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Molecular mechanisms of human T cell anergy induced by the cytoplasmic tail of CD45

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Abstract

The prototypic receptor-like protein tyrosine phosphatase CD45 is proteolytically cleaved in human monocytes and granulocytes upon activation via zymosan or PMA. The cytoplasmic tail of ct-CD45 (ct-CD45) is released via activation-induced cell death. Ct-CD45 acts as a cytokine-like factor on human T cells, thereby, potently reducing proliferation and cytokine production leading to an anergy-like state in T cells.

The aim of this work is to elucidate the molecular mechanisms governing ct-CD45-induced T cell anergy. A potential receptor candidate for ct-CD45 on T cells was identified to be PRAT4A. Here, we demonstrate that binding of ct-CD45 to a murine cell line transfected with human PRAT4A can be blocked by an antibody against PRAT4A. Induction of the anergic state is accompanied by an inhibition of T cell blast formation suggesting an early cell cycle arrest. Microarray analysis of T cells activated in the presence of ct-CD45 indicated upregulation of two factors not known to be associated with anergy before - Schlafen family member 12 (SLFN12) and Krueppel-like factor 2 (KLF2). While KLF2 is a transcription factor implicated in the maintenance of T cell quiescence, little is known about SLFN12. SLFN12 is a primate-specific factor which belongs to a gene family that is involved in thymocyte development and cellular growth regulation. The expression of both factors was confirmed and analyzed via quantitative real-time PCR identifying SLFN12 as a putative anergy factor. IL-2 strongly reduced SLFN12 mRNA whereas KLF2 expression was barely changed. Furthermore only SLFN12 showed early induction in ct-CD45-treated T cells. Transfection of a SLFN12 siRNA could partially restore proliferation of anergic cells. Expression profiling of cell cycle-associated factors supported an early cell cycle arrest as cyclin D1 showed impaired induction while other factors were unaltered.

Taken together, these results demonstrate that ct-CD45 induces anergy via PRAT4A subsequently leading to the induction of SLFN12. Inhibition of cyclin D1 results in an early cell cycle arrest characterized by reduced lymphoblast formation resembling cellular quiescence. Thus, our data supports a novel functional state for T cells.

Zusammenfassung

Die klassische Proteintyrosinphosphatase CD45 kann in humanen Monozyten und Granulozyten bei Aktivierung durch Zymosan oder PMA proteolytisch gespalten werden. Die Freisetzung der cytoplasmatischen Domäne von CD45 (ct-CD45) erfolgt durch aktivierungsinduzierten Zelltod. Diese Domäne bindet als löslicher Faktor an humane T-Zellen und reduziert Proliferation und Zytokinsekretion was zur Ausbildung eines anergieähnlichen Zustandes führt.

Das Ziel der vorliegenden Arbeit ist es die molekularen Mechanismen, welche hinter der von ct-CD45 verursachten Anergie stehen, zu analysieren. Mit PRAT4A hat unser Labor in einer früheren Studie einen Rezeptorkandidaten für ct-CD45 identifiziert. In dieser Studie demonstrieren wir, dass die Bindung von ct-CD45 an eine PRAT4A-überexprimierende Zelllinie durch einen Antikörper gegen PRAT4A unterbunden werden kann.

Es wird gezeigt, dass die Induzierung dieses Zustandes von einer verminderten Bildung von Lymphoblasten begleitet wird, was auf einen frühen Zellzyklusarrest hindeutet. Microarray-Analyse von humanen T-Zellen die in der Gegenwart von ct-CD45 aktiviert wurden, zeigte die Überexpression zweier Faktoren – Krüppel-like factor 2 (KLF2) und Schlafen family member 12 (SLFN12). Während KLF2 ein bekannter, mit der Regulation von T-Zellquieszenz assoziierter Faktor ist, weiß man wenig über SLFN12. Dieser Faktor wird nur in Primaten exprimiert und gehört einer Genfamilie an, die mit Thymozytenentwicklung und zellulärer Wachstumsregulierung in Verbindung gebracht wird. Diese Studie bestätigt die Überexpression von beiden Faktoren mittels quantitativer Realtime-PCR und identifiziert SLFN12 als möglichen Anergiefaktor. IL-2 reduzierte die Expression von SLFN12 mRNA wogegen KLF2 kaum reguliert wurde. SLFN12 wurde zudem bereits kurz nach Aktivierung in der Präsenz von ct-CD45 hochreguliert. Die Transfektion einer SLFN12 siRNA verbesserte die Proliferation von T-Zellen die in der Gegenwart von ct-CD45 aktiviert wurden. Die Expressionsmuster von Zellzyklusfaktoren unterstützen einen frühen Zellzyklusarrest durch ct-CD45, da sich die Expression von Zyklin D1, nicht aber von anderen Faktoren verringert zeigte.

Zusammenfassend zeigt diese Arbeit, dass ct-CD45 über die Bindung an PRAT4A T-Zellanergie induziert und zu einer Hochregulierung von SLFN12

führt. Die spezifische Inhibierung von Zyklin D1 führt zu einem frühen Zellzyklusarrest, der sich in einer verringerten Bildung von Lymphoblasten manifestiert, welche morphologisch ruhenden Zellen ähnlich sind. Unsere Daten unterstützen daher einen neuen funktionalen Differenzierungszustand von T-Lymphozyten.

Table of Contents

1. Introduction

The central role of the immune system lies in the prevention of infection and in the containment and subsequent eradication of established infections [1]. In order to achieve this, the immune system relies on two major defence mechanisms consisting of innate and adaptive immunity.

1.1. Innate immunity

Innate immunity conveys the immediate and the early immune response towards infection [2]. Innate immune defence mechanisms include physical barriers against pathogenic entry such as epithelia, molecular components like the serum proteins of the complement system and cellular effectors including phagocytes (i.e. neutrophil granulocytes and macrophages), professional antigen-presenting cells like dendritic cells (DCs) and natural killer (NK) cells [1].

The pathogen specificity of the innate immune system is based on the recognition of conserved molecular structures which are common to different classes of microbes, but are not found on cells of the host. These conserved structures, which are also termed pathogen-associated molecular patterns (PAMPs) are bound by germline-encoded pattern recognition receptors (PRRs) [1]. Among these receptors is, for instance, the mannose receptor on macrophages which recognizes terminal mannose residues, a typical feature of many microbial glycoproteins. Triggering the mannose receptor also mediates phagocytosis and subsequent degradation of pathogens within phagolysosomes [1].

Pattern recognition receptors also comprise the Toll-like receptor (TLR) family. While there are 10 TLR family members in humans, 12 functional TLRs have been identified in mice [3]. TLRs are expressed intracellularly as well as on the cell surface depending on the type of ligand being bound. For instance, TLR-3, TLR-7, TLR-8 and TLR-9, all of them binding nucleic acids, are localized in intracellular vesicles, including the endoplasmic reticulum (ER), endosomes, lysosomes or endolysosomes [3]. Their intracellular location is crucial to enable the recognition, for example, of viral nucleic acids but also to prevent contact to self nucleic acids which would potentially initiate autoimmune disease [3].

Conversely, TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 which recognize microbial membrane or cell wall components are located on the cell surface [2,3]. For example, TLR-4 (together with MD-2 and CD14) recognizes lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, while the TLR-2/TLR-6 heterodimer binds bacterial lipoproteins, lipoarabinomannan, peptidoglycan and zymosan, a component of yeast [2].

TLRs as well as other PRRs are widely distributed on immune cells and play an important role in their activation. DCs also require signalling through TLRs in order to fully mature and acquire the ability to migrate and present antigen taken up in the periphery to naïve T cells in the lymph nodes. Thus, they are important initiators of adaptive immune responses [2].

1.2. Adaptive immunity

Unlike innate immunity the specificity of the adaptive immune system relies on the recognition of distinct antigenic structures rather than conserved microbial patterns [1]. While PAMPs are conserved macromolecular structures, the antigens recognized by adaptive immune cells are highly specific enabling the detection of non-microbial antigens. The variety of the receptors of adaptive immunity is based on the somatic recombination of gene segments which leads to a much greater diversity than can be achieved for innate immune receptors. Adaptive immune receptors are distributed on lymphocytes, i.e. B and T cells which express B and T cell receptors, respectively [1]. A distinct feature of these receptors is their clonal distribution, only a few clones of B or T lymphocytes in the entire population are specific for a certain epitope of an antigen. Upon recognition of their cognate antigen, antigen-specific clones readily expand. Some of the clones persist as long-lived memory cells initiating even stronger responses upon reencounter with the antigen. Immunological memory is thus a hallmark of adaptive immunity [1].

B cell receptors are membrane-bound antibodies or immunoglobulins (Ig) which recognize either conformational or linear epitopes of macromolecules, like proteins, lipids or carbohydrates. Antigen-binding leads to the activation of a naïve B cell and differentiation to an antibody-secreting plasma cell. Secreted antibodies are found in the blood or are at mucosal sites binding and neutralizing extracellular pathogens or toxins. This type of immunity is termed

humoral immunity. Additionally, antibodies also mediate cellular effector functions carried out by innate immune cells expressing receptors for the Fc-part of antibodies. These functions include, for instance, phagocytosis of opsonized microbes by phagocytes or complement activation via the classical pathway. These effector functions are specific for antibody isotypes which are secreted as a result of immunoglobulin class-switch [1].

