipitated proteins could be detected by SDS-PAGE followed by western blotting.

Using this system, we showed that CBLB binds CD6 in a phosphorylation-dependent manner, given that the association was only detected on vanadate-activated Jurkat cells (fig. 2a). Moreover, we demonstrated that CBLB-CD6 binding is fully reliant on phosphotyrosine-mediated interactions, once substituting all tyrosines of the CD6 cytoplasmic tail by phenylalanines completely abolished CBLB coupling to CD6 (fig. 2a). We could further establish that the CD6-CBLB interaction is primarily mediated by the CD6 residues Y486, Y489 and Y629, as deletion of each of these tyrosine residue partially reduced the levels of interaction (fig. 2a, 2b).

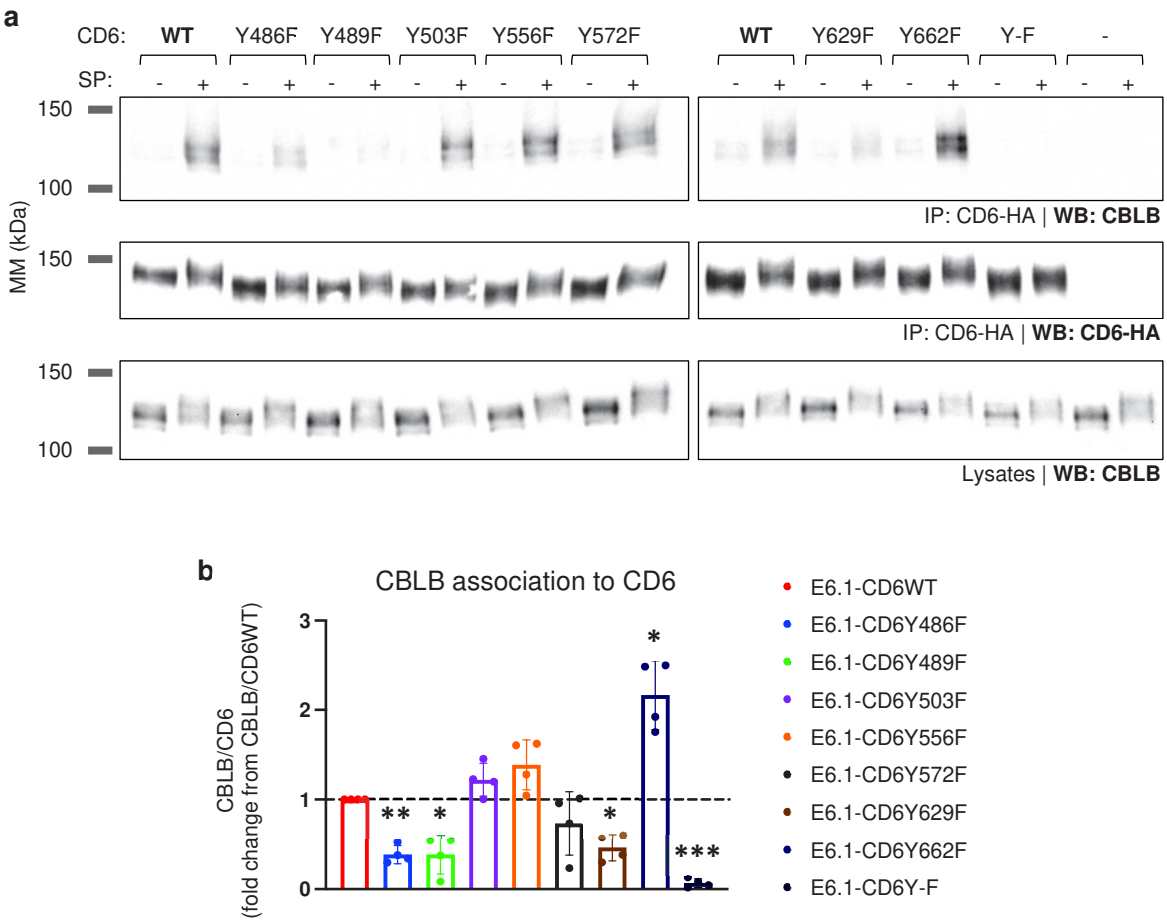

Figure 2. **CD6 associates with CD6 upon phosphorylation via Y486, Y489 and Y629**. E6.1 cells transfected with constructs encoding HA-tagged CD6WT, CD6 tyrosine point mutants or CD6Y-F, and untransfected E6.1 parental cells were activated with sodium pervanadate for 5 min. After activation, cells were lysed and CD6 was immunoprecipitated from lysates using an anti-HA mAb. **(a)** Precipitates were probed by WB for CD6 (positive control) and CBLB, and lysates were probed for CBLB to confirm uniform expression across cell lines. **(b)** WB results were quantified using Fiji, and co-precipitated CBLB levels were normalized to precipitated CD6. All conditions were further normalized to CBLB/CD6WT. Bars represent mean and SD for each condition, and each dot represents one of 4 independent experiments. Experimental conditions were compared using a one-way ANOVA (*P < 0.05; **P < 0.01, ***P < 0.001). MM, molecular mass; IP, immunoprecipitation; WB, western blotting.

On the other hand, we found that upon activation, the Y662F substitution approximately doubles the levels of CD6-bound CBLB, suggesting that an interactor to this tyrosine residue blocks the coupling of CBLB to the cytoplasmic tail of CD6 (fig. 2a, 2b).

We next investigated whether the association of CBLB to the cytosolic tail of CD6 could mediate ubiquitination of CD6 and/or associated molecules. The sets of Jurkat cells

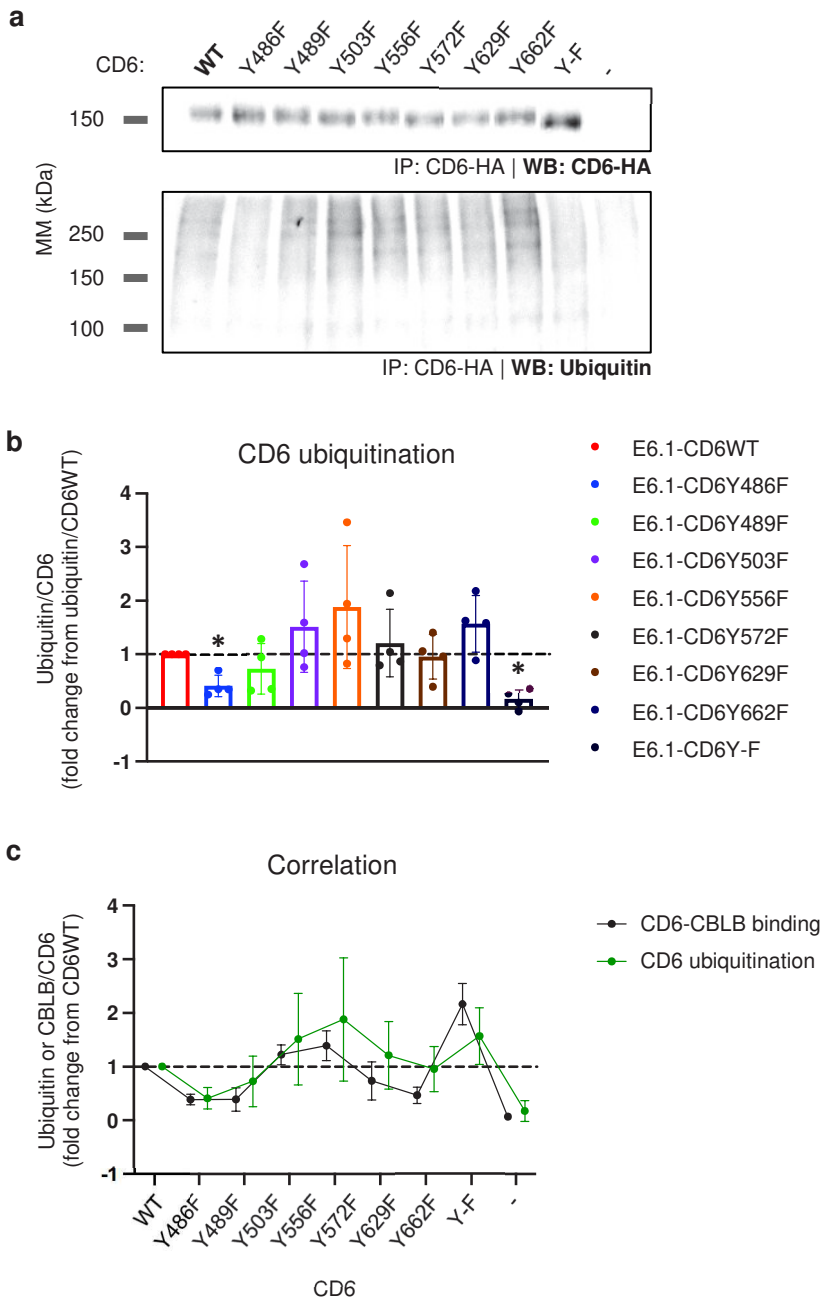

Figure 3. **The CD6 immune complex is ubiquitinated in activated Jurkat cells**. E6.1 cells were transfected with vectors encoding HA-tagged CD6WT, CD6 tyrosine point mutants or CD6Y-F. Cells were activated for 5 min with sodium pervanadate at 37 ºC and immediately lysed for 30 min. CD6 was then immunoprecipitated with an HA mAb. **(a)** CD6 and ubiquitin were detected by WB in CD6 immunoprecipitates, **(b)** and quantified using Fiji. Ubiquitin/CD6 ratios were normalized in all conditions to E6.1-CD6WT. Bars represent mean and SD for each condition, and each dot represents one of 4 independent experiments. Experimental conditions were compared using a one-way ANOVA (*$P < 0.05$). **(c)** Pearson's correlation was calculated between CBLB/CD6 association and ubiquitin/CD6 ratios and graphed. Points represent mean and SD for each condition of 4 independent experiments. MM, molecular mass; IP, immunoprecipitation; WB, western blotting.

were activated using vanadate, the different CD6 forms were immunoprecipitated from the activated cells, and co-precipitated material was probed for ubiquitin by WB. As seen in fig. 3a and 3b, we have observed extensive ubiquitination of CD6WT-immune complexes. While most mutant CD6-immune complexes were also extensively ubiquitinated, we have found that the signalosome of CD6-Y486F is significantly less ubiquitinated than its counterparts. Moreover, we have performed a Pearson's correlation analysis between the relative amount of CD6-bound CBLB and the ubiquitination of CD6 interactomes and found that the two variables are positively correlated (R^2 = 0.7117; fig. 3c). However, as there were still some residual levels of ubiquitination of the CD6Y-F interactome, it is possible that CBLB is not the only ubiquitination-mediating enzyme that assembles within the CD6 complex.

CD6 intracellular tyrosine residues differently modulate activation-induced upregulation of CD25 on Jurkat cells

We have previously established that the intracellular tail of CD6 has an overall restraining role on the activation of T cells [8, 40]. Still, the function of individual tyrosine residues in this process is yet to be dissected. To try to find a correlation between the association of individual CD6 tyrosines residues to CBLB (fig. 2), their role in promoting the ubiquitination of CD6 signalosome (fig. 3) and their potential inhibitory function, we performed T cell activation assays using the Jurkat cell lines expressing WT and mutant CD6 molecules as previously described [40]. Briefly, Jurkat cells were activated by superantigen (sAg)-loaded Raji cells for 24 h and, after that time, activation was assessed by measuring the upregulation of CD25 expression on the Jurkat cells (fig. 4a and fig. 5).

Compared with E6.1-CD6WT cells, CD25 levels were significantly increased in Jurkat cells where CD6 tyrosine residues 503, 556, 629 and 662 were substituted by phenylalanines. Among these, the cell line E6.1-CD6Y662F presented the most marked increase - 114%, while the other three cell lines showed percentage increases ranging from 23 to 34% (fig. 4b). Additionally, the percentage of CD25-positive cells upon activation was significantly increased in E6.1-CD6Y662F cells comparing to E6.1-CD6WT cells (fig. 4c).

Conversely, the cell line E6.1-CD6Y486F was 38% less activated than E6.1-CD6WT (fig. 4b). No differences were observed regarding CD69 upregulation (fig. 6).

These results indicate that while CD6 Y486 has an activating role, Y503, Y556, Y629 and Y662 are inhibitory residues. Among this group, Y629 is the only tyrosine that binds CBLB. Overall, it appears that interacting with CBLB and/or promoting the ubiquitination of the CD6 signalosome do not directly imply an inhibitory role for any given CD6 tyrosine residue.