Unlike B cells T lymphocytes are mediators of cell-mediated immunity towards intracellular pathogens such as viruses or intracellular bacteria. Different to the B cell receptor the T cell receptor (TCR) only recognizes processed forms of protein antigens which are presented by the body's cells on major histocompatibility complex (MHC) molecules [2].

1.3. T cell development

T cells arise from hematopoietic progenitors in the bone marrow which enter the thymus for final differentiation [4]. T cell development within the thymus consists of a series of signalling events which decide about the cellular fate of thymocytes and lineage commitment into either CD4 or CD8 co-receptor-bearing T lymphocytes. Thymocytes are initially generated via the interaction between haematopoietic stem cells and thymic stromal cells [5]. In this early phase the cells still-lack CD4 or CD8 expression and have to traverse a series of distinct double-negative stages during which the β-chain of the TCR undergoes rearrangement of its several variable (V), diversity (D) and joining (J) gene segments until a pre-TCR is assembled from the rearranged β-chain and a surrogate α-chain [2,5]. As soon as β-chain rearrangement is completed T cells start to express CD4 and the CD8αβ heterodimer. At this double-positive stage the TCR α-chain rearranges its V and J segments leading to the assembly of the fully rearranged TCR. [2, 104] Newly assembled TCRs are then selected via their avidity of binding of self-peptide:MHC complexes. Very weak to no interaction leads to "death by neglect". Conversely, a subsequent strong interaction induces cell death via "negative selection". Only intermediate interaction with self-peptide:MHC complexes leads to "positive selection" of thymocytes which become mature T cells [104]. Depending on the class of MHC-molecules recognized thymocytes either retain the expression of the CD4 or the CD8 co-receptor which gives rise to the two main T cell lineages.

1.4. Morphological and functional plasticity of T cell differentiation

Triggering of the TCR sets the stage for the functional differentiation of a T cell. Basic T cell differentiation states are accompanied by characteristic cellular phenotypes (Table 1). Quiescent lymphocytes are small with reduced metabolism but readily proliferate when receiving signals through the TCR [58]. TCR engagement in the absence of costimulation typically induces a tolerogenic or anergic T cell phenotype while full stimulation results in the differentiation into various effector cell subsets [102].

phenotype	quiescent lymphocytes	activated lymphocytes	anergic lymphocytes
cell size	small	large	large
rate of protein synthesis	low	high	low
response to activation	proliferation	expansion or apoptosis	none or apoptosis
sensitivity to FasL-dependent apoptosis	low	high	high

Table1: Morphological and functional characteristics of basic T lymphocyte differentiation states. Adapted from Yusuf and Fruman [58].

Activation results in dramatic increases in cellular size which is found in both fully activated as well as in anergic lymphocytes [58]. A key difference between these two functional states lies in their responsiveness to subsequent stimulation. While activated T cells typically show further expansion towards TCR stimulation anergic lymphocytes are hyporesponsive which is accompanied by reductions in cellular protein synthesis similar to quiescent T cells [58]. The anergic phenotype might also be accompanied by the acquirement of regulatory T cell (T_{reg}) characteristics [45]. T cell activation also renders T cell susceptible to activation-induced cell death and to apoptosis mediated via Fas ligand [58] which might be important for the contraction of immune responses and prevention of autoimmunity [103].

Differentiation of activated T lymphocytes into effector subsets depends on the cytokine environment as well as on the strength of the TCR stimulus [102].

Effector subsets of CD4^{+} T cells are generated and reinforced by a distinct milieu. However, this differentiation is not always a terminal fate and can be destabilized by changing conditions thus favoring differentiation towards another subset [107].

T cell differentiation and its plasticity are actively regulated on the molecular level involving antagonizing transcriptional programs which may be induced by external factors such as cytokines. Stability of a T cell differentiation state may also depend on transcriptional curcuits favoring its own expression via positive feedback loops while repressing another fate [107]. Examples for such processes are the mutual antagonism of the transcription factors T-bet regulating the development of Th1 cells and GATA-3 favouring Th2 cell development [107]. Highly stable states might depend on repeated cell divisions in order to be reversed such as clonal anergy which requires the T cell growth factor IL-2 to return to a state of full T cell activation [45]. Molecular factors and pathways involved in the functional differentiation of T lymphocytes are discussed in more detail in the following sections.

1.5. T cell lineages

CD4^{+} T cells

CD4^{+} T helper (Th) cells are crucial for the initiation and modulation of adaptive immunity [6]. Th cells recognize antigens processed and presented by antigen-presenting cells (APCs) on MHC class II molecules [2]. The main effector function of activated CD4^{+} T cells lies in cytokine and chemokine production [6]. Th cells may give help to primary CD8^{+} T cell responses. However, they have been shown to be essential for the generation of memory and initiation of secondary responses [14]. Similarly, there is a need for CD4^{+} T cells for the clonal selection of germinal center B cells to establish B cell memory and the regulation of antibody class switch as well as affinity maturation [6,9].

CD4^{+} T cell comprise a heterogeneous population with distinct functions [6]. Differentiation into subsets is regulated via the cytokine microenvironment but also depends on the strength of the TCR interaction with its cognate antigen [7]. Lineage choice is associated with the induction of a distinct transcriptional programme [6,7].

Differentiation into the Th1 lineage is achieved via the cytokines IL-12 and IFN-γ. The latter is the major cytokine produced by these cells and aids in the activation of macrophage-mediated phagocytosis of pathogens. Transcription factors expressed by these cells are STAT4, STAT1 and T-bet. [2, 7].

The Th2 subset requires IL-4 for its differentiation which is governed by the activity of GATA-3 and STAT6 transcription factors. Its physiological role lies in immunity against helminthic worms and other extracellular parasites by promoting antibody class switch especially to IgE. Th2 cytokines include IL-4, IL-5 and IL-13 [2, 7].

Immunity against extracellular bacteria and fungi at mucosal sites is mediated via Th17 cells which are producers of IL-17A, IL-17F and IL-22 [7]. These cytokines act proinflammatory by inducing cytokine production of epithelial, endothelial and fibroblast cells and help in neutrophil recruitment [2]. Polarization towards Th17 depends on signalling via TGF-β, IL-6, IL-21 and IL-23, ultimately leading to activation of the lineage-specific transcription factor (ROR)γt [7]. However, interspecies differences have been found between mice and human Th17 cells in several aspects. In contrast to mice, the human Th17 cell lineage not only expresses (ROR)γt but also T-bet as central transcription factors. Furthermore these cells show the propensity to secret IFN-γ besides IL-17A in the presence of IL-12 suggesting a close relationship to Th1 cells. There is also indication for a different origin of human Th17 cells from CD161^{+} precursors with constitutive (ROR)γt and IL-23R expression [8].

Another possible T helper cell subset was discovered recently. Th22 cells were found to produce IL-22 in the absence of other lineage-specific cytokines [105]. Similar to IL-17, IL-22 exerts its action on non-hematopoietic cells rather than immune cells. IL-22 production seems to be linked to inflammatory states of the skin during which Th22 cells probably develop from the Th17 subset [105].

T follicular helper cells (Tfh) are a subset of CD4^{+} T cells which regulates antigen-specific B cell immunity (7,9). Tfh cells are located to B cell follicles through their expression of the chemokine receptor CXCR5 [9]. Their differentiation requires IL-6 and IL-21 leading to the expression of a specific transcriptional program including Bcl-6 and STAT-3 [9].

Another important T helper cell population are regulatory T cells. T_{regs} express FOXP3, a transcription factor critical for regulatory T cell function. Two major

groups of regulatory T cells have been characterized depending on their site of induction [7]. While naturally occurring $CD4^+CD25^+$ Tregs (nT_{regs}) arise from the thymus [7], T regulatory cells can also be induced in the periphery via TGF-β signalling (iT_{regs}) leading to the expression of FOXP3 [7,10]. Both types of cells are assumed to play an essential role in peripheral immune tolerance in order to prevent autoimmunity [7] with their suppressive activity mainly relying on the action of TGF-β [11]. However, different to iT_{regs} the suppressor activity of nT_{regs} seems to be blocked by IL-6 [11].

Another subset of $CD4^+$ T cells are Natural killer T cells, although these cell type can also have a $CD4^-CD8^-$ phenotype [106]. NKT cells are generated during thymic development of T cells at the double-positive stage via recognition of CD1d which is a non-classical class I antigen-presenting molecule for glycolipids [106]. Characteristic features of NKT cells are the expression of a TCR with limited α-chain diversity while also having NK cell receptors. Interestingly, NKT cells seem to acquire a memory T cell phenotype already during thymic selection and produce both, Th1 and Th2 cytokines upon activation [106].

CD8$^+$ T cells

Cytotoxic T lymphocytes (CTLs) are essential for the immune response against viruses. They express the CD8 co-receptor and thus recognize peptide:MHC class I complexes [1,2,12-14]. Antigen processing for display on MHC class I typically involves the uptake of cytosolic proteins into the endoplasmic reticulum (ER). However, $CD8^+$ T cells can also be presented with extracellular antigens via a mechanism termed cross-presentation. Antigens derived from virus-infected cells or tumor cells taken up via phagocytosis by an antigen-presenting cell can be translocated from the ER to the cytoplasm and thus be alternatively loaded on MHC class I molecules [2]. Cross-presentation is most efficiently performed by dendritic cells [15].