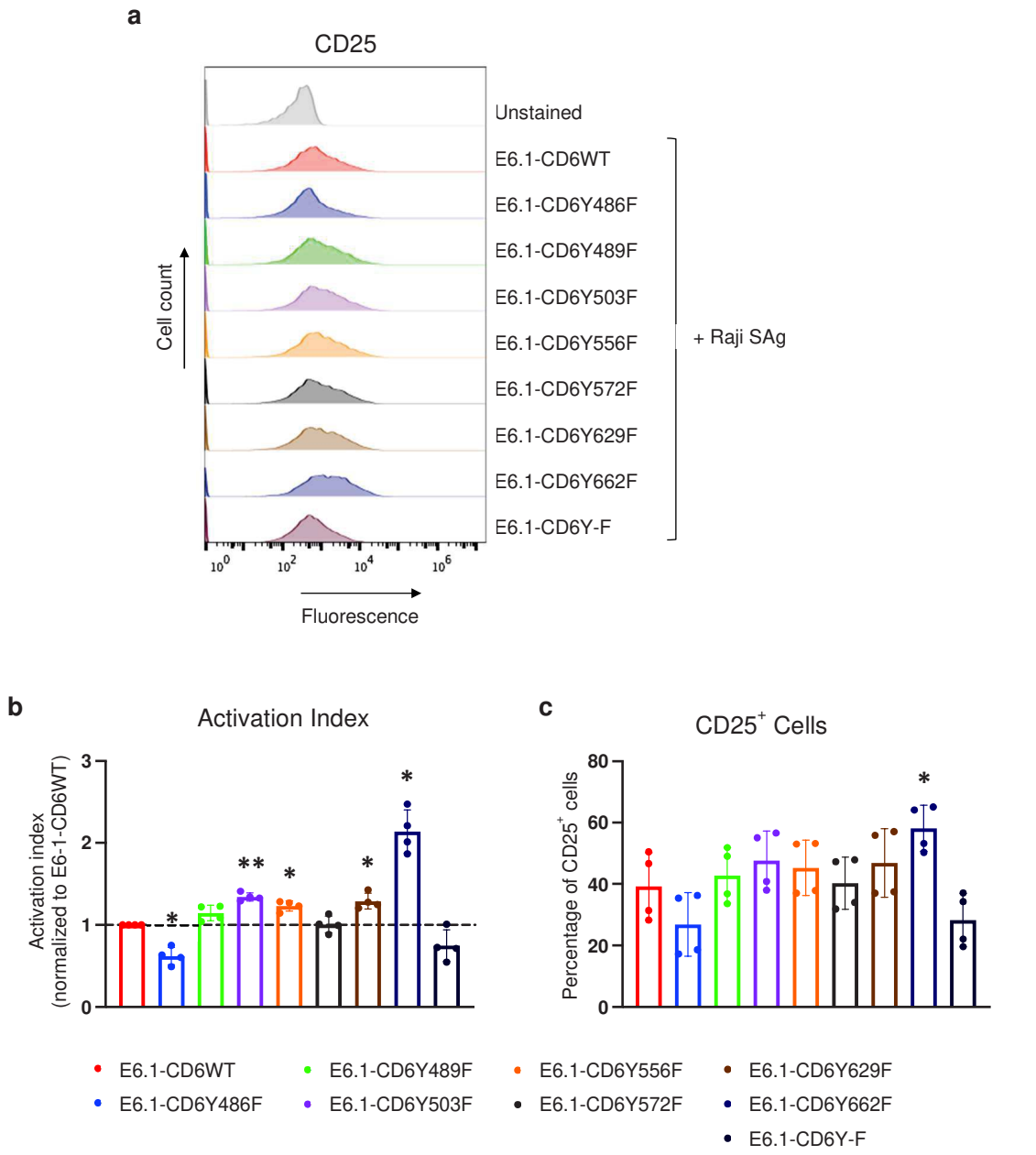

Figure 4. **CD6 intracellular phosphotyrosine residues have distinct signaling properties**. E6.1 Jurkat cells were stably transduced with vectors encoding CD6WT, CD6 tyrosine point mutants or CD6Y-F. Jurkat cells were then activated for 24 h by sAg-loaded Raji cells and stained with an APC-conjugated anti-CD25 mAb. **(a)** Histograms are representative of CD25 expression profiles in T cells activated with sAg-loaded Raji cells. **(b)** Activation indexes for each condition were calculated as the ratio between CD25 median fluorescence intensity (MFI) in Jurkat cells activated with sAg-loaded Raji cells and CD25 MFI in Jurkat cells activated with unloaded Raji cells. For each experiment, the activation indexes of all conditions were normalized to that of E6.1-CD6WT cells. **(c)** Percentage of CD25-positive cells in sAg-Raji-activated Jurkat cells. Bars represent mean and SD for each condition, and each dot represents one of 4 independent experiments. Experimental conditions were compared using (b) a one-way ANOVA or (c) a Friedman test (*$P < 0.05$, **$P < 0.01$).

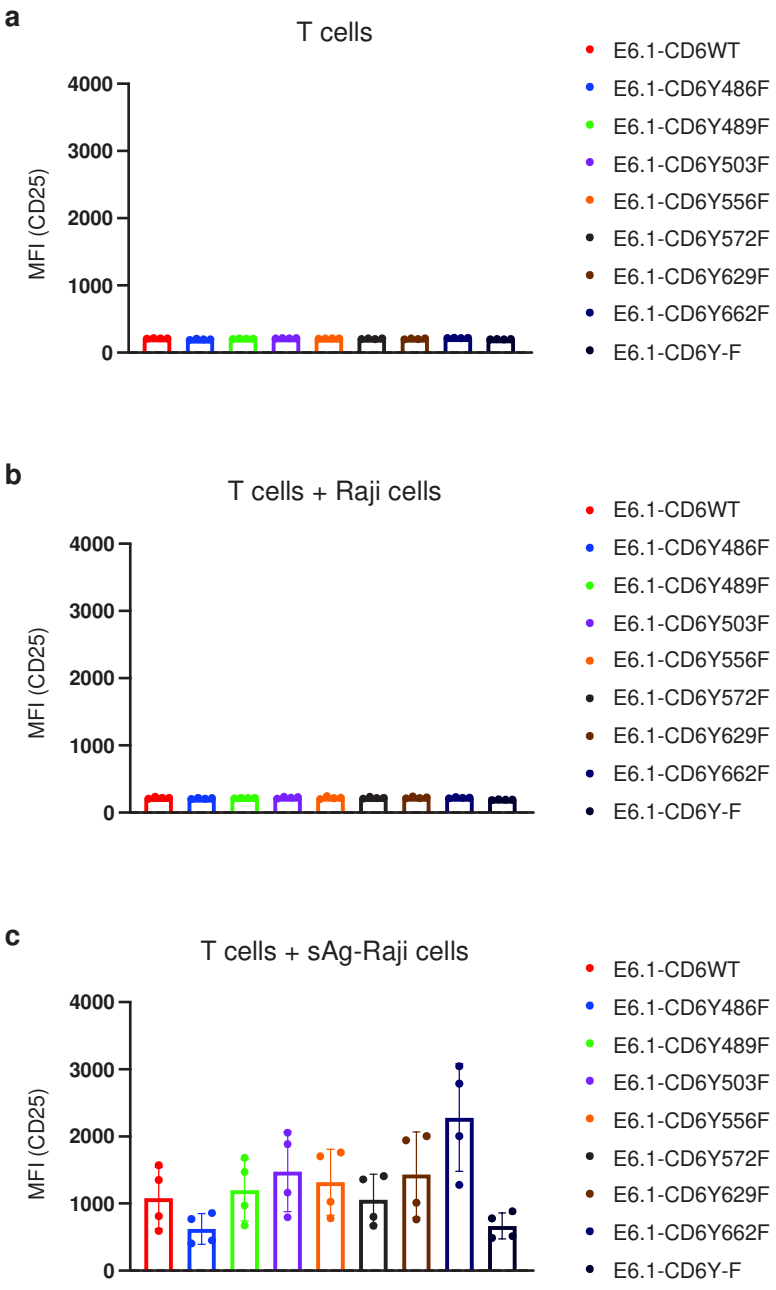

Figure 5. **CD25 expression in resting and activated Jurkat cells.** E6.1 Jurkat cells were stably transduced with vectors encoding HA-tagged CD6WT, CD6 tyrosine point mutants or CD6Y-F. They were cultured **(a)** alone, **(b)** with Raji cells or **(c)** Raji cells previously loaded with sAg for 24 h. After that period, cells were washed and stained with APC-conjugated anti-CD69 mAb. Bars represent mean and SD for each condition, and each dot represents one of 4 independent experiments. MFI, Median Fluorescence Intensity.

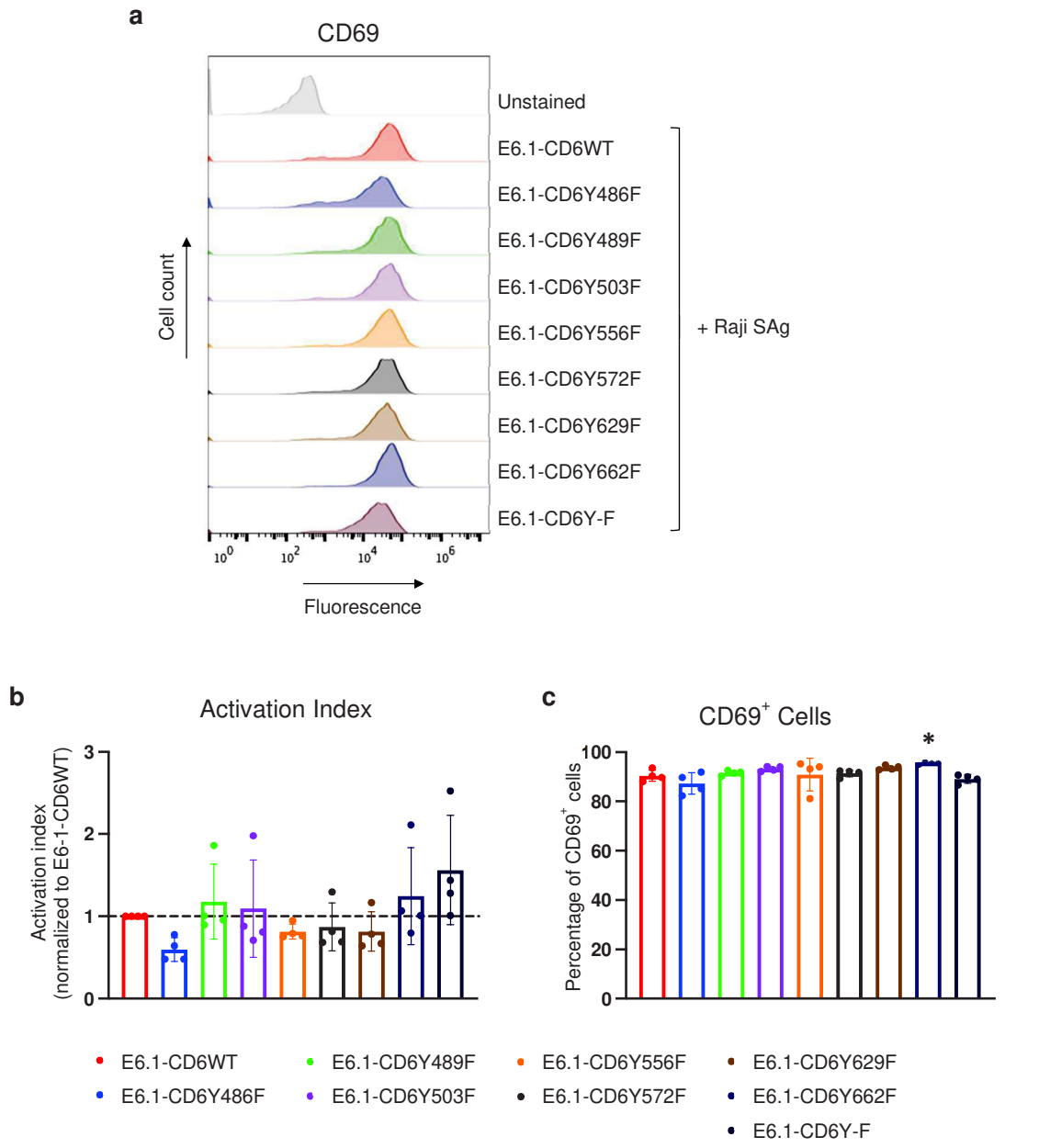

Figure 6. **Activation-induced increase of CD69 expression in CD6-expressing Jurkat cells**. E6.1 cells were transfected with vectors encoding either CD6WT, CD6 tyrosine point mutants or CD6Y-F, and activated for 24 h with Raji cells pre-loaded with sAg. Subsequently, cells were washed and stained with APC-conjugated CD69 mAb. **(a)** Representative histograms of CD69 expression profile in Jurkat cells co-cultured with sAg-loaded Raji cells. **(b)** Activation indexes of all conditions were determined as the ratio between CD69 median fluorescence intensity (MFI) in Jurkat cells activated by sAg-loaded Raji cells and CD69 MFI in Jurkat cells activated by unloaded Raji cells. In individual experiments, activation indexes were normalized to E6.1-CD6WT cells. **(c)** Percentage of CD69-positive Jurkat cells, compared to unstained E6.1 cells. Bars represent mean and SD for each condition, and each dot represents one of 4 independent experiments. Experimental conditions were compared using a Friedman test (*$P < 0.05$,).

CBLB association to CD6 is not indispensable for CD6 ubiquitination and restraining properties

Once the CBLB-CD6 interaction is mediated by three tyrosine residues, there may be redundancy in these associations, which precludes the observation of differences in activation or ubiquitination assays. To assess the impact of completely eliminating CBLB binding sites to CD6, we developed a triple mutant E6.1 cell line where all CBLB-binding tyrosine residues were mutated (E6.1-CD6Y486/489/629F). As expected, in this cell line the interaction of CD6 with CBLB is completely abrogated (fig. 7a). However, ubiquitination assays demonstrated that in E6.1-CD6Y486/489/629F cells the CD6 interactome is ubiquitinated to the same extent as in E6.1-CD6WT cells (fig. 7a). The activation index and the percentage of CD25-postive cells are also comparable in both cell lines (fig. 7b, 7c). Similarly, no differences were observed regarding CD69 upregulation (fig. 8).