Upon activation in the lymph nodes via dendritic cells CTLs rapidly expand undergoing cell division every 4 to 6 hours [16]. During this phase they also gain effector functions and the capacity to migrate to reservoirs of infection [16,17]. The effector functions of CTLs mainly rely on their cytoxic or cytolytic activity whereby target cells are killed upon recognition either via the release of lytic

granules containing perforins and granzymes or through the engagement of death receptors [17]. Perforins are calcium-dependent pore-forming proteins which are involved in the delivery of other granule contents, while granzymes are serin-proteases which cleave the pro-apoptotic proteins such as Bid leading to cytochrome c release and caspase-activation [18]. However, cell death can also be induced independent of granzymes. CTLs express ligands for receptors of the tumor necrosis factor (TNF) superfamily which are expressed on the target cells including Fas, TNFR1, TNFR2 or TRAIL-R [18]. The FasL-Fas interaction leads to the direct recruitment of pro-caspase 8 via adaptor molecules subsequently initiating apoptosis in a caspase-dependent fashion [18].

1.6. T cell activation

The process of T cell activation is initiated in secondary lymphoid organs such as lymph nodes where naïve T lymphocytes search the surfaces of dendritic cells for specific peptide-MHC complexes [19]. Recognition of less than 10 specific pMHC complexes has been found sufficient to induce long-lasting contacts between T cells and dendritic cells ranging between 6 and 18 hours [19]. These contacts ("immunological synapses") finally lead to the activation of the T cells which subsequently enter the cell cycle and differentiate into effector cells [19]

Central to this process is the ligation of the T cell receptor with its cognate pMHC complex which determines antigen specificity and initiation of T cell activation. However, additional signals are needed for the full activation of T cells. Signaling through the TCR alone was not only found to be insufficient for activating T cells but also to result in a hyporesponsive state termed anergy [20]. Thus, a two-signal-model was established. "Signal 1" refers to the engagement of the TCR, while "signal 2" implies the ligation of additional costimulatory molecules presented by antigen-presenting cells [20].

The immunological synapse

The term "immunological synapse" (IS) broadly refers to any functional structure formed between APC and T cell, although its original meaning was restricted to a characteristic "bull's-eye" structure formed by concentric rings of molecules

[21]. The inner circle or central supramolecular activation cluster (cSMAC) consists of the TCR, the co-receptors (CD4 or CD8) as well as of receptors engaged during co-stimulation such as CD28 or CD2. The outer circle is termed peripheral supramolecular activation cluster (pSMAC) containing integrins, especially LFA-1, which are important for cell-cell adhesion [19, 21]. Outside the pSMAC lies an area of active membrane movement called distal supramolecular activation cluster (dSMAC) [19].

Initially, the function of the cSMAC was assumed to lie in the delivery of the activating signals. However, it was found that this central ring rather serves TCR internalization and degradation while active TCRs are mainly located within the pSMAC [19]. Thus, the function of the cSMAC might be linked to the regulation of long-term T cell responses. Besides T cell priming, the IS might also function in mediating the contact between activated CTLs and their target cells focussing the content of cytolytic granules on the target cell to avoid causing damage to bystander cells [19].

However, the bull's-eye structure is not required per se to initiate T cell activation. Contact of T lymphocytes with dendritic cells is characterized by the establishment of multiple TCR clusters giving rise to multifocal immunological synapses resulting in efficient activation of the T cells [21].

TCR signal transduction

The TCR consists of an αβ heterodimer which does not contain any intrinsic signaling activity. TCR signal transduction requires the association with the CD3 complex consisting of dimeric δε, γε; and ζζ chains [22]. Binding of cognate pMHC complexes activates protein tyrosine kinases (PTKs) of the Src family which are associated either with the TCR or the co-receptors [22].

Upon activation, Src kinases such as Lck or Fyn phosphorylate immunoreceptor tyrosin-based activation motifs (ITAMs) within the CD3 chains which serve as recruitment domains for ZAP-70 kinase. ZAP-70 phosphorylation via Lck activates the former leading to further downstream signalling events [22]. Targets of ZAP-70 are the transmembrane adaptor protein LAT and the cytosolic SLP-76 which contains a Src homology 2 (SH2) domain. The two adaptors form the basis of a protein complex from which multiple signalling pathways originate [22]. Phoshorylation of LAT tyrosine residues via ZAP-70

leads to the recruitment of other proteins including phospholipase Cγ1 (PLCγ1), phoshoinositide-3-kinase and the adaptor proteins Grb2 and Gads [22].

Activation of PLCγ1 upon TCR ligation leads to the enzymatic hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP(4,5)P$_2$) into phosphoinositol-1,4,5-trisphosphate (PIP$_3$) and diacylglycerol (DAG) both serving as second messengers [22,23].

PIP$_3$ binds to ion channel receptors of the endoplasmic reticulum (ER) which are permeable for Ca^{2+} thus initiating release of Ca^{2+} ions into the cytosol. Ca^{2+} released from the ER additionally stimulates the inflow of extracellular Ca^{2+} ions via Ca^{2+}-dependent channels (CRAC) of the plasma membrane [22]. The increase in cytosolic Ca^{2+} leads to the activation of various Ca^{2+}- and calmodulin-dependent transcription factors as well as signalling factors such as calcineurin, a serin/threonin phosphatase [22,24].
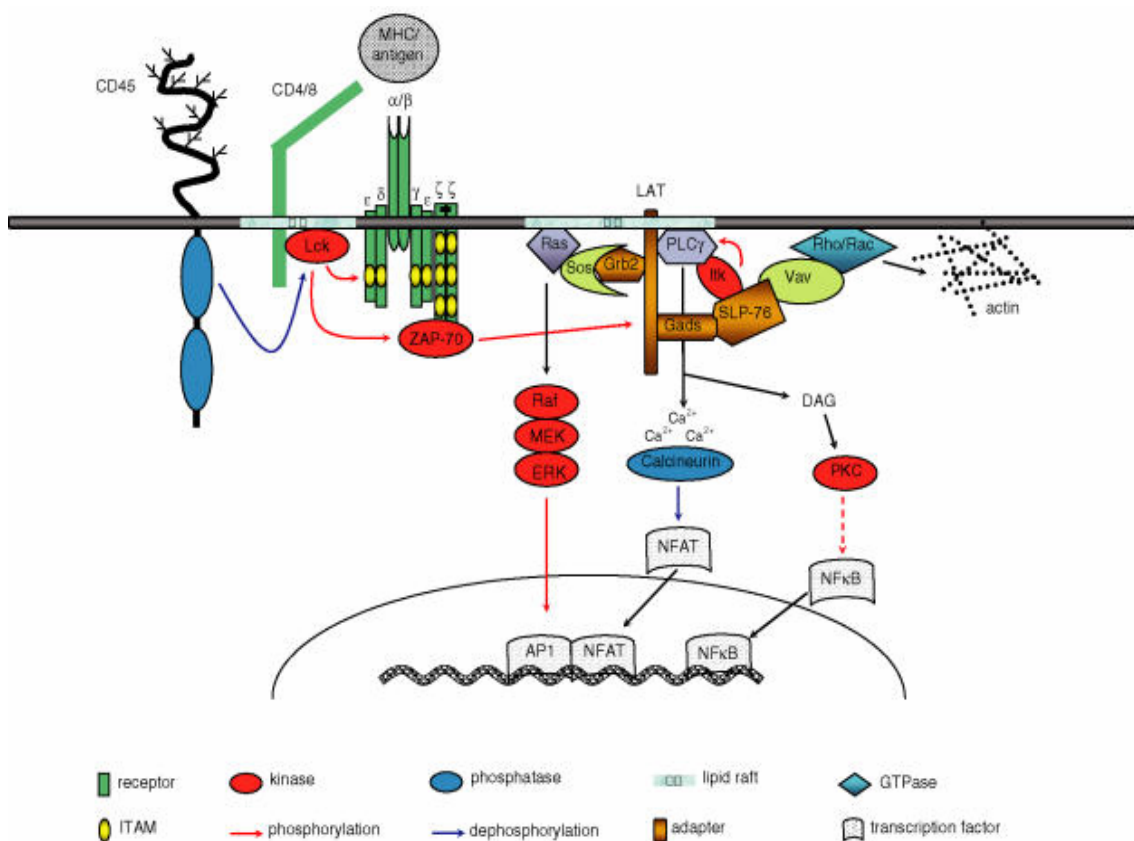
Fig. 1: Simplified scheme of TCR signalling pathways. Major components involved in the signal transduction upon TCR ligation and costimulation (not shown). Signalling results in the recruitment of transcription factors central to T cell function including NFκB and AP1 which forms heterodimers with NFAT [22, 30]. Figure was adapted from Razzaq et al. [30].

Calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) transcription factor family, namely NFAT1, NFAT3 and NFAT4 [24]. Dephosphorylation of N-terminal serine residues within NFAT proteins reveals a nuclear import sequence subsequently mediating their translocation to the nucleus [24]. Within the nucleus NFAT forms cooperative complexes with other transcription factors thus linking several signalling pathways [22,24]. These interactions depend on additional signals besides the one released via the TCR [22], for example, on the presence of IL-2 signalling [25]. One of these interactions is the formation of NFAT/AP-1 complexes [22]. The transcription factor activator protein 1 (AP-1) is a dimeric protein consisting of either Fos-Jun heterodimers or Fos-Fos homodimers. The complex formed by NFAT/AP-1 combines the signals of the Ca^{2+}/calcineurin and of the mitogen-activated protein kinase (MAPK) pathways [24]. Interaction of NFAT with AP-1 is important for the expression of genes regulating for T cell proliferation and effector function including IL-2, IFN-γ and IL-4 [22, 24]. Conversely, NFAT activity without the action of AP-1 has been shown to result in T cell anergy [26]. DAG, the second product of $PIP(4,5)P_2$ hydrolysis serves as the activator of two signalling pathways, namely the Ras-Raf-MAPK pathway and the PKCθ pathway [22]. Ras is a GTP-binding protein which is required for the activation of the serine/threonine MAPK kinase kinase (MAPKKK) Raf-1. Raf-1 phosphorylates MAPK kinases (MAPKK) which themselves activate the MAP kinases (MAPK) Erk-1 and Erk-2. The latter activate the transcription factor Elk-1 subsequently inducing the expression of AP-1 [22].

Protein kinase Cθ (PKCθ) is activated by DAG via binding to its specific lipid-binding domain which recruits the kinase to the plasma membrane [22]. PKCθ induces then the activation and nuclear translocation of the nuclear factor NF-κB (NF-κB) by promoting the assembly of a complex of the adaptor proteins CARMA1, Bcl-10 and MALT1 [27]. Serine phosphorylation of CARMA1 induces complex formation which subsequently activates the inhibitor of NF-κB kinase (IKK) complex. IKK then promotes degradation of inhibitor of NF-κB (IκB) which sequesters the transcription factor in the cytosol. NF-κB release results in its nuclear translocation [27]. NF-κB is a transcription factor essential for the function of immune cells. In lymphocytes, it regulates cell proliferation by induction of cell cycle-associated genes such as cyclin D1, cyclin D2 or c-Myc

as well as cell survival by the upregulation of the anti-apoptotic genes Bcl-2 and BCL-XL [28]. NF-κB also regulates cytokine genes including the T cell growth factor IL-2 or pro-inflammatory IL-6. Another NF-κB target gene is the costimulatory molecule CD40 ligand (CD40L) [28].

T lymphocyte activation through TCR signalling is also associated with profound cytoskeletal changes which are necessary to mediate various tasks important for T cell function, including cell growth, migration, cell-cell adhesion to APCs as well as to endothelial cells and extravasation into tissues [29]. TCR stimulation induces the formation of receptor clustering and of immunological synapses given the presence of costimulation. These events require the linkage to the actin and myosin cytoskeleton which convey these dynamic changes. A key molecule for linking TCR signals to the cytoskeleton is the guanine nucleotide exchange factor Vav1 which is linked to the LAT signalosome via SLP-76. Vav1 functions to activate the Rho-like GTPases cdc42, Rac and Rho which mediate cytoskeletal changes [29].

Signalling pathways originating from TCR stimulation are summarized in Fig. 1.

T cell costimulation

Costimulation serves as "signal 2" to TCR stimulation and is required for the full activation of T cells. Although soluble factors such as cytokines can augment T cell activation the term "costimulation" is typically restricted to the interaction of membrane-bound receptor-ligand pairs. Costimulation can promote both, activating as well as inhibitory pathways depending on the nature of the costimulatory molecule engaged [31].

The most intensively investigated costimulatory interaction is the one of the T cell receptor CD28 with its ligands of the B7 family. CD28 is a homodimeric glycoprotein which is linked via disulfide bonds. It spans the plasma membrane displaying type I transmembrane protein topology [20].

Human T cells do not uniformly express CD28. While about 80% of CD4^{+} T cells are positive for CD28, it can be detected on roughly half of all CD8^{+} T cells [20]. Signalling through CD28 has profound effects on the threshold of TCR triggering which is significantly lowered. The increased sensitivity also results in enhanced cytokine production, most importantly of IL-2 [20].

CD28 engages with two ligands on APCs, namely B7-1 (CD80) and B7-2 (CD86). However, these ligands interact with another receptor on T cells termed cytotoxic T lymphocyte associated antigen 4 (CTLA-4) which transduces inhibitory signals into T cells [31,32]. Although structurally similar, CTLA-4 has higher binding affinities for both ligands. The two receptors display differential distribution on T cells. While CD28 is expressed on both, resting as well as activated cells, CTLA-4 is induced during the process of T cell activation [32] in order to prevent excessive T cell responses [31]. However, its expression is constitutive on T_{regs} [32]. Lack of CTLA-4 results in uncontrolled T cell activation and autoimmune disease [33].

CD28, CTLA-4 and their ligands CD80 and CD86 belong to the B7-CD28 superfamily which is generally accepted as the major group of T cell costimulatory molecules [31]. Other members of this family include inducible costimulator (ICOS) and its ligand (ICOSL) which are associated with the differentiation of T helper cells, the production of Th lineage cytokines and the regulation of B cell responses [31] by promoting the induction of follicular T helper cells [33]. CD28 and ICOS were shown to act synergistically in T cell activation as only the absence of both pathways resulted in the loss of T cell effector function [34].

Another receptor-ligand interaction of the CD28-B7 superfamily is the one of programmed cell death 1 (PD1) with its ligands PD-L1 and PD-L2. Its primary action is inhibitory, although an alternative activating function has been described in mice [31].

The Tumor necrosis factor receptor (TNFR) superfamily also contains several costimulatory molecules the most potent of which is the 4-1BB/4BBL interaction. Other important interactions within this family are the ones of OX40 with its ligand or of CD27 with CD70 [31].

Besides the two major pathways mentioned other costimulatory pathways exist including the interaction of members of the CD2 superfamily, integrins, tetraspanins or the TIM family [31].

Integrins comprise a large protein family which function as receptors as well as adhesion molecules. Their structure is heterodimeric consisting of α and β subunits [35]. Integrin are expressed on cell surfaces typically in an inactive form. Signalling via other receptor such as the TCR is required to achieve an

active conformation which is dependent on linkage to the cytoskeleton [35] The main T cell integrin is lymphocyte-function associated antigen 1 (LFA-1) which interacts with intercellular adhesion molecule 1 (ICAM-1) [31,35] as well as with ICAM-2 and ICAM-3 [31]. This interaction functions on the one hand to promote arrest of T cells on endothelial surfaces inside blood vessels enabling subsequent transmigration and on the other one to establish contact and synapse formation with APCs [35]. In the latter interaction, LFA-1 initiates costimulatory pathways similar to CD28 [31].
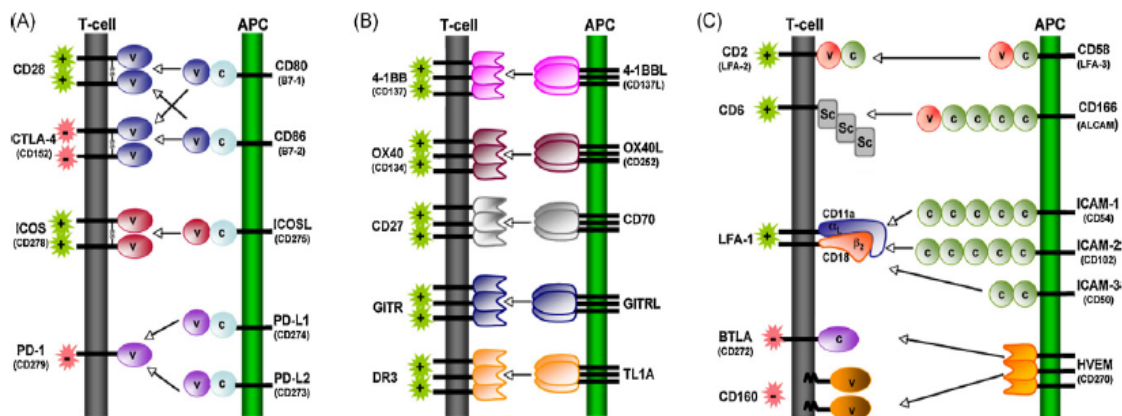
Fig. 2: T cell costimulatory molecules. (A) Members of the CD28-B7 family, (B) Costimulatory molecules of the TNF receptor family, (C) Members of other pathways. Symbols (+) and (-) within the cytoplasmic tails of the receptor molecules depict costimulatory and coinhibitory function, respectively. Figure was adapted from Leitner et al. [31].

Tetraspanins are cell surface proteins which contain four characteristic membrane-spanning domains [36]. Many tetraspanins are regulated during activation of immune cells involving either up- or downregulation. On the molecular level some tetraspanins have been shown to interact with integrins, the T cell CD4/CD8 coreceptors, but also with members of their own family [36]. In the context of costimulation, the function of tetraspanins has usually been studied using antibody-mediated ligation or via loss-of-function studies, even though for some family members interaction partners have been found, e.g. HCV-E2 interacts with the tetraspanin CD81 [31]. Another tetraspanin which is targeted in this study for costimulatory reasons is CD63. A monoclonal antibody binding to CD63 was found to act costimulatory if bound to a plate resulting in T cell proliferation, cytokine production and induction of anti-apoptotic pathways similar to CD28 costimulation [37]. Additionally, it was shown that T cells

primarily activated via CD3 and CD63 antibodies respond even more readily to restimulation than in the context of a primary CD3/CD28 stimulus thus indicating a potent costimulatory role for this tetraspanin [37].