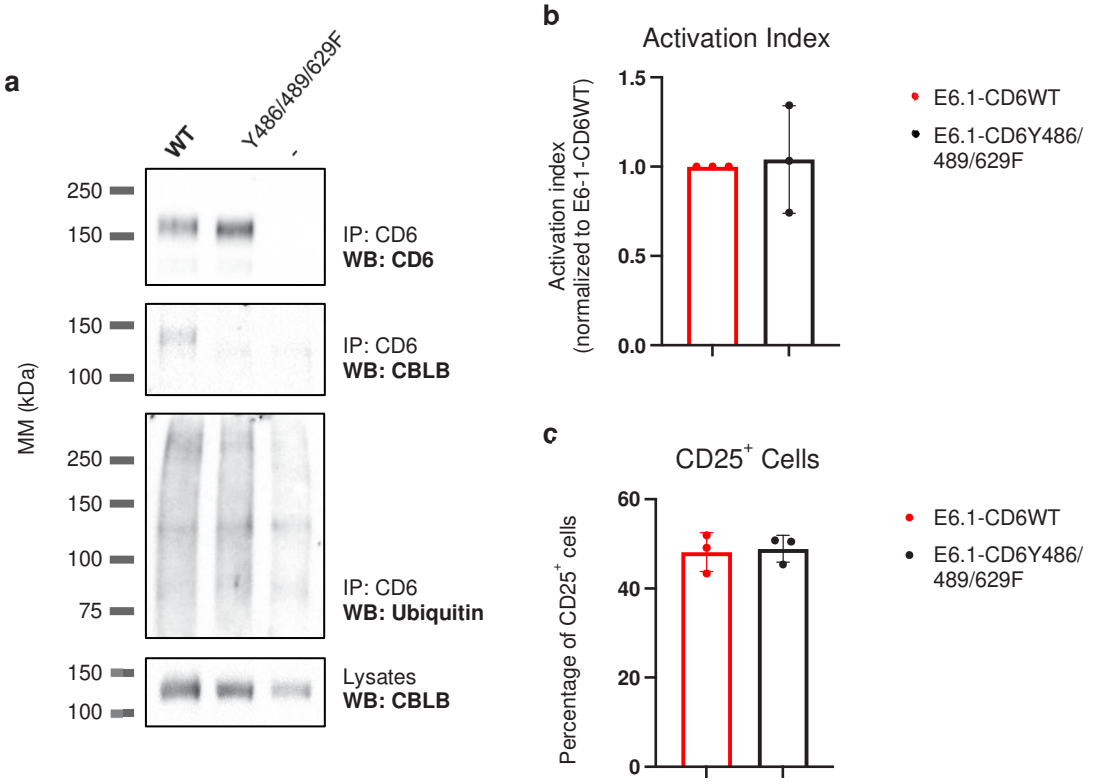

Figure 7. **Characterization of the signaling properties E6.1-CD6Y486/489/629F cells.** E6.1 cells were transfected with vectors encoding HA-tagged CD6WT or CD6Y486/489/629F. **(a)** Jurkat cells were activated for 5 min with sodium pervanadate, lysed, and CD6 was immunoprecipitated with an HA mAb. CD6, CBLB and ubiquitin were detected by WB in immunoprecipitates and lysates. Images are representative of 3 independent experiments. MM, molecular mass; IP, immunoprecipitation; WB, western blotting. **(b)** Jurkat cells were co-cultured for 24 h with Raji cells previously loaded with sAg and stained with APC-conjugated anti-CD25 mAb. Activation indexes were computed as the ratio between CD25 median fluorescence

intensity (MFI) in Jurkat cells activated with sAg-loaded Raji cells and CD25 MFI in Jurkat cells activated with unloaded Raji cells. The activation index of E6.1-CD6Y486/489/629F cells was normalized to E6.1-CD6WT for each experiment. **(c)** Percentage of CD25-positive Jurkat cells. Bars represent mean and SD for each condition, and each dot represents one of 3 independent experiments. Experimental conditions were compared using a paired *t* test.

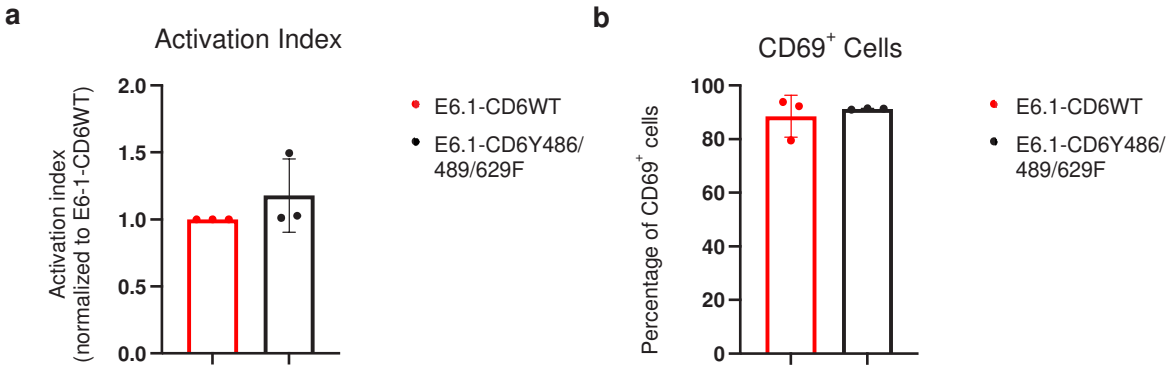

Figure 8. **CD69 upregulation in E6.1-CD6Y486/489/629F cells upon cell activation.** Jurkat cells were stably transduced with vectors encoding HA-tagged CD6WT or CD6Y486/489/629F. Jurkat cells were activated for 24 h with sAg-Raji cells stained with APC-conjugated anti-CD69 mAb. **(a)** The ratio between CD69 median fluorescence intensity (MFI) in Jurkat cells activated by sAg-Raji cells and CD69 MFI in Jurkat cells activated by Raji cells was calculated as the activation index. The activation index of E6.1-CD6Y486/489/629F cells was normalized to E6.1-CD6WT for each experiment. **(b)** Percentage of CD69-positive activated Jurkat cells. Bars represent mean and SD for each condition, and each dot represents one of 3 independent experiments. Experimental conditions were compared using a paired *t* test.

In summary, E6.1-CD6Y486/489/629F cells have similar levels of CD6 ubiquitination and restraining activity as E6.1-CD6WT cells, suggesting on one hand that CD6 may interact with ubiquitination related enzymes other than CBLB, and that the absence of CBLB in the CD6 signalosome does not have any net impact on T cell signaling.

CD6 is not required for translocation of CBLB to the immunological synapse

The CD6-related transmembrane receptor CD5 has been described as a key scaffold for CBLB-mediated ubiquitination [18]. Additionally, CD5 and CD6 on one hand, and CBLB on the other, are recruited to the IS upon T cell-APC engagement [46, 47]. Taking this into consideration, we hypothesized that either CD5 or CD6 (or both) could play a critical role on the re-localization of CBLB to the IS following TCR triggering. To investigate this hypothesis, we developed four Jurkat cell lines, each of them excluding or including the two

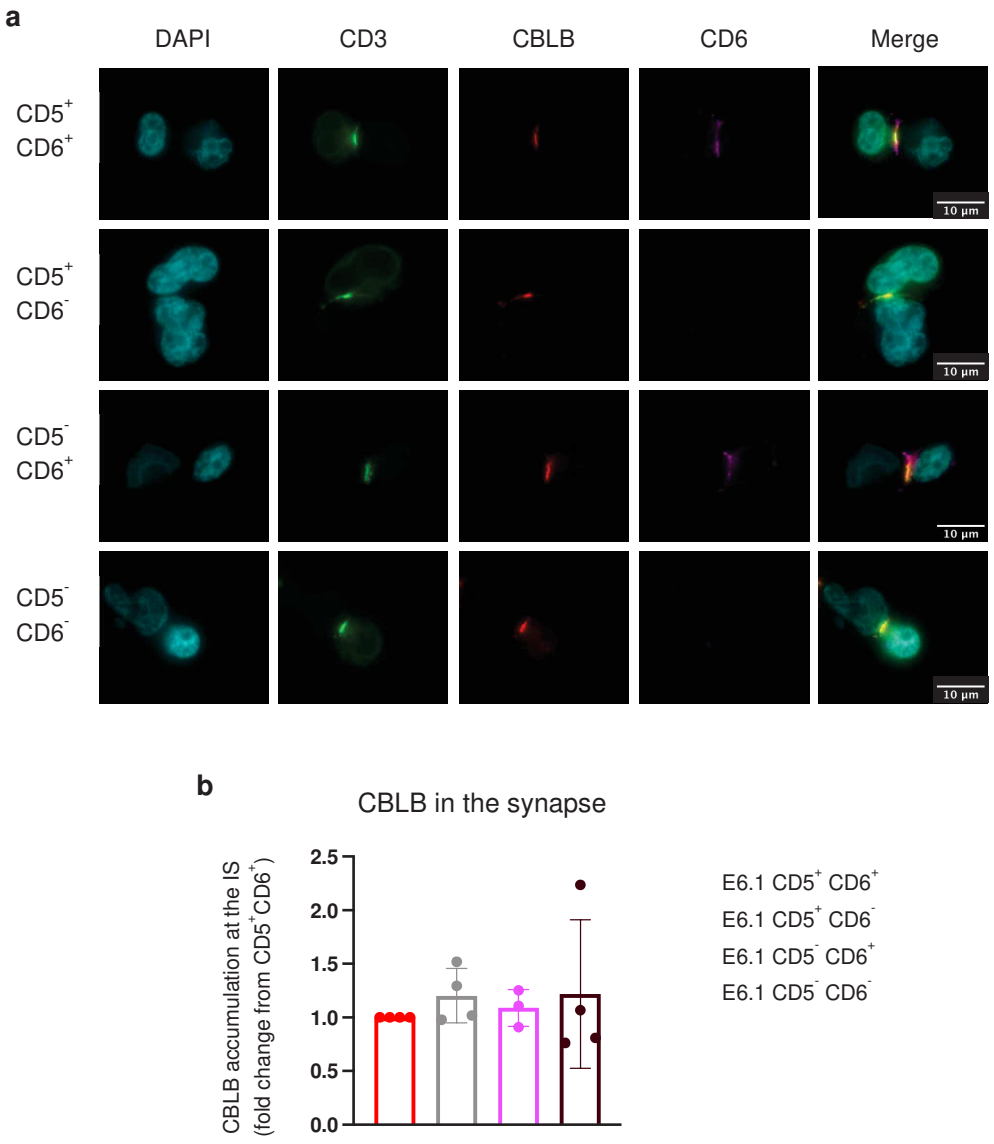

Figure 9. **CD5 and CD6 are not indispensable for CBLB relocation to the IS**. E6.1 cells were depleted of CD5 by CRISPR and/or transfected with a vector encoding CD6WT. **(a)** Jurkat cells were allowed to interact with sAg-loaded Raji cells for 30 min, and then fixed and stained for CD3, CBLB and CD6, to assess the distribution of CBLB in Jurkat cells upon IS formation. **(b)** The accumulation of CBLB at the IS was calculated as the ratio of CBLB in the IS *vs.* CBLB in the whole cell, and all conditions were normalized to E6.1-CD5$^+$ CD6$^+$ cells. Bars represent mean and SD for each condition, and each dot represents one of 3-4 independent experiments. Experimental conditions were compared using a one-way ANOVA.

receptors in all possible combinations (CD5$^+$CD6$^+$, CD5$^+$CD6$^-$, CD5$^-$CD6$^+$ and CD5$^-$CD6$^-$) and evaluated the role of CD5 and CD6 in the recruitment of CBLB to the IS. To that end, all Jurkat cell lines were activated with sAg-loaded Raji cells for 30 min, fixed and stained.

The synapses were identified by the accumulation of CD3 in the contact areas between Jurkat and Raji cells (fig. 9a). However, in all four conditions CBLB was recruited to the IS independently of either CD5 or CD6 expression (fig. 9b). This suggests that other membrane-associated receptors or scaffolds are able to accommodate CBLB within other signalosomes at the IS.

CD6 as a scaffold ubiquitination hub

We next explored other alternative roles for the CD6-CBLB interaction, including whether CBLB could impact the ubiquitination of different sets of substrates depending on the presence or absence of CD6. In order to isolate and quantify ubiquitin-tagged proteins upon T cell triggering, we activated parental CD6-negative E6.1 and E6.1-CD6WT cells with sodium pervanadate, followed by ubiquitin precipitation and mass spectrometry analysis of those samples. The abundance ratio (AR) of each detected protein was calculated as the ratio between its prevalence in E6.1-CD6WT cells and its prevalence in E6.1 cells. In fig. 10, we list the proteins found to be significantly enriched (AR>1) or impoverished (AR<1) in ubiquitin precipitates from E6.1-CD6WT cells, when compared with E6.1 parental cells.

The list of differentially ubiquitinated proteins was processed using the ToppGene Suite software (at https://toppgene.cchmc.org [48-50]), an online tool used to probe protein lists for functional enrichment, in order to understand whether ubiquitination of any specific signaling pathway or group of proteins was particularly targeted in the presence of CD6. Among the proteins with AR>1, three clusters were detected: cluster 1) proteins Inhibitor of nuclear factor-κB kinase regulatory subunit ɣ (IKBKG) and ubiquitin-conjugating enzyme E2 D3 (UBE2D3), linked to the canonical NF-κB pathway and to IKK complex recruitment mediated by receptor-interacting serine/threonine-protein kinase 1 (RIP1); cluster 2) proteins patatin-like phospholipase domain-containing 2 (PNPLA2) and 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2), linked to triacylglyceride synthesis; and cluster 3) proteins IKBKG and immunoglobulin lambda-like polypeptide 1 (IGLL1), clustered and linked to primary immunodeficiency.