1.7. T cell tolerance

Immunological tolerance is defined as a state of unresponsiveness towards self or foreign antigens [38]. While protective immune responses are essential for clearance of pathogens immunological tolerance is required towards self antigens [1, 2]. T cell unresponsiveness towards the latter plays a central role for the establishment and maintenance of tolerance. Mechanisms involved are clonal deletion via apoptosis, the induction of anergy, active suppression of T cell responses and antigen sequestration. T cell tolerance is achieved in central and peripheral lymphoid organs, thus being termed central and peripheral tolerance, respectively [38, 39].

T cell central tolerance

The establishment of T cell central tolerance is based on the selection of T lymphocytes during their development in the thymus [2, 5, 8, 39].

The selection process of thymocytes takes place at the CD4$^+$/CD8$^+$ double-positive stage after the rearrangement of the TCR. After this step, immature cells are destined to undergo apoptosis or "death by neglect" unless they are rescued by a weak signal through the TCR by interaction with self-peptide-MHC complexes provided by cortical thymic epithelial cells (cTECs) [38,39]. Most of the peptides interacted with during positive selection are not unique to the epithelial cells, but involve peptides from other tissues of the body. Explanations for the necessity for immature cells to weakly react to self antigens involve the requirement for self-MHC restriction, i. e. T cells have to be able to recognize foreign peptides displayed on self-MHC molecules. Another aspect might be that T cells might require low levels to stay alive in the periphery as cells transferred into MHC-deficient hosts gradually disappear [38].

During the overall process of positive selection more than 95% of thymocytes undergo apoptosis due to the lack of reactivity of their TCR. After positive selection the cells enter the single-positive stage. Depending on the reactivity to MHC class I or class II either the CD8 or CD4 coreceptors are retained [38,39].

Furthermore, the expression of chemokines is induced which leads the selected thymocytes to the thymic medulla for further maturation [39].

Negative selection is performed towards T cells reacting strongly to self antigens which are abundantly expressed in the medulla. However, medullary thymic epithelial cells (mTECs) seem to express a wider array of self-antigens than cTECs [39]. Generally, expression of self-antigens is controlled by the transcription factor autoimmune regulator (AIRE) the loss of which was shown to result in excessive autoimmunity [40].

Negatively selected T cells undergo apoptosis or clonal deletion. This process involves specific dendritic cell subsets which are only found in the medulla [38]. It is assumed that DCs acquire self-antigens from mTECs involving phagocytosis of dead cells as well as obtaining material from living cells, e.g. via exosomes [39].

Apotosis induced upon negative selection also seems to be dependent on costimulation or Fas. While moderate self-reactivity seems to require costimulation while being independent of Fas, strong TCR avidity for self-peptides initiates apoptosis in a Fas-dependent form [39].

The thymus is also the induction site for $CD4^+CD25^+FOXP3^+$ natural T regulatory cells. T_{regs} display moderate to high avidity for self-antigens and are a means for non-deletional tolerance induction in the periphery. However, it is unclear how T_{regs} are rescued from apoptosis in order to find a different fate [39].

T cell peripheral tolerance

Although most autoreactive T cells are efficiently deleted in the thymus some cells which are reactive towards self-antigens escape to the periphery [41]. Typically, these cells display relatively weak TCR avidity towards self antigens. However, even in the absence of active immunosuppression these cells may not necessarily act autoreactive due to the phenomenon of immunological ignorance. Naïve T lymphocytes show a restricted circulation pattern involving migration from the blood to the lymph nodes which is subsequently re-entered via efferent lymphatics [41]. Tissue-specific expression of some antigens limits the access of naïve T cells to their respective self-antigen, thus avoiding the encounter with self-peptide-MHC complexes. Effector-memory T lymphocytes

display a more efficient entry into peripheral tissues. However, they only pose a serious threat in terms of autoimmunity if TCR re-ligation occurs under inflammatory conditions [41].

A major cell type involved in peripheral T cell tolerance mechanisms are dendritic cells. While being highly efficient in activating T cells in a mature state, immature DCs are poorly immunogenic [42].

Dendritic cell populations undergoing migration from peripheral tissues to the draining lymph nodes in the steady-state (i.e. in the absence of inflammation) were found to act tolerogenic on T cells. Characteristically, these cells display a semi-mature phenotype and are capable of inducing either FOXP3$^+$ adaptive T regulatory cells (aT$_{regs}$) or FOXP3$^-$ Tr1 cells which are IL-10 producers [43].

The induction of tolerogenic dendritic cells (tDC) is thought to be influenced by factors in their local microenvironment. For example, DCs in the gut are typically associated with tolerance induction which is assumed to be dependent on anti-inflammatory factors like TGF-β, retinoic acid, IL-10 or thymic stromal lymphopoietin (TSLP) [42].

Negative costimulatory or coinhibitory molecules also play a major role in the maintenance of T cell tolerance, including CTLA-4 and PD-1. Knockout studies in mice indicate an early role for CTLA-4 in control of homeostatic T cell proliferation as mice deficient in CTLA-4 die soon after birth. PD-1 deficiency requires several months for the onset of autoimmunity which is restricted to certain tissues. Although not directly pro-apoptotic, signalling through both receptors antagonizes the induction of survival factors which favours activation-induced cell death [44]. Coinhibitory signals are also associated with the induction of T cell anergy [33, 44].

1.8. T cell anergy

T cell anergy is a mechanism of peripheral tolerance induction where T lymphocytes are still alive but hyporesponsiveness to antigenic stimulation [45]. Basically, two forms of anergy are distinguished; "clonal anergy" and "adaptive tolerance" which is also termed "in vivo anergy". Clonal anergy mainly represents a state of growth arrest while the latter state also involves inhibition of T cell effector functions [45].

Clonal T cell anergy can be induced via a strong TCR signal without costimulation or via a weak signal in the presence of costimulation. The state is associated with reduced production of IL-2 reflecting the growth arrest but not necessarily of other cytokines. Typically, clonal anergy can be reversed by addition of exogenous IL-2 [45].

Adaptive tolerance has been described in vivo in the persistence of antigen involving rapid T cell expansion followed by a gradual decline of cell numbers. Remaining cells are hyporesponsive to restimulation and display a reduction of several other cytokines besides IL-2. Upon antigen removal most T cells lose their anergy within a week. This is different to clonal anergy where the unresponsive state can last for months. However, in most models of in vivo anergy IL-2 was not sufficient to reverse the hyporesponsive state [45].

Molecular factors maintaining clonal T cell anergy

Although in principal a growth arrest state, clonal anergy is associated with the induction of a distinct transcriptional program involving signalling molecules, transcription factors [46] and cell cycle regulators [47]. Additionally, clonal anergy might also be maintained via chromatin remodelling [33].

The initial signalling process promoting the induction of anergy is usually described as a defect in TCR signalling pathways. While calcium signals promoting the nuclear translocation of NFAT are intact, defective activation of the Ras/MAPK pathway leads to the diminished activation of AP-1 (Fig. 3). The resulting imbalance of signals impairs the transcription of the genes dependent on the NFAT-AP1 heterodimer while favouring NFAT-dependent genes which include anergy-associated genes [46]. Clonally anergic T cells also display impaired activation of NF-κB which is characterized by a lack of IκB degradation upon T cell activation [97].

E3 ubiquitin ligases

Many cellular processes are regulated by ubiquitylation, where the small protein ubiquitin is linked to lysine residues of target proteins [48]. Depending on the type of ubiquitylation different outcomes are achieved including for example proteasome-mediated degradation or cellular relocation of a protein. The ubiquitylation process is achieved by the action of three enzymes: the ubiquitin-

activating enzyme E1 binds ubiquitin via a thioester linkage which is then transferred to the ubiquitin-conjugating enzyme E2 forming a complex with the ubiquitin ligase E3. E3 binds to degradation signal sequences of target proteins and aids in the transfer of the polyubiquitin chain to lysines [48].

E3 ubiquitin ligases have been shown to play important roles for the induction and maintenance of T cell anergy [46].
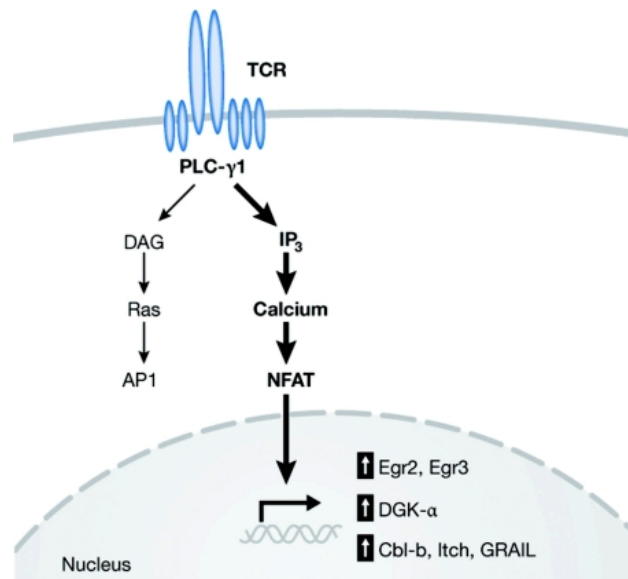
Fig. 3: Signalling pathways involved in clonal anergy induction. Defective activation of the Ras/MAPK pathway and excessive mobilization of Ca^{2+} leading to NFAT activation are assumed to be the main pathway in the induction of anergy. Fig. was adapted from Zheng et al. [46].