Discussion

CD6 is a transmembrane glycoprotein expressed in mature peripheral T cells and thymocytes. Although initial reports suggested that CD6 potentiates T cell activation, it has become evident in recent years that CD6 expression, *per se*, inhibits lymphocyte activation [51, 52]. Although CD6 has no intrinsic enzymatic activity, it includes several signaling motifs within its 245 aa-long cytoplasmic tail able to mediate CD6-induced restrain on T cell

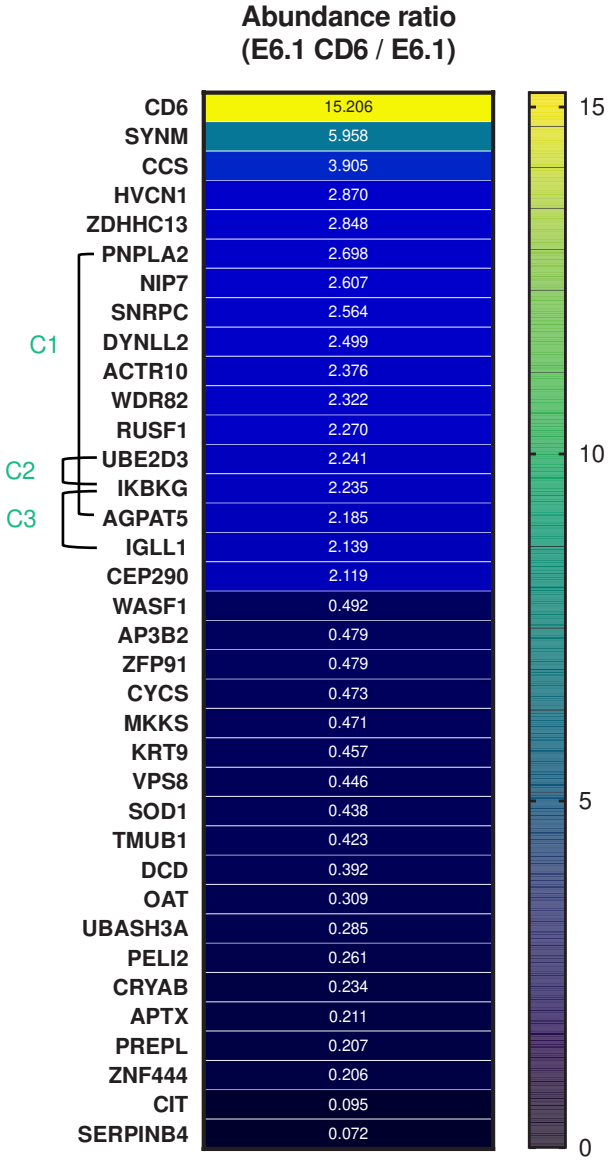

Figure 10. **CD6 modulates the ubiquitination dynamics of several proteins in activated Jurkat cells**. Parental E6.1 and E6.1-CD6WT cells were activated at 37 ºC for 5 min with sodium pervanadate and lysed for 30 min on ice. Ubiquitin was precipitated using a mAb and immunoprecipitates were analyzed by mass spectrometry to identify and quantify co-precipitated molecules. The proteins found to have a significantly increased or decreased abundance ratio are listed above. The abundance ratio equals the abundance of each protein in E6.1CD6WT cells over the abundance of that protein in E6.1 cells. The proteins found to be enriched or impoverished in ubiquitin-immune complexes from E6.1 CD6WT cells (as compared to their prevalence in E6.1 cells) were functionally clustered using the ToppGene Suite software. The identified clusters are signaled (as C1, C2 and C3).

triggering, such as proline-rich motifs, CK2 and PKC phosphorylation motifs, and potentially phosphorylated serine and tyrosine residues [10, 12]. As such, the impact of CD6 expression on lymphocyte signaling is likely facilitated by intracellular protein interactions.

Over the years, different proteins have been identified as part of the CD6 signalosome, and the nature of these proteins is both stimulatory and inhibitory. Specifically, LCK, FYN, ZAP70, SLP76 and CD3 have been co-precipitated with CD6 [6, 13, 14, 46]. This demonstrates that not only CD6 associates with activating proteins, but it also associates with the earliest molecules involved in TCR triggering, including CD3 itself [6, 46]. On the other hand, CD6 has also been described to associate with SHP-1, SHIP-1 and CD5, all of them molecules known to downplay T cell activation [13, 16, 17, 53-55]. The complexity of CD6 signalosome has recently been further illustrated when Mori and colleagues investigated the high-confidence network of CD6, finding in it both stimulatory (like ZAP70 and Vav) and inhibitory (like UBASH3A and SHIP-1) molecules [17]. This diverse signalosome aligns with the complex nature of CD6-mediated signaling. As mentioned, most of the initial reports of CD6 function pointed to co-stimulation, because the use of antibodies or recombinant proteins that block the CD6-CD166 interaction systematically decreased T cells activation [4-6], but most of those observations can just as well be the result of a co-capping phenomenon. This seems to be the case as later studies where CD6 was ablated from T cells demonstrated its inhibitory potential [2, 8, 9]. In summary, unlike CD5, an established inhibitory protein, CD6 appears to have a more complex role, which may depend on the physiological context of activation.

In this study, we report for the first time that CD6 binds CBLB upon phosphorylation of its intracellular tyrosine residues, suggesting a new signaling pathway regulated by CD6. CD6 binds CBLB via tyrosines 486, 489, and 629, and while none of them is solely responsible for this association, when all three residues are mutated CBLB can no longer bind CD6. CBLB has been described as an attenuator of T cell signaling [34-36], as its expression holds TCR activation dependent on CD28 engagement [35, 36], therefore increasing the threshold for T cell triggering. Specifically, CBLB expression decreases T cell proliferation, interleukin 2 (IL-2) production, TCR aggregation, AKT, Vav phosphorylation and activation and PLCG1 phosphorylation. Although its mechanism of action is not completely resolved, CBLB has been shown to bind and promote the ubiquitination of PI3K, a co-stimulatory enzyme, interfering with its recruitment by both CD28 and the TCR rather than triggering its degradation [56]. Another study, however, reported that CBLB is not able to ubiquitinate PI3K, instead hindering downstream signaling by binding neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4). NEDD4 ubiquitinates and tags for degradation phosphatase and tensin homolog (PTEN), an enzyme with an opposite role to PI3K. That way, when CBLB ubiquitinates

NEDD4, the latter is no longer available to promote PTEN degradation [57]. Altogether, these findings support the notion that CBLB-mediated inhibition functions by promoting PTEN activity instead of suppressing PI3K activity. In an analogous manner, CBLB ubiquitinates Crk-like protein (CRKL) and, instead of impacting expression levels, this modification seems to regulate C3G binding and downstream Rap1 activation [58]. As such, the current knowledge on CBLB signaling suggests that, while CBLB acts as an inhibitor of T cell activation, its E3 activity is mostly directed at modulating protein-protein associations and not at promoting protein degradation, which may also apply to CD6.

The tyrosine residues that we demonstrate to mediate binding to CBLB are Y486, Y489 and Y629, not coincident with what was suggested by our systematic analysis of the binding kinetics between CD6 tyrosine resides and the SH2 domain of diverse effectors, which had shown that CBLB has the highest affinity to CD6 Y556. This discrepancy between results from an isolated system, where interactions between individual peptides are characterized, and a more physiological system, although nevertheless flawed, illustrates how they can complement each other. On one hand, a simplified system allows to identify potential CD6 interactors and to compare the affinity of different effectors on a residue level. On the other hand, demonstration of these interactions is still necessary on a more physiological system once factors other than affinity are not considered on a peptide-based setup. For instance, the signalosome assembled around one tyrosine can hinder binding of effectors to a nearby residue, regardless of their relative affinities. This can explain, for instance, why mutating CD6 Y662 increases CBLB association – as CD6 Y629 is one of CBLB binding tyrosines, removing the signalosome nucleated around Y662 can remove the hindrance to further CBLB binding.

To investigate whether the residues responsible for CBLB association promoted inhibitory signaling, we assessed the signaling potential of the seven C-terminal tyrosines of CD6 and found that different tyrosines may have different roles. Although Y486 conveys stimulatory signals, Y503, Y556, Y629 and Y662 are inhibitory residues. Y662, in particular, seems to be the most relevant for CD6-mediated T cell inhibition, in contradiction with a previous report on the signaling potential of this residue [14]. In our experimental setup, the Y662F mutation nearly doubled the activation index of Jurkat cells, compared with E6.1-CD6WT cells. Although experimental conditions were similar for both reports (ours and [14]), activation in the present study involved cell lines of human origin expressing human proteins, while previous experiments assessed activation of human T hybridoma cells (expressing human CD6) by APCs from CBA mice that, in turn, expressed mouse CD166 [14]. Moreover, while we assessed CD25 and CD69 upregulation after 24 h, Hassan and colleagues quantified IL-2 secretion. In any case, activation readouts are usually in accordance and, most likely, that is not the fundament for this difference.

The finding that Y662 is overall an inhibitory residue is particularly thought-provoking, as one the few residue-specific interactions described for CD6 is that between CD6 Y662 and SLP76, a co-stimulatory adaptor crucial for the progression of T cell signaling [14]. However, given that Jurkat cells are less activated when CD6 Y662 is present, its net effect is inhibitory. An explanation for this can be found on the aforementioned SPR-based assay, where we found the CD6 Y662 has a high binding affinity not only to SLP76, but also to Ras GTPase-activating protein 1 (RasGAP). Accordingly, we have recently described that CD6 binds RasGAP when phosphorylated [40]. Future mapping of this interaction can shed some light on the net restraining role of CD6 Y662.

Among the tyrosine residues that directly bind CBLB, we found that only Y629 mediates inhibitory signals. To rule out possible a possible redundancy between residues on CBLB-mediated inhibition, we analyzed the activation index of cells expressing CD6Y486/489/629F and found that these cells were activated similarly to those expressing CD6WT. However, as previously stated, removal of three anchorage points from CD6 likely depletes several other interactors besides CBLB and, for that reason, it is difficult to say with certainty that CBLB is not relevant for CD6-mediated inhibition. In any case, reconciliating the results from our activation assays, it is plausible to reason that, because Y486 and Y629 are stimulatory and inhibitory, respectively, mutating those tyrosines simultaneously does not alter activation levels.

Exploring further functional consequences of CD6-CBLB binding, we demonstrated for the first time that CD6 is ubiquitinated on activated T cells, and that this process is partially mediated by CD6 Y486. Although further experiments will be necessary to show that CBLB mediates CD6 ubiquitination via binding to Y486, we found a significant positive correlation between CBLB binding and CD6 ubiquitination, strengthening that hypothesis. Because CD6 Y486 is the only stimulatory residue in the conditions we analyzed, this information means one of two things: 1) CD6 ubiquitination is a stimulatory PTM, promoting signal propagation; or 2) CD6 ubiquitination is in fact an inhibitory PTM, counterbalanced by other stimulatory proteins that bind CD6Y486 as well.

Nevertheless, CBLB is not the only E3 ligase targeting CD6, once the mutant CD6 Y486/489/629F is as ubiquitinated as CD6WT. Given that CD6Y486F is less ubiquitinated, it can be reasoned that mutating Y489 and Y629 increases CD6 ubiquitination, although further studies are required to substantiate this hypothesis. Still, the finding that CD6 is ubiquitinated, *per se*, is significant because as CD6 is not degraded upon T cell activation, this PTM emerges as a potential new mechanism to modulate signaling [59, 60]. Yet, much is still unclear regarding which proteins mediate CD6 ubiquitination, and which cytoplasmic motifs are relevant to that process.

In our experiments, we have also found that CD6 expression contributes to increases in the ubiquitination of three protein clusters: 1) IKBKG and UBE2D3, two proteins positively involved in NF-κB activation; 2) PNPLA2 and AGPAT2, two enzymes that mediate the degradation and synthesis of tryacylglicerides, respectively, regulating the levels of these molecules; and 3) IKBKG and IGLL1, clustered together in a primary immunodeficiency pathway. Among these three groups of proteins, cluster 2 encompasses enzymes involved in the metabolism of membrane lipids [61, 62], and cluster 3 is more relevant to the context of activation during B cell development [63, 64]. Cluster 1, however, includes two enzymes that directly contribute to T cell activation by modulating activation and translocation of NF-κB transcription factors. IKBKG, on the one hand, is a subunit of IKK, a kinase complex that phosphorylates the NF-κB-associated protein IκB, allowing NF-κB to translocate into the nucleus [65, 66]. UBE2D3, on the other hand, is a ubiquitin-conjugating enzyme that promotes ubiquitination and degradation of NFKBIA, a member of the IκB family [67]. It is still unclear at this point whether the increased ubiquitination of IKBKG and UBE2D3 in the presence of CD6 is related to CBLB association or not – however this finding further demonstrates that CD6 may not only be a direct target for ubiquitination, but also a modulator for the ubiquitination of other effector proteins. Further experiments will be necessary to evaluate 1) whether CD6-mediated ubiquitination of these proteins is co-stimulatory or inhibitory; 2) whether CBLB is the E3 enzyme promoting this process; and 3) which CD6 residues are involved.

Lastly, we have also demonstrated, using newly generated E6.1 cell lines expressing CD5 and/or CD6 (or neither), that ablation of these molecules does not preclude CBLB translocation to the IS. As the molecular mechanisms underlying CBLB translocation to the IS are yet undescribed, and both CD5 and CD6 are now known to bind CBLB, these two closely related glycoproteins could, together or individually, mediate this transportation. Although our assays have not excluded the involvement of CD5 and CD6 in CBLB translocation to the IS, as redundancy may occur, we have observed that neither of them is indispensable for this action.

In summary, we have identified a new CD6 interactor – CBLB – and mapped this interaction at a residue level. We have also, for the first time, investigated the signaling role of each of the seven C-terminal tyrosine residues of the CD6 cytoplasmic tail, expanding the current knowledge on the CD6 signalosome and its complex role in T cell triggering.

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Results

Chapter 3: CD6-mediated regulation of RhoA activity

Introduction

The cytoskeleton is a key component of most cells. In eukaryotes, the cytoskeleton not only sustains cell shape but it also regulates processes such as migration, proliferation and activation [1]. Cytoskeletal elements can be divided into three major groups: microtubules, intermediate filaments and actin filaments [2]. Microtubules are tubular structures assembled from dimers of α-tubulin and β-tubulin. The tubulin dimers linearly polymerize into protofilaments that will, in sets of thirteen, assemble one microtubule that is extended or retracted by the addition or removal of other tubulin dimers (although at different rates) [3, 4]. Microtubules radiate from microtubule organizing centers (MTOCs) and have a central role in intracellular transport, organelle distribution and cell division [5]. Intermediate filaments, on the other hand, are assembled from a wider range of proteins, *e.g.*, keratins, vimentins or neurofilaments, and their composition varies with the cell type where they are present [6]. Structurally, each filament is composed of sixteen individual rod-type linear strands, and they are generally more stable than microtubules or actin filaments. In accordance with those characteristics, intermediate filaments have a mostly mechanic function, providing the cell with physical support and tensile strength [7]. Lastly, actin filaments [or filamentous actin (F-actin)] are similar to microtubules in the sense that a) they are comprised of a single protein type, in this case glomerular actin (G-actin); and b) they are highly dynamic structures that expand or retract based on the actin turnover at its extremities [8]. F-actin has several functions: it mediates contractility by associating with motor proteins (crucial in muscle cells), responds to cues from the extracellular medium (mechanosensing) and modulates shape remodeling [9-11]. In addition to determining cell shape in the basal state, it also mediates processes where shape needs to readjust, such as proliferation and cell motility [11-13].