The most well-known E3 ubiquitin ligase is Casitas B cell lymphoma b (CBL-b). CBL-b has a decisive role in the negative regulation of TCR signals as it targets the p85 subunit of phosphoinositide-3-kinase which thus cannot associate with the TCR or CD28 [49]. This subsequently leads to the inhibition of NFκB activation via PKCθ and Akt and prevents cytoskeletal rearrangements via Vav1. CBL-b deficiency in mice results in severe autoimmunity [49].

Deletion of the E3 ligase Itch leads to a dermatitis-type inflammation of the skin accompanied by constant itching [33]. Itch ubiquitylates the Th2-specific transcription factor JunB leading to its degradation. Itch deficiency is associated with an increased production of IL-4 and IL-5 as well with elevated IgG1 and IgE serum levels [33, 49]. Other targets of Itch within TCR signalling pathways are PLCγ and PKCθ which are subsequently degraded within the proteasome [33].

Gene related to anergy in lymphocytes (GRAIL) is a type I transmembrane protein which is localized in endosomes [49]. GRAIL is strongly induced in anergized T cells and is sufficient to render T cells anergic in overexpression experiments [49]. A putative target of GRAIL is the Rho guanine dissociation inhibitor which is stabilized by ubiquitylation and thus inhibits activation of the RhoA GTPase, another important factor in TCR signalling. Other likely targets are transmembrane proteins such as CD40L and the tetraspanins CD151 and CD81 [49]

Transcription factors

The early growth response genes Egr2 and Egr3 are zinc finger transcription factors which are highly implicated in the induction of the anergic state [46]. Both *Egr* genes are strongly transcribed upon TCR stimulation. However, costimulation via CD28 significantly reduces the level of Egr mRNA [50]. Overexpression of Egr proteins results in decreased synthesis of IL-2 by T cells and seems to regulate the transcription of other anergy factors including CBL-b and diacylglycerolkinase α (DGK-α). Conclusively, deficiency in Egr3 was shown to confer resistance to anergy induction [46].

Proteins expressed in quiescent cells may also serve the maintenance of anergy. DNA binding zinc finger proteins including Ikaros, Blimp-1, p50 homodimers of NFκB and Tob-Smad heterodimers are highly expressed in resting cells and mediate the transcriptional repression of the *il2* gene via recruitment of histone deacetylases (HDAC) [51]. HDAC activity maintains histones at the *il2* promoter in a hypoactetylated form which is transcriptionally inactive. Converting the promoter into an active conformation via chromatin remodelling is dependent on CD28 costimulation [51].

Diacylglycerol kinases

Diaclglycerol (DAG) is an important activator of the Ras/MAPK and PKCθ-dependent NFκB activation pathways. Diacylglycerol kinases (DGK) mediate conversion of DAG into phosphatidic acid thus impairing activation of both pathways [52]. DGK-α as well as DGK-ζ have been associated with anergy induction since deficiency in either gene has been shown to result in resistance to anergy induction. Conversely, DGK-α overexpression was found sufficient to

induce anergy in T cells [52]. Although T cells deficient in either DGK are hyperresponsive to TCR stimulation autoimmune disease only develops in double-knock out mice suggesting a potential redundant function of these kinases [52].

Cell cycle-associated factors

Anergy induction seems to be closely linked to cell cycle progression of activated T cells. Although costimulation via CD28 during a primary response is commonly associated to be sufficient for escaping anergy this only holds true for cells undergoing several rounds of cell division while expressing high levels of IL-2 and IFN-γ [47]. Cells who fail to divide during the primary response do not produce IL-2 and are hyporesponsive to secondary stimulation even though provided with both, a full signal 1 and signal 2 [47].
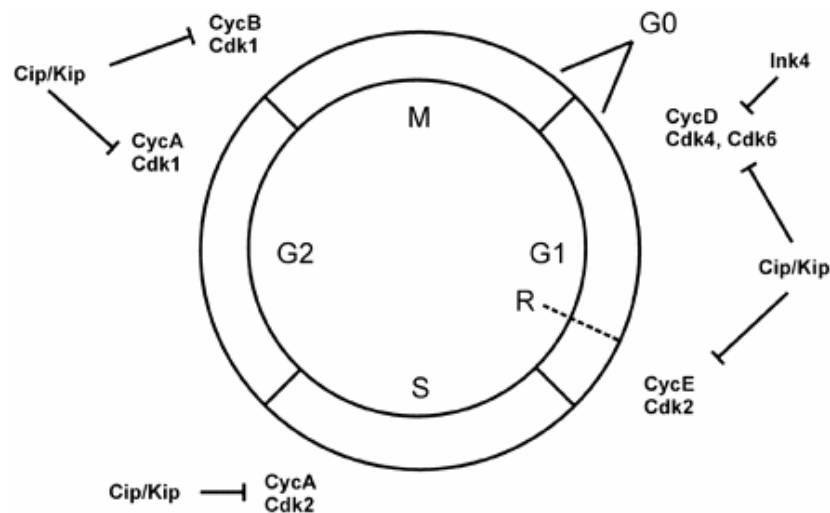
Fig. 4: Phases of the cell cycle and their regulation via cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors. CDK activity is regulated via expression of associating cyclins at different stages of the cell cycle. CDK activity is antagonized via inhibitors of the Cip/Kip and Ink4 family. Figure was adapted from Schmetsdorf et al. [53]

In terms of cell cycle progression the transition from G1 into S phase seems to be essential for anergy avoidance, as the use of pharmacological inhibitors for G1 progression renders T cells anergic even in the presence of full stimulation while a block in the S phase fails to do so [47].

On the molecular level cell cycle progression is regulated by the activity of cyclin-dependent kinases which are associated with different cyclins depending on the phase of the cycle (Fig. 4). While CDK levels remain relatively constant

throughout the cell cycle, cyclins display an oscillating expression pattern. Additionally, the activity of CDK inhibitors is required for the regulation of different cell cycle stages [54].

In order to traverse the G1 phase of the cell cycle activated cells require CDK4 and CDK6 kinases which are both regulated by D-type cyclin activity as well as CDK2 which is dependent on cyclins of the E-type [47]. CDK activity results in the hyperphosphorylation of the retinoblastoma protein (Rb) which promotes the release and activation of E2F transcription factors ultimately leading to the synthesis of S phase proteins [55]. While activation of CDK6 and cyclin D2 is unaffected in anergic T cells, cyclin E as well as CDK2 and CDK1 activity cannot be induced [47]. Anergic T cell clones do assemble Cyclin D2-CDK4 holoenzymes. However, there is a lack of kinase activity which is due to the association with p27^{kip1}, a CDK inhibitor [55]. p27^{kip1} presumably plays a dual role. While aiding in the cell cycle entry of naïve quiescent T cell by protecting D-type cyclin-CDK4/6 complexes from degradation via ink4 CDK inhibitors in the first cell division, it acts as negative regulator by additionally inhibiting CDK1/2 activity in ensuing cell divisions [47]. Loss of p27^{kip1} was shown to uncouple T cell activation from the need of costimulation as silencing of the *il2* locus does not occur [47]. However, its role as an essential anergy factor has been challenged by models where anergy could be induced despite lack of p27^{kip1}expression [56, 57].

1.9. T cell quiescence

The term quiescence corresponds to the G0 phase of the cell cycle, a state where cells display markedly reduced size, metabolism, protein synthesis and transcriptional activity [58, 59]. Unlike anergy quiescence is characterized by responsiveness to activation as well as resistance to apoptosis. Quiescence is governed by a distinct transcriptional program by factors which are actively suppressed upon activation and cell cycle entry [58, 59].

A classical factor in T cell quiescence is Krüppel-like factor 2 (KLF2) or lung KLF (LKLF) the latter name being derived from its high expression levels in lung tissue [60]. KLF2 is a zinc finger transcription factor which is profoundly expressed in naïve T cells and resting memory cells. Activation mediates rapid downregulation of KLF2 [58].

KLF2 deficiency in mice leads to pronounced defects in T cell development resulting in dramatically reduced T cell numbers. Remaining T cells bear activation markers leading to spontaneous proliferation and subsequent Fas-dependent apoptosis [58]. Consistent with this is the observation that KLF2 overexpression is sufficient to induce growth arrest accompanied by a decrease in size and metabolic activity and transferrin receptor expression in the T cell leukaemia line Jurkat. Further studies identified the proto-oncogene *Myc* as a target of KLF2 which is negatively regulated upon overexpression of the latter [58]. Putative targets of c-Myc involve p27^{kip1}, complexes of CDK4/CDK6 with cyclin D1 as well as the phosphatase cdc25. The latter dephosphorylates CDK2 at inhibitory sites [59].

Other factors involved in T cell quiescence involve Tob, a member of the anti-proliferative (APRO) family and forkhead box O (FOXO) family members [58, 59] and Schlafen 2 (slfn2) [66].