Actin polymerization usually begins with the nucleation phase where actin monomers form a first stable association, followed by the elongation phase where actin is added to the previously formed nucleus at a higher rate than it dissociates, leading to filament growth, and lastly the steady state phase where the rate of association and dissociation are similar and filaments have reached a dynamic equilibrium [8]. Actin polymerization, *i.e.*, the assembly of F-actin from G-actin, or depolymerization are regulated by actin-binding proteins (ABPs) [14]. While some ABPs promote nucleation and elongation of new filaments [*e.g.*, formin and the actin related protein 2/3 (ARP2/3) complex] [15-19], others promote disassembly (*e.g.*, actin-depolymerizing factors (ADF)/cofilin family of proteins) [20-22] or have a nuanced role, like profilin, simultaneously facilitating and hindering F-actin generation [23]. The activity of ABPs is regulated by, among others, Rho GTPases. When active, Rho GTPases can bind and activate downstream effectors and

modulate intracellular signaling [24]. All three of its most investigated members - RhoA, Rac and Cdc42 - have been linked to cytoskeletal rearrangements [25, 26]. RhoA, specifically, binds effectors Rho-associated protein kinase (ROCK) and diaphanous-related formin-1 (DRF1/mDia) [27, 28] and, in doing so, regulates different elements of the cytoskeleton, increasing actin polymerization, promoting contractility and mediating disassembly of intermediate filaments [29-31].

T cell activation triggered by binding of the TCR to its cognate antigen presented by APCs leads to phosphorylation of LAT and activation of diverse signaling pathways [32, 33]. Upon assembly of the IS, RhoA has been shown to accumulate locally and to regulate its diameter, as well as cytoskeletal rigidity, while potentiating activation responses [34-36]. RhoA can further regulate thymopoiesis and transendothelial migration [36-38], and its depletion effectively diminishes T cell migration (and its associated polarized phenotype), proliferation and response to TCR-mediated activation [36, 37, 39]. Conversely, RhoA has also been implicated in negative regulatory mechanisms for the activation of T lymphocytes [40].

CD6 was shown to bind RasGAP and to downmodulate the activity of Ras upon T cell activation (Results, Chapter 1), showing for the first time that CD6 can participate in the regulation of GTPases [41]. Additionally, CD6 also binds syntenin-1, an adaptor protein that mediates the association of transmembrane proteins to actin filaments [42], and Rho GTPase-activating protein 45 (ARHGAP45), an enzyme that inactivates RhoA [43]. As such, we asked whether CD6 could regulate the activity of RhoA and, if so, whether CD6 would regulate cytoskeletal properties of the cell via RhoA. In the present study, we applied a FRET-based biosensor to live microscopy in order to monitor RhoA activity in real-time in E6.1 Jurkat T cells, over the course of IS formation. We demonstrate that, although RhoA activity is not significantly altered after T cell activation, this GTPase is significantly more active in E6.1 cells expressing full-length CD6 than in E6.1 cells expressing a cytoplasmic-truncation mutant of CD6. We further show that expression of full-length CD6 results in a decreased number of cells adhering to an activating surface. Although further studies are necessary to determine whether CD6 controls actin remodeling (via RhoA or not), the results strengthen the notion that CD6 interacts with and modulates cytoskeletal regulators.

Materials and methods

Cell culture

Cell lines Jurkat E6.1 and Raji were cultured in RPMI-1640 (Cytiva), complemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin and 1% (v/v) sodium pyruvate (Thermo Fisher Scientific). HEK293T cells were cultured with similarly supplemented Dulbecco's Modified Eagle Medium (DMEM, Cytiva). All cell lines were grown inside a controlled incubator (BINDER) kept at 37 °C and 5% CO_2.

Constructs and cell lines

Sequences for CD6WT and CD6Δcyt were described previously [41]. The RhoA FRET biosensor used for live imaging of RhoA activity was obtained from the pCAGGS-Raichu-RhoA-CR plasmid [44]. pCAGGS-Raichu-RhoA-CR was a gift from Michael Lin (Addgene plasmid #40258; http://n2t.net/addgene:40258; RRID:Addgene_40258). The sequence for the RhoA FRET biosensor within the pCAGGS-Raichu-RhoA-CR plasmid (here named RhoA-CR) was isolated via PCR, and RhoA-CR-T19N was generated via site-directed mutagenesis of RhoA-CR, also by PCR. The cDNAs of CD6WT, CD6Δcyt, RhoA-CR and RhoA-CR-T19 were digested and ligated into the lentiviral expression vector pHR via *Bam*HI, *Mlu*I or *Not*I restrictions sites. All resulting plasmids were sequenced and verified prior to cell transfection.

Using different combinations of the transducing plasmids, we obtained the following E6.1-derived cells lines: E6.1-RhoA-CR, E6.1-RhoA-CR-T19N, E6.1-CD6WT-RhoA-CR, E6.1-CD6WT-RhoA-CR-T19N, E6.1-CD6Δcyt-RhoA-CR, E6.1-CD6Δcyt-RhoA-CR-T19N, E6.1-CD6WT, E6.1-CD6Δcyt.

Live cell imaging of immunological synapses

5×10^4 E6.1-CD6WT and E6.1-CD6Δcyt cells expressing RhoA-CR or RhoA-CR-T19N were washed twice, resuspended in RPMI without phenol red (Cytiva) and plated in µ-slides (ibidi) previously coated with poly-L-lysine (Merck). In parallel, 1×10^5 Raji cells were incubated with SEE (Toxin Technology) at 1 µg/ml for 45 min at 37 °C and posteriorly resuspended in RPMI without phenol red. After sample preparation, Jurkat cell lines alone were transferred to the microscope stage and filmed for 5 min, to ascertain the basal RhoA activity. The microscope stage was kept in controlled conditions at 37 °C and 5% CO_2,

employing an Okolab microscope incubator system (Okolab). At that point, Raji cells were gently added to the preparation and samples were filmed for 30 min.

Images were sequentially acquired every minute with an inverted motorized Nikon Ti widefield microscope (Nikon) equipped with an Andor iXon888 EMCCD camera (Andor), a PL APO LAMBDA 60x/1,4 Oil DIC objective, a Lumencor SpectraX light source, and controlled through the NIS-Elements AR software (version 5.02). To image the Clover channel, the lumencor Spectra X cyan filter 470/24 was used for excitation with a GFP/DsRed—bs 493/574, and a 514/30 emission filter. For the mRuby2 channel, the lumencor Spectra X green filter 550/15 was used for excitation with a GFP/ DsRed—bs 493/574, and a 617/73 emission filter. Lastly, for the FRET channel, the lumencor Spectra X Cyan filter 470/24 was used for excitation, a GFP/DsRed—bs 493/574, and a 617/73 emission filter. LEDs were applied at 10%, and images in all channels were acquired without binning and at 100 ms exposure. To track cell movements and interactions, an additional brightfield image was acquired with the lamp at 6.5 V and an exposure time of 10 ms.

Imaging of fixed Jurkat cells on a mAb-coated surface

Ibidi dishes were coated with either anti-CD3 mAb (UCHT1, BioLegend) at 1 µg/ml and anti-CD28 mAb (CD28.2, BioLegend) at 5 µg/ml, or only with anti-CD28 mAb. After careful washing of the plate with PBS (Thermo Fisher Scientific), 1 x 10^5 Jurkat cells resuspended in complete medium were allowed to interact with the plate surface at 37 °C. After 30 min, cell medium was gently aspired and cells were fixed for 10 min with 2% PFA (Electron Microscopy Sciences) in PBS at RT, washed and permeabilized with 0.1% (v/v) Triton X-100 (Thermo Fisher Scientific) in PBS for 10 min. Cells were simultaneously stained with HCS CellMask™ Deep Red stain and DAPI (both from Thermo Fisher Scientific) for 30 min at RT, washed and resuspended in PBS. Images of adhered cells were acquired using the IN Cell Analyzer 2000 imaging system (GE Healthcare), and analyzed using the CellProfiler™ software [45].

Activation of Jurkat cells and flow cytometry analysis

0.5 x 10^6 Jurkat cells were activated with anti-CD3 mAb at 3 µg/ml and anti-CD28 mAb at 5 µg/ml for min at 37 °C. Medium was removed and cells were fixed with 2% PFA in PBS for 10 min at RT and washed with FACS buffer [0.2% (w/v) BSA (NZYTech), 0.1% (v/v) sodium azide (Merck) in PBS], prior to permeabilization with 0.5% (v/v) saponin in PBS for 5 min at RT. Samples were stained with Alexa Fluor™ 488-conjugated phalloidin in 0.5% saponin for 30 min at 4 °C, washed, and filtered to FACS tubes through 100 µm nylon filters

(Corning Life Sciences). Data were acquired on a BD Accuri™ C6 Flow Cytometer (BD Biosciences) and posteriorly analyzed using the FlowJo™ v10 Software (BD Biosciences).

Alternatively, for assessment of protein expression in resting Jurkat lines, these were only washed with FACS buffer and stained with specific antibodies prior to filtration and data acquisition. Antibodies used were: anti-CD3 mAb (UCHT1, BioLegend), anti-CD6 mAb (MEM98, EXBIO) and Alexa Fluor™ 647 goat anti-mouse IgG (Thermo Fisher Scientific).

Statistical analyses

Statistical analyses were performed on GraphPad Prism version 9.5.1 for (GraphPad Software, Inc.; www.graphpad.com).

Results

Transduction and validation of new E6.1 Jurkat cell lines

To track RhoA activation spatially and quantitatively in real-time, we used a fluorescent FRET biosensor for RhoA previously validated and optimized, RhoA-CR [44, 46]. Briefly, RhoA-CR encompasses a pair of fluorophores (Clover on one end and mRuby2 on the opposite end), interspaced by RhoA (amino acids 1-189) and the RhoA-binding domain (RBD) of protein kinase N (PKN), as well as the C-terminal region of KRas after mRuby2 [44, 46]. When RhoA is inactive, fluorophores in the sensor are apart and Clover stimulation will only result in Clover emission. On the other hand, when RhoA is active it binds RBD-PKN, bringing Clover and mRuby2 together and consequently increasing FRET emission upon Clover stimulation.

Although FRET biosensors are a powerful tool to study GTPases, the reported variations in protein activity can sometimes arise from experimental artifacts [47]. To overcome this difficulty, we generated a dominant negative FRET biosensor from RhoA-CR where we replaced threonine 19 with an asparagine – RhoA-CR-T19N. This substitution constitutes a fundamental negative control in live imaging experiments, as we demonstrated previously in a similar experimental setup, where Ras activity was monitored in E6.1 cells during synapse formation [41].

E6.1 cells were transduced with vectors encoding RhoA-CR or RhoA-CR-T19N to obtain E6.1-RhoA-CR and E6.1-RhoA-CR-T19N cells. These cells were then transduced with vectors encoding CD6WT or the cytoplasmic truncated mutant CD6Δcyt to obtain the set of cells indicated in fig. 1. The expression patterns for the RhoA FRET biosensor sensors, CD6 and CD3 were confirmed by flow cytometry (fig. 1).

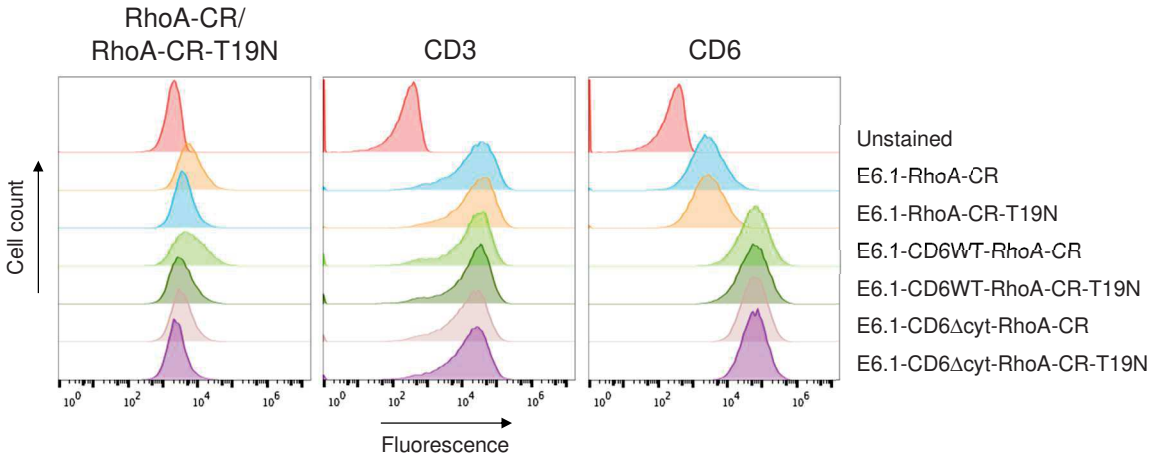

Figure 1. **Characterization of transduced E6.1 Jurkat cells**. E6.1 cells were initially transduced with RhoA-CR- or RhoA-CR-T19N-encoding vectors and, posteriorly, with CD6WT- or CD6Δcyt-encoding vectors, using lentivirus. After recovery, cells were briefly washed and stained for flow cytometry analysis with CD6 or CD3 mAbs and Alexa Fluor™ 647-conjugated goat anti-mouse IgG. Depicted histograms illustrate the expression of RhoA biosensors, CD3 or CD6 in each analyzed cell line.