1.10. The Schlafen gene family

The Schlafen gene family was first identified in the mouse as a family of growth regulatory genes which are preferentially expressed in lymphoid tissues and whose members were found to be differentially regulated during thymocyte development [61]. Already in the initial study a link between Schlafen genes and growth control was established since Slfn1 was reported to induce a cell cycle arrest in fibroblasts before the transition from G1 to S phase [61]. The mechanism of growth arrest seems to occur via suppression of cyclin D1 expression [62]. However, the in vitro growth inhibitory action of Slfn1 as well as of Slfn2 was not always confirmed by others [63]. Schlafen genes have also been shown to be regulated during T cell activation [61, 64, 65]. A role for Schlafen family members in immune cell quiescence was suggested by a study characterizing a mutation in *Slfn2* which lead to lineage-specific defects in monocytes and T cells. The latter were found to reside in a semi-activated state and cells of both lineages rapidly died in response to activation signals suggesting a loss of cellular quiescence [66]. Schlafen-like proteins have also been identified in orthopoxviruses implicating importance for viral immune evasion [67].

All proteins of the Schlafen family share a common core reagion which harbours a divergent AAA (ATPases associated with various cellular activities) domain (Fig. 5A) functioning as an ATP-binding site and a conserved domain signature (COG2865) which is found in transcriptional regulators and helicases [65-67]. The AAA motif is a highly conserved domain found in eukaryotes as well as in prokaryotes. Although AAA proteins are very homologous within this domain other sequences show little conservation. The diverse cellular functions exerted include, for example, fusion of membranes during vesicular transport, protein quality control as well as functions as microtubule-associated motor proteins [101]. The ATPase domain common to this class of proteins is assumed to provide energy through ATP hydrolysis enabling remodelling of substrates. Additionally, ATP-binding might aid in the formation and stabilization of ring-shaped hexamers which is a typical feature of AAA proteins [101]. SLFN family members are divided into three subgroups based on their length and domain composition. Members of subgroup III within the SLFN family share motifs within their C-terminal domain homologous to superfamily I RNA/DNA helicases [65].

Little is known about the function of the six human Schlafen family members. A study investigating the expression of SLFN genes in primary human melanocytes and malignant melanoma cell lines showed basal expression of human SLFNs in both cell types [68]. All SLFN genes were found to be inducible in primary human melanocytes upon treatment with IFN-α. However, only SLFN5 was consistently suppressed in all melanoma cell lines observed and was the only IFN-α-inducible SLFN gene in these cells. Knock-down of SLFN5 resulted in increased anchorage-independent growth and invasion of malignant melanoma cells [68].

Growth inhibitory function or implication in T cell quiescence has not been shown for human SLFNs so far. Although having no direct orthologue in the mouse SLFN12 seems to be the most similar of human SLFN genes to the novel T cell quiescence factor Slfn2 [66]. According to its domain structure SLFN12 can be classified as a member of SLFN subgroup II (Fig 5B). Expression data for this factor is limited. Similar to other SLFN genes, SLFN12 was shown to be upregulated in primary human melanocytes upon IFN-α treatment and seems to be suppressed in some melanoma cell lines [68].

Another study showed upregulation of SLFN12 during the LPS stimulation of human monocyte-derived macrophages [70]. To date, expression of SLFN12 has not been characterized in T cells or other lymphocytes.

A
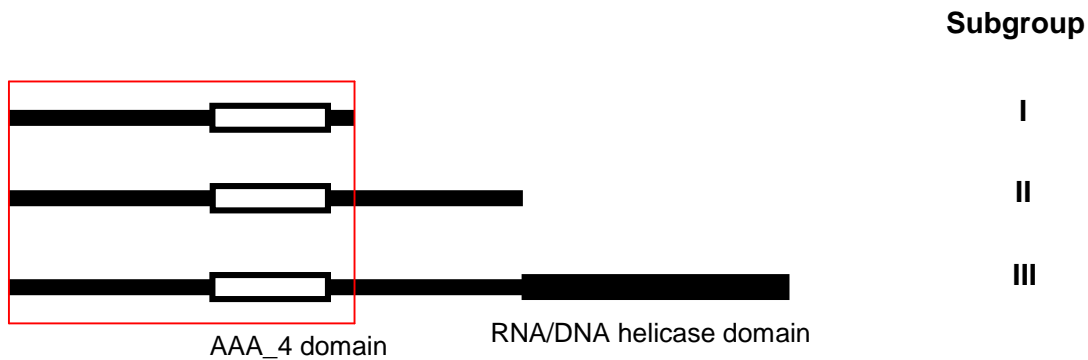
B

Subgroup	Murine Slfns	Human SLFNs
I	slfn1, slfn2	
II	slfn3, slfn4, slfn14	SLFN12, SLFN12L, SLFN14
III	slfn5, slfn 8, slfn9, slfn10	SLFN5, SLFN11, SLFN13

Fig. 5: Domain structure of SLFN proteins and division into subgroups. (A) Scheme of domain structures of SLFN family members. (B) Subgroups of human and murine SLFN family members. Based on Bustos et al. [67] and on search results in NCBI conserved domain search [69].

1.11. CD45 as a regulator of TCR signalling

The common leukocyte antigen CD45 is the prototypic receptor-like protein tyrosine phosphatase (PTP) and has been shown to be essential in the regulation of signal transduction pathways in immune cells [71]. CD45 is abundantly expressed on all nucleated hematopoietic cells and their precursors and displays type I transmembrane topology [72]. While the cytoplasmic part of CD45 appears to be highly conserved across mammalia, its extracellular domain reveals little sequence homology. Alternative splicing of the extracellular domain gives rise to various isoforms the expression of which is dependent on cell type, developmental stage and activation state [71] The extracellular domain shows three conserved regions involving carbohydrate linkage sites followed by a region rich in cysteine residues and three fibronectin type III domains [73].

The cytoplasmic tail of CD45 consists of two tyrosine phosphatase homology domains, designated D1 and D2. Only D1 has enzymatic activity, while D2 might serve as a stabilizer of D1 [71].

CD45 seems to regulate signal transduction of the T cell receptor in a positive as well as in a negative fashion. Its main substrates are Src family protein tyrosine kinases (SFKs) like Lck or Fyn [71].

SFKs contain two sites of tyrosine phosphorylation for the regulation of kinase activity. While the inhibitory site is near the C-terminus, an activating site is located within the kinase domain [74]. However, despite hyperphosphorylation of the negative regulatory tyrosine residue in cell lines and thymocytes deficient in CD45, average SFK activity was found to be increased [71]. This observation implies that the activating residue also serves as a substrate for CD45 and is further supported by the abnormal adherence of CD45-deficient macrophages and T cells which is dependent on integrin receptor signalling regulated via SFKs [71].

The function of the extracellular domain of CD45 lies in the regulation of phosphatase activity via differential dimerization of isoforms [71]. Dimerization seems to be regulated via sialylation and O-glycosylation of the alternatively spliced exons. As a result, the smallest isoform RO which is the least modified homodimerizes more readily than larger isoforms, resulting in decreased TCR signalling [75].

1.12. The soluble cytoplasmic tail of CD45 and its action on human T cells

Recently, an alternative function for CD45 has been discovered showing that CD45 is proteolytically cleaved in activated human monocytes and granulocytes upon stimulation with the fungal cell wall component zymosan or phorbol 12-myristate 13-acetate (PMA) but not by any other microbial stimulus [76]. Both stimuli are potent inducers of the respiratory burst in phagocytes, which was found necessary for CD45 cleavage. A critical step involved in burst formation via zymosan might be the ligation of Dectin-1 [76]. Following activation and recruitment of the NADPH oxidase CD45 is sequentially cleaved by serine/metalloproteases and γ-secretase leading to the shedding of the ectodomain and the generation a 95 kDa fragment of the cytoplasmic tail of

CD45 (ct-CD45). This fragment was found to be released upon activation-induced cell death [76].
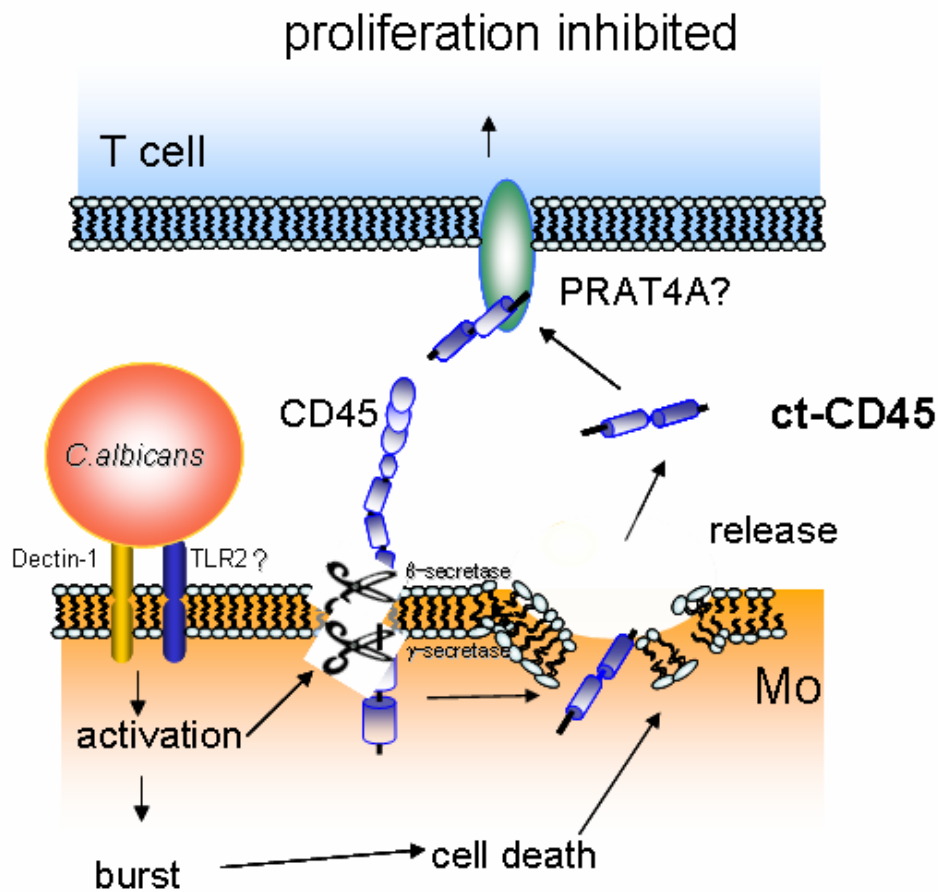
Fig. 6: ct-CD45 generation upon activation of human granulocytes and monocytes. Upon activation-induced cell death ct-CD45 is released and binds to human T cells via the putative receptor PRAT4A.