CD6 expression increases RhoA activity

To measure RhoA activation in the different E6.1 Jurkat cell lines using real-time FRET microscopy, we used the experimental setup previously established by our laboratory [41]. Briefly, E6.1 cells expressing RhoA-CR or RhoA-CR-T19N, with CD6WT or CD6Δcyt, were plated on poly-L-lysine-coated dishes and imaged for 5 min, unperturbed, to ascertain basal RhoA activity. After that time, Raji cells previously loaded with SEE were added to the preparation and the formation of cell conjugates between Jurkat and Raji cells was monitored during the following 30 min (fig. 2a). For each cell line, we quantified the ratio between FRET and Clover emissions at the single cell level (FRET ratio) (fig. 3a). RhoA activation was calculated by normalizing the FRET ratio in E6.1 cells expressing RhoA-CR by the FRET ratio in E6.1 cells expressing RhoA-CR-T19N (fig. 2b). Additionally, fold changes in RhoA activation for both cell lines were also calculated as a function of the first three time points imaged (fig. 3b).

Quantification of RhoA activity during activation by sAg-primed Raji cells was performed for both E6.1-CD6WT and E6.1-CD6Δcyt cells. We found that, in both cell lines, APC-mediated cell activation did not significantly alter basal RhoA activity levels (fig. 2b and fig. 3b). On the other hand, RhoA activity was significantly higher in E6.1-CD6WT than in E6.1-CD6Δcyt cells (fig. 2b and 2c). Additionally, RhoA recruitment to the IS was

increased in E6.1-CD6WT cells comparing to RhoA recruitment in E6.1-CD6Δcyt cells (fig. 2a).

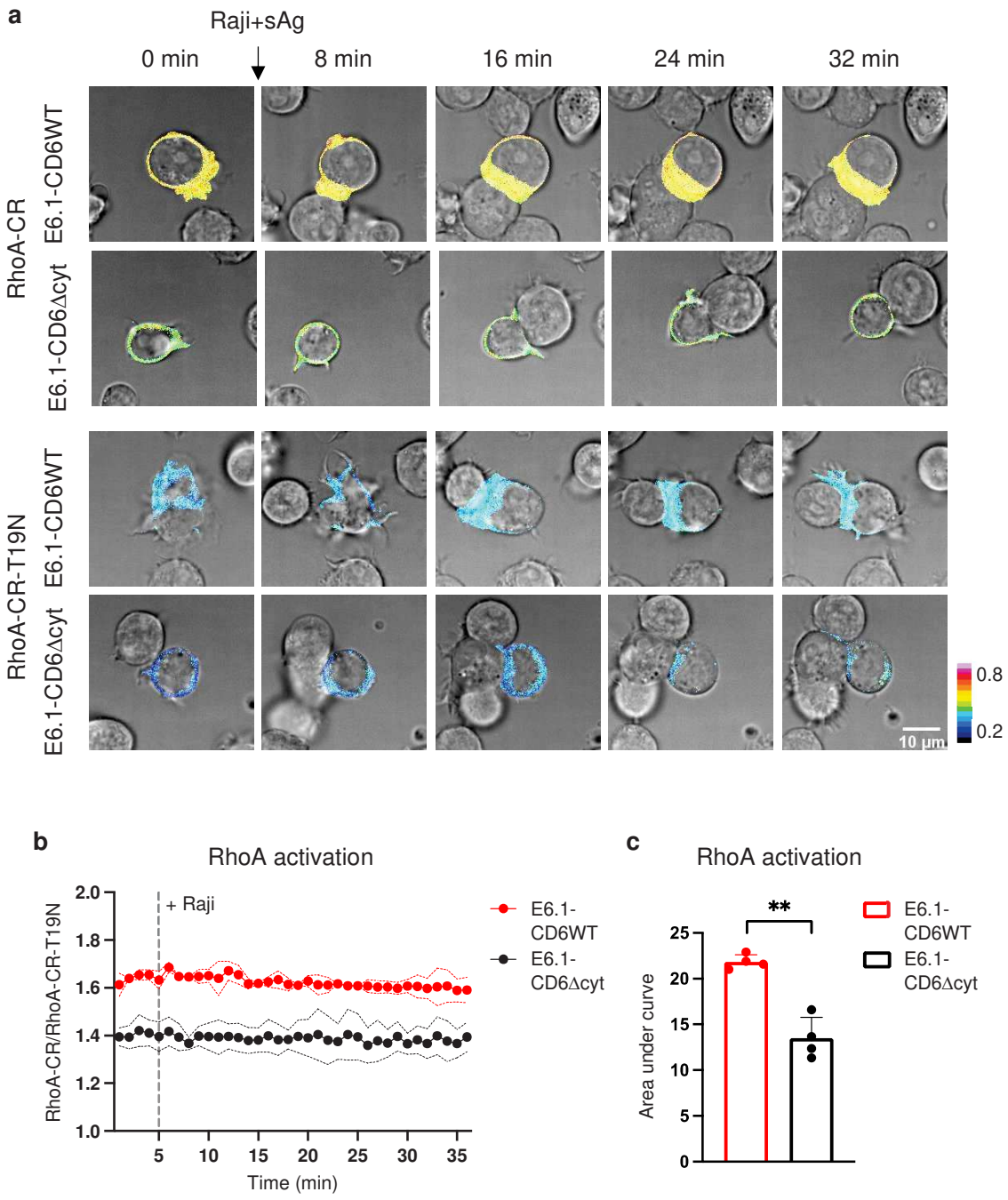

Figure 2. **The cytoplasmic tail of CD6 is responsible for increased RhoA activity in resting and activated E6.1 cells.** E6.1-RhoA-CR and E6.1-RhoA-CR-T19N cells expressing CD6WT or CD6Δcyt were imaged before (5 min) and during (30 min) interaction with SEE-loaded Raji cells. **a)** Images are representative of immunological synapses formed over time between Raji cells and E6.1-CD6WT or E6.1-CD6Δcyt cells expressing the functional or the unproductive version of the RhoA FRET biosensor. Each image presents an overlap of the brightfield and the FRET/Clover ratio calculated for each cell (thermal scale in the bottom right). **b)** The average FRET/Clover ratio

of RhoA-CR-expressing cells was divided by the ratio from RhoA-CR-T19N-expressing cells (see fig. 3) to calculate RhoA activity in E6.1-CD6WT or E6.1-CD6Δcyt cells. Dots and lines represent mean ± standard deviation of four independent experiments. **c)** Area under the curve of graph (b). Bars and error bars represent mean ± standard deviation of four independent experiments. 7-10 cells were analyzed in each cell line per experiment. Comparisons were performed using a paired t test. ** $P < 0.01$.

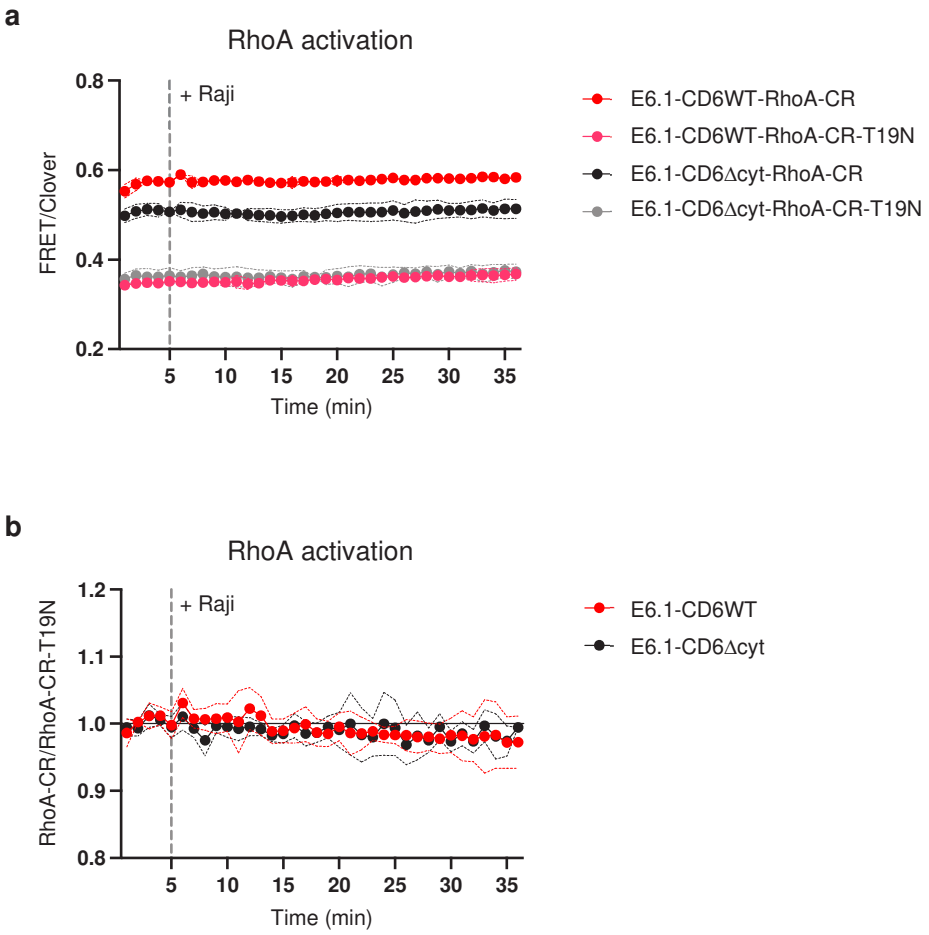

Figure 3. **Monitoring RhoA activity in E6.1 cells during IS formation.** E6.1 cells expressing RhoA-CR/RhoA-CR-T19N and CD6WT/CD6Δcyt established synapses with SEE-loaded Raji cells and RhoA activity was monitored for 35 min. **a)** FRET/Clover ratio was initially determined for all cell lines. **b)** To estimate RhoA activation, the FRET/Clover ratio in RhoA-CR cells was divided by the ratio in RhoA-CR-T19N cells for both CD6WT- and CD6Δcyt-expressing E6.1 cells (see fig. 2b) Subsequently, to calculate the fold change of RhoA activation upon E6.1 cell triggering, all time points were normalized to basal RhoA activity (average of the first three timepoints). Dots and lines represent the mean ± standard deviation of four independent experiments. 7-10 cells were analyzed in each cell line per experiment.

CD6 does not impact on overall F-actin levels

RhoA mediates diverse cellular processes in activated T lymphocytes, including cell rigidity and actin polymerization [34]. In order to examine the functional effects of CD6 on regulating RhoA activity, we initially examined whether CD6 expression modulated actin polymerization. To quantify actin polymerization in E6.1-CD6WT vs. E6.1-CD6Δcyt cells, we activated both cell lines with CD3 and CD28 mAbs for 5 min (at 3 and 5 µg/ml, respectively), fixed them and stained polymerized actin with fluorophore-conjugated phalloidin, followed by flow cytometry analysis (fig. 4a and 4b).

We observed that the levels of polymerized actin were similar between E6.1-CD6WT and E6.1-CD6Δcyt cells in both resting and activated conditions, despite the tendentially lower geometric mean fluorescence intensity (gMFI) of phalloidin staining in E6.1-CD6Δcyt cells (fib. 4c). Additionally, we observed no significant changes in F-actin accumulation in either cell line upon T cell activation (fig. 4c). Morphometric analysis of both cell lines by flow cytometry further revealed that although E6.1-CD6Δcyt cells tend to be smaller than E6.1-CD6WT cells, this difference is not statistically significant as well (fig. 4d). Overall, these results suggest that CD6 expression does not alter the accumulation of F-actin in resting or activated Jurkat T cells, as assessed by this technique.

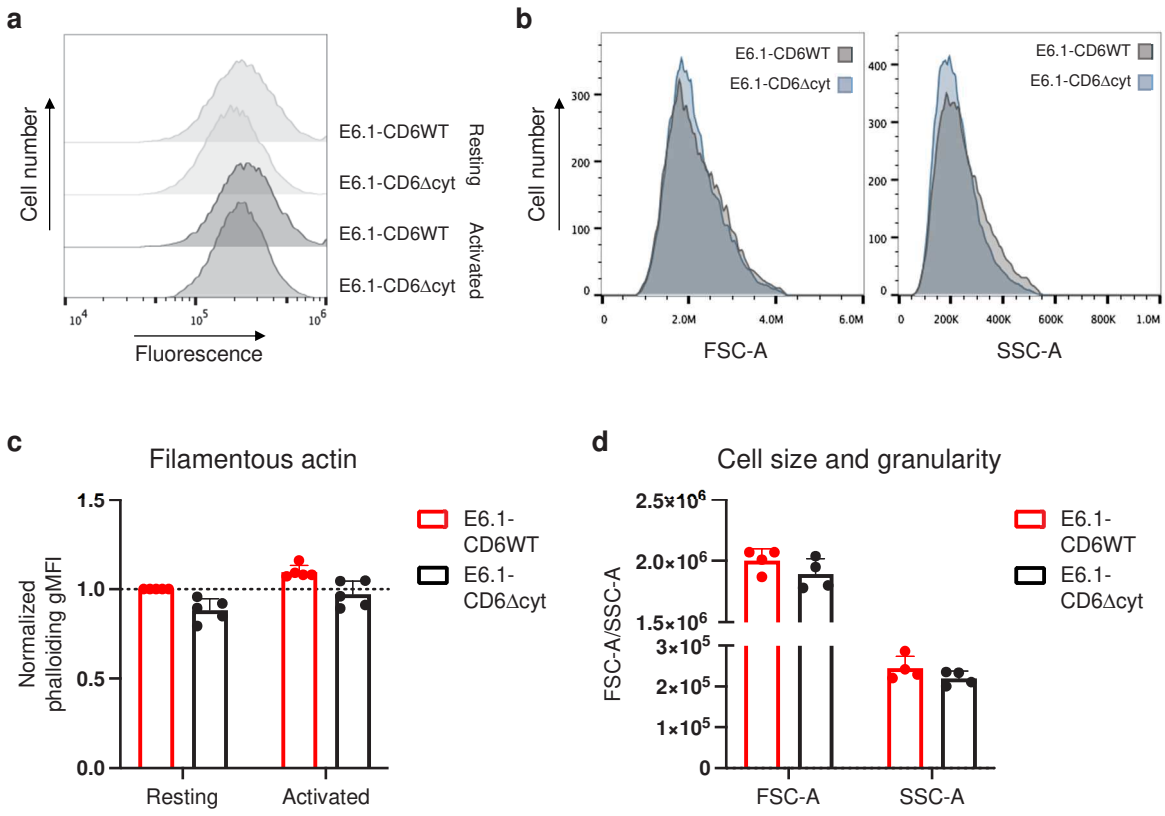

Figure 4. **CD6 expression does not overall impact on intracellular actin polymerization, as assessed by flow cytometry.** E6.1-CD6WT and E6.1-CD6Δcyt cells were activated for 5 min with anti-CD3 (3 µg/ml) and anti-CD28 (5 µg/ml) in RPMI at 37 °C. After activation, cells were fixed and stained for flow cytometry analysis with Alexa Fluor™ 488-conjugated phalloidin. **a)** Representative histograms of phalloidin staining in resting (incubated with medium alone) or activated E6.1-CD6WT or E6.1-CD6Δcyt cells. **b)** Representative histograms of FSC-A or SSC-A values in resting E6.1-CD6WT or E6.1-CD6Δcyt cells. **c)** Geometric mean fluorescence intensity (gMFI) of phalloidin staining. Phalloidin gMFI values were normalized, within in each experiment, to resting E6.1-CD6WT cells. Bars and error bars represent mean ± standard deviation of five independent experiments. **d)** FSC-A and SSC-A values for resting E6.1-CD6WT or E6.1-CD6Δcyt cells. Bars and error bars represent mean ± standard deviation of four independent experiments. Comparisons were performed using a Friedman test (for filamentous action) or a paired t test (for size and granularity analysis).

CD6 decreases adhesion of T cells to an activating surface

To further explore the functional effects of the elevated RhoA activity in CD6WT-expressing T cells, we next investigated whether CD6 expression modulates cell spreading and adhesion. For that, we plated E6.1-CD6WT and E6.1-CD6Δcyt cells on CD3/CD28 or CD28 mAb pre-coated dishes for 30 min prior to fixation and staining with an intracellular dye. Cells were then imaged and analyzed using automated systems (fig. 5a). No differences were detected regarding the spreading area of both cell lines onto the activating or non-activating surfaces (fig. 5b). On the other hand, upon T cell activation there was decreased cell adhesion dependent on the expression of CD6WT, as the number of E6.1-CD6Δcyt cells stably adhering to CD3/CD28-coated plates was higher than for E6.1-CD6WT cells (fig. 5c and fig. 6). Summarily, this suggests that CD6 decreases adhesion of T lymphocytes to activating surfaces.

Discussion

The cytoskeleton is a key element in nearly all cells, including T lymphocytes. Upon activation, the actin cytoskeleton is fundamental in the physical response of these cells to different stimuli [48]. For non-specific stimuli, such as the case of chemokine gradients, the actin cytoskeleton remodels and reorganizes in order to allow cell migration [48]. While resting T cells have uniformly distributed microvilli and flat surfaces all around, migrating T cells quickly polarize and create a leading edge and a trailing edge [49]. In the leading edge of the cell, pre-existing actin filaments will disintegrate and make way for new actin-rich structures like lamellipodia and filopodia. These structures contain highly mobile and

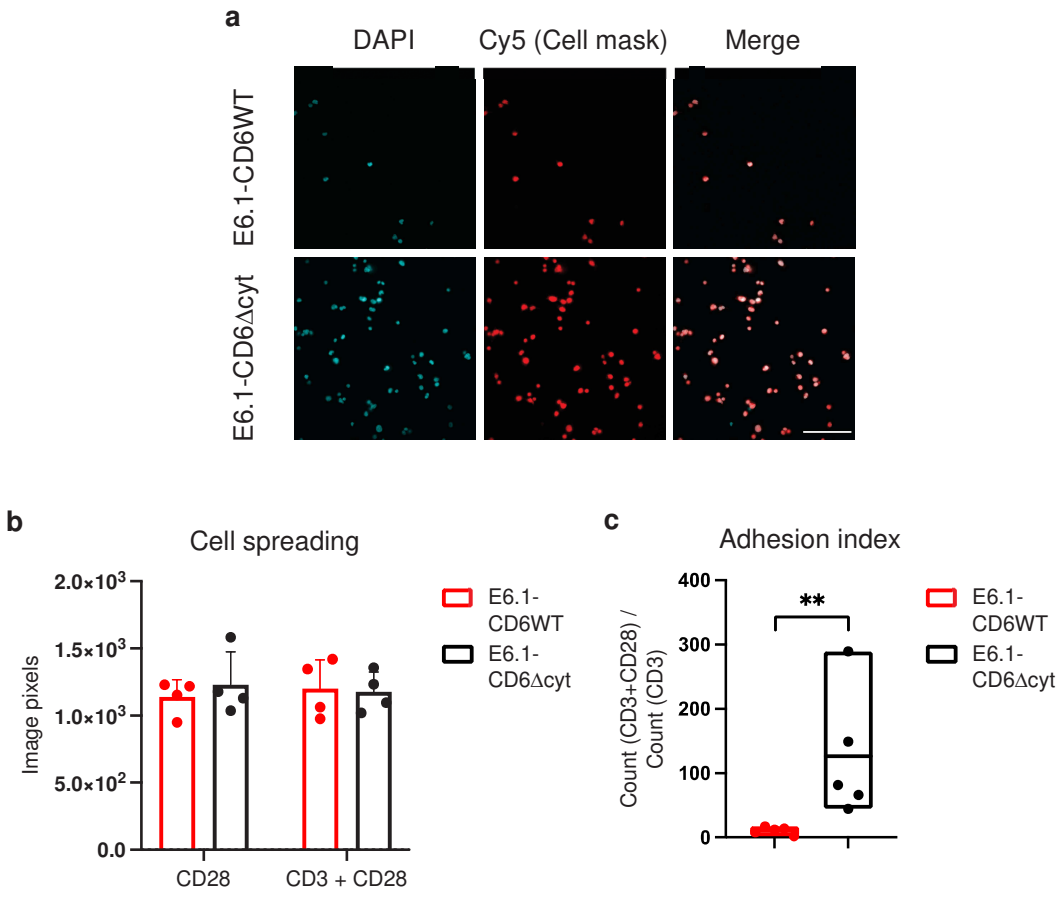

Figure 5. **The cytoplasmic tail of CD6 is responsible for decreased T cell adhesion to an activating surface.** E6.1-CD6WT and E6.1-CD6Δcyt cells were allowed to interact for 30 min at 37 °C with a plate pre-coated with mAbs for CD3 (1 µg/ml) and CD28 (5 µg/ml) or for CD28 alone (5 µg/ml). Cells were then fixed, stained with HCS CellMask™ Deep Red and DAPI and imaged. **a)** Representative images of E6.1-CD6WT or E6.1-CD6Δcyt cells attached to a CD3 + CD28 mAb-coated plate. Scale bar = 100 µm. **b)** Average area of cells attached to plates pre-coated with CD28 mAbs, or CD3 and CD28 mAbs. Bars and error bars represent mean ± standard deviation of four independent experiments. Comparisons were performed using a one-way ANOVA. **c)** Adhesion index of E6.1-CD6WT and E6.1-CD6Δcyt cells to an activating surface. The adhesion index was calculated by dividing the number of cells adherent to a plate pre-coated with CD3 and CD28 mAbs by the number of cells adherent to a plate precoated with CD28 mAb alone. Boxes represent the mean and variation of five independent experiments. Comparisons were performed using a paired t test. ** $P < 0.01$.

responsive receptors that, upon engagement, quickly trigger stabilization of cytoskeletal elements to arrest movement [50-52]. In the trailing edge, a structure called the uropod will form around the MTOC, where adhesion receptors and ezrin, radixin and moesin (ERM) proteins are concentrated [49, 53-56]. Rho GTPases are crucial in migration-induced T cell polarization: Rac1 and Cdc42 accumulate at the leading edge [57, 58] and RhoA, while

initially thought to accumulate only at the uropod contributing to the phosphorylation of ERM proteins and LFA-1 activation, is active both at the leading and trailing edges [37, 59-63].

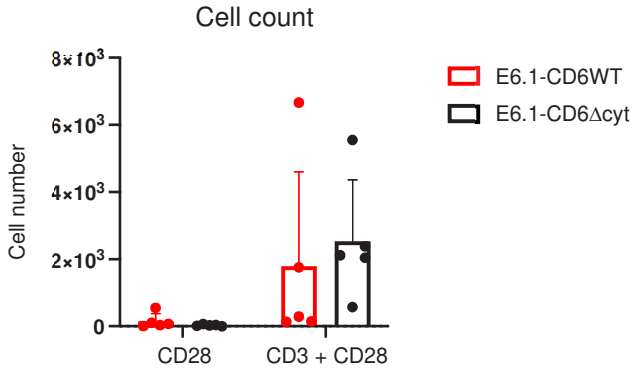

Figure 6. **Adhesion of E6.1 cells to mAb-coated plates.** E6.1 cells expressing CD6WT or CD6Δcyt were incubated for 30 min at 37 °C on a plate previously coated with CD3 (1 µg/ml) and CD28 (5 µg/ml) mAbs, or CD28 mAbs alone. After incubation, samples were fixed, stained with HCS CellMask™ Deep Red and DAPI and imaged. Shown are the total number of cells attached to the plate surfaces. Bars and error bars represent mean ± standard deviation of five independent experiments.

The actin cytoskeleton also mediates polarization during formation of the IS. In this context, the filamentous actin network will reorganize the necessary molecular complexes and act as a physical scaffold to hold them in place [12, 64]. When the TCR binds its cognate antigen, T cell migration is arrested and TCR subunits quickly agglomerate at the IS, surrounded by a second ring of integrins - a process dependent on an inward flow of actin and on myosin II [65-69]. One recent report has conducted a comprehensive analysis of the spatiotemporal distribution of several key effectors in T cell activation and described that, in activated T cells, active RhoA accumulates at the IS as well [35]. This analysis, however, was based on the distribution of a molecular reporter based on a single RhoA effector (Rhotekin), instead of the RhoA protein itself. As such, although this evidence suggests that active RhoA accumulates at the synapse, this has not yet been indisputably demonstrated.

The results presented here further suggest that RhoA accumulates at the IS and, moreover, that this accumulation is decreased when CD6 lacks its cytoplasmic tail. This observation suggests that CD6 contributes non-redundantly to the recruitment of RhoA to the IS. Although we observe that RhoA activity is not increased (or decreased) upon T cell activation, RhoA is constitutively more activated in E6.1 cells expressing full-length CD6, comparing to E6.1-CD6Δcyt cells. Considering this information, it is likely that the cytoplasmic tail of CD6 recruits, directly or indirectly, RhoA regulators. Two potential candidates have so far been described: Vav1 and ARHGAP45. Vav1, which has been

shown to inducibly bind CD6 upon T cell activation [70], is an enzyme with GEF activity towards RhoA, activating it [71]. However, considering that CD6 expression regulates RhoA activity constitutively, and that their distribution patterns seem comparable, an inducible interaction is unlikely to mediate this effect. The second candidate, ARHGAP45, has recently been described to be constitutively present in the CD6 signalosome [43]. ARHGAP45 has the opposite effect of Vav1 on RhoA, inactivating it [72]. One can speculate that, in T cells, CD6-ARHGAP45 binding is a sequestering mechanism that maintains ARHGAP45 unable to bind and/or inactivate RhoA, thereby increasing its basal activation. This hypothesis is yet to be demonstrated because although ARHGAP45 can inactivate RhoA, the relevance of this process in vivo has been questioned for Jurkat cells specifically [72]. A second concern with this hypothesis is that it is physically challenging for CD6 to simultaneously recruit RhoA (whether directly or indirectly) to the IS and to sequester its negative regulator. A third hypothesis, and a simpler one, is that CD6 regulates RhoA activity via an unknown positive regulator, bringing the two molecules closer in both resting and activating states.

To understand the relevance of CD6-mediated regulation of RhoA, we initially decided to investigate whether actin polymerization was comparable in E6.1 cells expressing CD6WT or CD6Δcyt. We chose to address this question using a simplified system where we quantified the overall amount of polymerized actin in different cell lines using flow cytometry. We found that filamentous actin is present in comparable amounts in E6.1-CD6WT and E6.1-CD6Δcyt cells and that T cell activation did not significantly increase actin polymerization in either cell line. Despite the absence of any statistically significant differences, identifiable trends were present in our results. Firstly, E6.1-CD6WT cells consistently presented slightly elevated amounts of F-actin when compared to E6.1-CD6Δcyt cells. Secondly, we found that E6.1-CD6WT cells tend to be slightly larger than E6.1-CD6Δcyt cells. These two concurrent observations suggest, in our opinion, that the tendentially higher amount of F-actin in E6.1-CD6WT cells likely reflects the expansion that results from expressing full-length CD6 in Jurkat cells. In cells expressing CD6Δcyt, this expansion is not required (and not observed) as this molecule has only a residual cytoplasmic tail. Taking into consideration that we were unable to see any meaningful activation-induced increase in actin polymerization, which is documented for lymphocytes [48, 73], we conclude that this technique is likely lacking in capacity to assess actin (de)polymerization processes. Such analysis will most likely require a finer system, such as the regularly used confocal microscopy, which we plan to establish and optimize in the future.

Lastly, we studied whether the observed differences in RhoA activity could translate into differences in the spreading of E6.1 cells onto an activating surface. It has previously

been reported that increased activity of RhoA in naïve T cells underlies a cofilin-mediated increase in stiffness of the actin cytoskeleton, generating in turn smaller synapses [34]. We considered that possibility by performing a large scale-assay where E6.1 cells interacted with a CD3/CD28 mAbs-coated plate for 30 min. After that time, cells were fixed and imaged. We chose to analyze spreading on a large-scale setup to identify differences that, even small, could be easily detected by large sampling. We found that the number of E6.1-CD6WT cells adhering to the activating surface was lower than that in E6.1-CD6Δcyt cells. Considering that the only proteins engaged in our experimental setup are CD3 and CD28 (by their respective mAbs), these differences can be potentially explained by the distinct rigidity of the cytoskeleton in both cell lines. E6.1-CD6WT cells have, as we showed, higher RhoA activity and can, as such, present with a more rigid actin cytoskeleton that forms smaller spreading regions, hindering permanent attachments and facilitating their elimination by experimental manipulation. To complement our observations, we believe that follow up studies are required taking advantage of other techniques. Specifically, assessment of spreading area using conventional confocal microscopy and of cytoskeletal rigidity using atomic force microscopy can provide further insights into the mechanisms behind the marked effect we observed.

Overall, we have demonstrated for the first time that CD6 expression constitutively regulates RhoA activity. This observation, along with our previous study identifying the RasGAP-mediated regulation of Ras by CD6 [41], positions CD6 as an important regulator of GTPases. Accordingly, it has been described that, in the absence of LAT, CD6 can function as an alternative platform for signal branching in T cells [70]. This can be facilitated by governance of GTPases, which are gatekeepers for numerous cell pathways. Although we have begun an analysis on the role of CD6 on cytoskeletal remodeling in the present study, we have not conclusively established any clear effect of CD6 expression on cytoskeletal elements. Still, many reports including our own point to that possibility and, as such, future detailed analysis of the structure of the cytoskeleton in T cells using more powerful techniques than the ones we employed, such as confocal microscopy and atomic force microscopy, are key to clarifying whether CD6 can indeed tune actin polymerization.

Acknowledgements

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General discussion and conclusions

Ever since the first description of lymphocytes, growing understanding of the role of these cells has demonstrated how central they are to the immune system and how their deregulation can trigger disease. T lymphocytes, specifically, are generated in the thymus and circulate through the human body surveilling it for the presence of pathogens. When pathogens are encountered, T cells quickly proliferate and work synergistically with other immune cell populations, to eliminate them [1].

The importance of T cells in health and disease has been recently highlighted by the attribution of the 2018 Nobel prize in Physiology or Medicine to Dr. James Allison and Dr. Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation" [2]. Both Allison and Honjo have developed new cancer therapies based on targeting immune checkpoint receptors present on the surface of T lymphocytes: CTLA-4 and PD-1, respectively. These molecules are responsible for downmodulating the response of T lymphocytes and, when they are blocked by antibodies that prevent ligand binding (called immune checkpoint inhibitors), T cell-mediated immune response to cancer is significantly enhanced. Outstanding results in cancer remission have been observed in patients treated with immune checkpoint inhibitors, and these results can be further potentiated when both CTLA-4 and PD-1 are targeted. For the reasons above and many others, T cell biology in health and disease remains a relevant research subject [3-6].

CD6 is a protein expressed mostly in T cells and thymocytes. This transmembrane molecule was first described in 1981 [7, 8] and it has been clear from the beginning of studies on its function that CD6 expression and engagement could regulate T cell activation. Initial studies have deemed its effect co-stimulatory, when several authors observed that interfering with CD6 binding to its ligand CD166 decreased T cell activation [9]. However, the real nature of CD6 net signaling potential was later clarified to be inhibitory by the depletion of CD6 in T cells. Our laboratory was the first to put these findings forward and, during later years, other researchers reached the same conclusion, strengthened by the development of CD6 knockout (KO) mice [10-12]. The present thesis constitutes one of the the first deep functional analyses of CD6-mediated inhibition of T cell activation.

CD6 recruits intracellular inhibitory enzymes

The signalosome of CD6 began to be dissected 20 years ago, when CD6 was discovered to bind CD5, a closely related inhibitory protein [13]. CD5 is, much like CD6, an adaptor protein that modulates signaling by recruiting suppressive enzymes like Cbl proto-oncogene B (CBLB) and GTPase activating protein of Ras (RasGAP) [14]. Other inhibitory proteins were also shown to be a part of the CD6 signalosome, namely ubiquitin associated and SH3 domain containing A (UBASH3A) and tyrosine-protein phosphatase non-receptor

type 6 (PTPN6) [15, 16], both of them enzymes that directly act to suppress T cell activation [17, 18]. Nevertheless, neither of these studies has investigated the mechanisms downstream of these interactions. In the present document, we have identified two new major players from the CD6 signalosome, RasGAP and CBLB, and examined the functional role of these associations.

RasGAP is a GTPase-activating protein (GAP) that inactivates Ras, a GTPase that regulates several signaling pathways. GTPases cycle between a GDP-bound inactive state and a GTP-bound active state. Active Ras can bind its effectors and initiate different signaling pathways. The canonical Ras effector is the serine/threonine-protein kinase Raf-1. When activated, Raf-1 phosphorylates mitogen-activated protein kinase kinase (MEK), which will in turn activate extracellular signal-related kinase (ERK)-1 and -2, in a signaling cascade termed the mitogen-activated protein kinase (MAPK) pathway [19]. The MAPK pathway has been extensively studied and it is crucial for cell survival and proliferation. Accordingly, Ras is activated upon T cell triggering, mediating proliferation [20]. The key role of Ras in proliferation is further illustrated by the fact that 25% of cancers are described to have Ras mutations [21, 22].

In a physiological context, T cell proliferation is essential for the role of lymphocytes in the immune system. Whenever one T cell encounters and recognizes its cognate antigen, it quickly proliferates to propagate the clonal population that can recognize said antigen. We have linked, for the first time, CD6 to a signaling pathway that promotes proliferation. Specifically, we have shown that CD6, when phosphorylated, recruits RasGAP and diminishes Ras activation, downmodulating the MAPK pathway. These results mechanistically elucidate how CD6 downmodulates proliferation in activated T cells. Additional studies are still needed to clarify whether recruitment of RasGAP is solely accountable for the role of CD6 in decreasing proliferation. It is possible that CD6 recruits other proteins to tune Ras activation, or to regulate other pathways in parallel. The second aspect to be clarified is whether CD6-mediated tuning of Ras activation impacts on the activity of other proteins, taking into consideration that enzymes other than Raf-1, like phosphoinositide-3-kinase (PI3K) and Ral guanine nucleotide dissociation stimulator (RalGDS), are directly activated by Ras [23, 24].

The second interactor of CD6 that we have identified is, in keeping with CD6 inhibitory function, the E3 ubiquitin-protein ligase CBLB. This inhibitory enzyme increases the threshold for T cell activation by holding CD28 co-stimulation as a requirement [18, 25, 26]. While the precise signaling mechanism behind CBLB-mediated inhibition is not totally clear, some downstream targets have been identified and two of them bind CD6. The first one is Vav1, an enzyme the promotes both calcium mobilization and the cytoskeletal rearrangements that follow T cell activation [25]. The co-stimulatory nature of Vav has been

difficult to reconcile with CD6 inhibitory role, along with that of other CD6 interactors like lymphocyte cell-specific protein-tyrosine kinase (LCK), 70 kDa zeta-chain associated protein (ZAP70) or lymphocyte cytosolic protein 2 (LCP2/SLP76) [13, 27, 28]. Regarding Vav1 specifically, our results make it tempting to speculate that CD6 recruits both Vav1 and CBLB to facilitate CBLB-mediated inhibition of Vav1. The second enzyme downmodulated by CBLB and recruited by CD6 (unpublished data) is PI3K. Whether CBLB downregulates PI3K activity directly or indirectly [via phosphatase and tensin homolog (PTEN)] is still a debate but, in any case, the approximation of CBLB to its target proteins can once again be potentially facilitated by CD6 [29, 30].

Reconciling CD6 inhibitory and co-stimulatory interactions

As mentioned, the presence of co-stimulatory proteins in the signalosome of CD6 is challenging to interpret in the context of its net inhibitory effect. For CD5, a protein closely related to CD6 both genetically and structurally, this was not the case. All CD5 interactors identified so far are inhibitory molecules (like CBLB, RasGAP or UBASH3A) and, for that reason, its inhibitory effect is easily explained by the recruitment of such enzymes [15]. For CD6, on the other hand, the signalosome assembled upon activation of T cells is more diverse in nature, including the inhibitory proteins CBLB and RasGAP, but also the co-stimulatory enzymes Vav1 and ZAP70, for example [15]. Three hypotheses can be raised to explain this diversity: 1) the recruitment of co-stimulatory molecules by CD6 serves the purpose of bridging the gap between them and their negative regulators, as suggested previously for CBLB; 2) the association of positive molecules in fact triggers positive signaling; or 3) the previously two points are both true but applicable to different contexts.

Although the second hypothesis (the mediation of positive signaling by CD6) appears to contradict the net inhibitory role of CD6 during T cell activation, this hypothesis becomes more realizable when considering the broader view of all lymphocytic processes. The work described in the present document was focused nearly entirely on T cell signaling during TCR-mediated activation. However, outside-in signaling in lymphocytes is crucial for other responses, like T cell migration. One group has recently addressed the role of CD6 in the progression of experimental autoimmune encephalomyelitis (EAE) in mice. EAE is an animal model for multiple sclerosis, a disease where autoreactive lymphocytes destroy the myelin sheath of neurons after crossing the blood–brain barrier, a process called transendothelial migration [31]. Looking into the role of CD6 in this process, researchers have found that CD6 potentiates transendothelial migration [11]. Accordingly, two of CD6-associated positive regulators (ZAP70 and Vav1) potentiate chemokine-triggered T cell migration [32, 33]. Although no study has yet been performed on the signaling mechanisms

via which CD6 promotes T cell migration, and ZAP70 and Vav1 have been shown to bind CD6 only upon TCR triggering [28, 34], it is possible that these associations at least contribute to CD6-mediated increase in motility.

A second dimension to be considered in the complexity of CD6-mediated signaling is the occurrence of CD6 isoforms arising from alternative splicing. Although there is only one gene for CD6, several extracellular and intracellular isoforms have been described. Extracellularly, CD6 can occur either in its full-length form or lacking its CD166-binding domain, an isoform identified by our group and increasingly expressed upon activation [35]. Intracellularly, five different isoforms have been described [36, 37]. Although these isoforms were identified long ago, there has not been sustained research following up on their variable expression. The present work has explored, for the first time, the signaling potential of several CD6 intracellular tyrosine residues. We have found that, while most of them mediate inhibitory signaling, one tyrosine residue (tyrosine 486) has a net activating effect. Interestingly, one of the few CD6 interactions mapped to the residue level is with SLP76, a co-stimulatory adaptor that binds CD6 tyrosine residue 662. Nevertheless, the net effect of that residue is negative, strongly suggesting that other protein associations are more relevant in an activation context than SLP76. Although the analysis we performed was detailed and informative, it lacks in that: 1) it considers only seven out of nine CD6 intracellular tyrosine residues, and 2) it only assesses the signaling potential of tyrosine residues, excluding other signaling motifs. Still, it demonstrates the eclectic signaling repertoire of CD6 and shows that different splicing variants of the CD6 gene could generate molecules with distinct signaling properties. As such, further studies on CD6 alternative splicing could shed some light on whether specific isoforms are increasingly expressed depending on the extracellular stimulus, endowing CD6 with either an inhibitory or activating role.

CD6 and the cytoskeleton

The present work also shows, for the first time, that CD6 expression basally potentiates RhoA activation, a GTPase polarized during T cell activation and required for transendothelial migration [38-40]. This finding aligns with previous studies showing that CD6 associates with two RhoA regulators: Vav1 and Rho GTPase-activating protein 45 (ARHGAP45) [15, 28].

To assess the role of CD6 in the regulation of RhoA activity, we monitored it in real-time during the formation of T cell-B cell immunological synapses. Besides a significant increase in the basal RhoA activity of Jurkat cells expressing CD6, our experimental setup

has further allowed us to show that RhoA activation is not altered upon T cell triggering, which had previously been suggested [38].

This new signaling axis positions CD6 as a master regulator of GTPases, once it has now been shown to modulate two GTPases from distinct families and with different functions, ranging from survival and proliferation to cytoskeletal rearrangement. A second larger implication of this finding is the existence of a link between the transmembrane protein CD6 and regulator proteins of the cytoskeleton. Nevertheless, we have not yet been able to demonstrate neither an effect of CD6 expression on cytoskeleton organization, nor a functional outcome for the regulation of RhoA activity by CD6. To address both these issues, a deeper assessment of cytoskeletal elements using more sophisticated techniques that the ones we employed here will be necessary. Regardless, the fact that CD6 increases RhoA activation is undoubtedly promising in the context of T cell migration, as CD6 and RhoA have been shown to increase transendothelial migration of lymphocytes [11, 39, 40].

Repositioning CD6 as a crucial inhibitory T cell adaptor

More than four decades have now passed since CD6 was first described. Initially described as a co-stimulatory molecule, and later clarified to be an inhibitory receptor, its influence on T cell activation has always been recognized throughout. The body of knowledge collected so far has shown us that CD6 is, in fact, a complex and versatile adaptor, able to recruit diverse enzymes and other adaptors. The present thesis adds to the complexity and the reach of CD6-mediated signaling, controlling several classes of proteins and signaling pathways. We have established in the present work that CD6, in the context of T cell activation, functions as an inhibitory adaptor that keeps activation levels in check by recruiting enzymes such as RasGAP and CBLB. We have further opened the door connecting CD6 role in T cell motility and its signaling mediators and raised the possibility that a new post-translation modification (*i.e.*, ubiquitination) can take effect on both CD6 and its signalosome, adding once more a completely new dimension to its grasp.

Understanding and fully mapping CD6 signaling pathways will be an essential facet of the future of research in T cell biology. This will allow us not only to have a deeper understanding of the mechanisms at action in T lymphocytes during homeostasis, but also to apply that information to the study of the pathophysiology of diseases such as multiple sclerosis, where T cell activation is central. Moreover, as the use of Itolizumab, a CD6 monoclonal antibody, gains ground on the treatment of inflammatory diseases [41-44], unveiling the action mechanisms of this therapy will support not only the optimization of its use, but also its potential expansion to other pathologies.

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