Binding studies determined that ct-CD45 acts as a cytokine-like factor on human T cells and was shown to inhibit the proliferation of these cells upon in vitro upon stimulation with CD3 and CD3/CD63 antibodies as well as in allogeneic mixed leukocyte reactions [76, 77]. Inhibition of T cell proliferation was shown to be independent of phosphatase activity as fusion proteins consisting of D2 or a phosphatase-dead D1 showed inhibitory action. The reduced proliferation of these cells is not due to cell death [76]. Furthermore it was shown that even T cells activated via CD3/CD28 antibodies do not show impaired proliferation upon ct-CD45 challenge. However, most strikingly, it was demonstrated that even these cells display reduced production of all cytokines tested as well as an impairment in the induction of T cell activation markers [77].

Further experiments showed that ct-CD45-treated T cells are hyporesponsive to restimulation which could be partly reversed by exogenous IL-2 [77]. A potential receptor candidate for ct-CD45 was identified by a cDNA library screen showing binding of ct-CD45 to a protein associated with TLR4 (PRAT4A) on the cell surface [77]. PRAT4A was originally described as an endoplasmic reticulum resident protein involved in the regulation of TLR4 surface expression [78].

Following studies found that PRAT4A is important for immune responses via several TLRs by regulating maturation of TLRs within the ER including TLR1 and TLR4 as well as the subcellular distribution of TLR7 and TLR9 [98]. Since prior reports suggest a role for PRAT4A as an ER chaperone surface expression of this molecule including a receptor function for ct-CD45 appeared to be quite surprising. Nevertheless, evidence for this was provided by showing cells surface expression of PRAT4A on a Bw 5417 clone overexpressing human PRAT4A as well as on human T cells following activation [77]. A siRNA-mediated knock-down of PRAT4A in the Bw clone showed reduced binding of ct-CD45 to the cells which also supported its receptor function as obtained by the screen [77]. However, a physical blockade of ct-CD45 binding via PRAT4A antibodies could not be achieved so far. Besides, the question regarding molecular factors regulating this anergic state of human T cells still remained.

2. Aim

The pronounced inhibition of T cell proliferation by ct-CD45 accompanied by hyporesponsiveness to restimulation suggested an anergy-like state bearing similarities as well as differences to classical anergy. Since this anergy-like state is regulated by biochemical events the major aim of this study was to elucidate the molecular mechanisms involved in its maintenance. T cell anergy is based on a distinct transcriptional program [56-57] involving actively regulated factors such as signalling molecules, transcription factors and cell cycle regulators. Therefore, the specific aims of the study were:

1. To broadly identify responsible factors for this anergy-like state via the microarray technology.
2. Then to confirm and further analyze differentially expressed factors by quantitative real-time PCR.
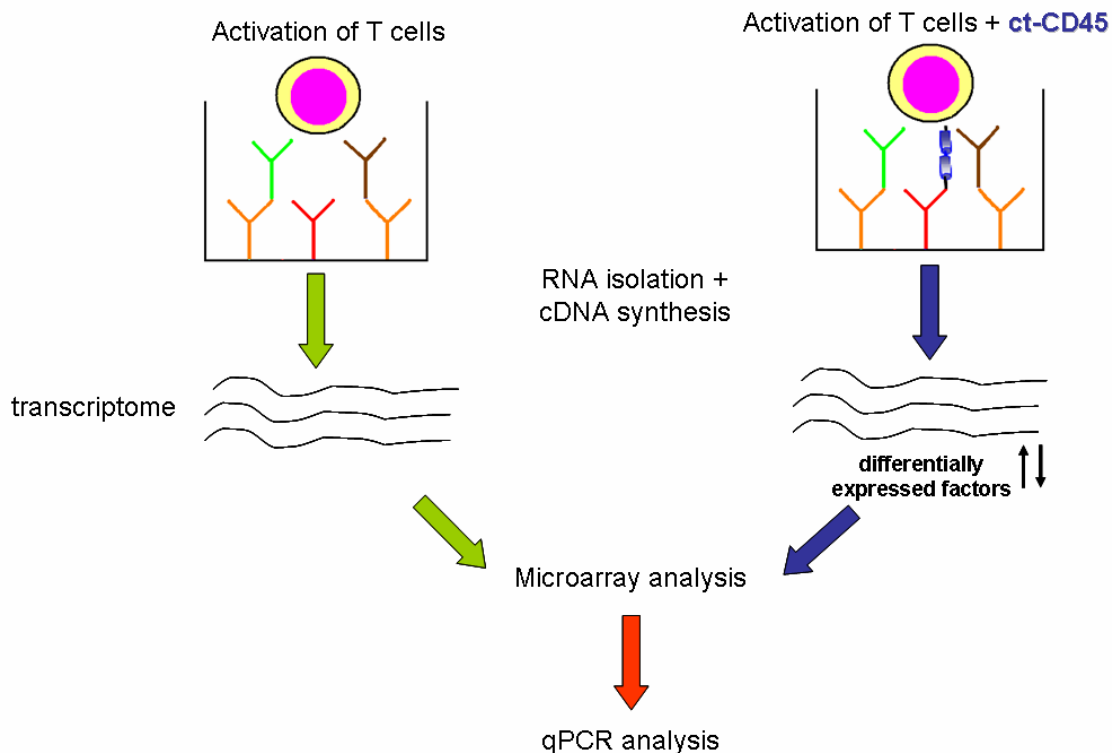
Fig. 7: Experimental procedure for the identification of molecular factors induced by ct-CD45 in human T cells creating an anergy-like state.

3. Materials and Methods

3.1. Antibodies, fusion proteins and oligonucleotides

Antibodies

species	specificity	clone	isotype	source
mouse	hu CD3	OKT3	IgG2a	Jansen-Cilag, Vienna, AT
mouse	hu CD28	15E8	IgG1	Caltag Laboratories, Burlingame, CA
mouse	hu CD63	CD63 - 11C9	IgG3	Otto Majdic, Institute of Immunology
rabbit	SLFN12 (N-term.)	polyclonal	IgG	Abcam, Cambridge, UK
rabbit	SLFN12 (C-term.)	polyclonal	IgG	Abcam, Cambridge, UK
rabbit	hu KLF2	polyclonal	IgG	Sigma-Aldrich, St. Louis, MO
rabbit	mu+hu PRAT4A	polyclonal	-	Kensuke Miyake, Tokyo, JP
goat	human IgG	polyclonal	IgG	Jackson Immunoresearch Lab., West Grove, PA
goat	murine IgG	polyclonal	IgG	Jackson Immunoresearch Lab., West Grove, PA
goat	human IgG-PE	polyclonal	IgG	Jackson Immunoresearch Lab., West Grove, PA
goat	rabbit IgG-HRP	polyclonal	IgG	Dako Diagnostics AG, Glostrup, DK

Abbreviations: hu……human

mu…. murine

term…terminus

Fusion proteins

fusion protein	source
ct-CD45-Fc	Stefan Hopf and Alexander Puck, Institute of Immunology, Vienna
CTLA-4-Fc	Bristol-Meyers Squibb, NY

Primers used for qPCR

primer	sequence 5'->3'
ACTB_F	AGAGCTACGAGCTGCCTGAC
ACTB_R	AGCACTGTGTTGGCGTACAG
β2-m_F	GATGAGTATGCCTGCCGTGTG
β2-m_R	CAATCCAAATGCGGCATCT
CBL-b_F	ATGCTGAATGGAACACATGG
CBL-b_R	ACTATGCCTTGCAGGAGGTG
CD3E_F	TGAGGGCAAGAGTGTGTGAG
CD3E_R	TCCTTGTTTTGTCCCCTTTG
CDK1_F	GGTCAAGTGGTAGCCATGAAA
CDK1_R	TCCTGCATAAGCACATCCTG
CDK2_F	TTGTCAAGCTGCTGGATGTC
CDK2_R	TTTAAGGTCTCGGTGGAGGA
CDK4_F	TTCTGGTGACAAGTGGTGGA
CDK4_R	CTGGTCGGCTTCAGAGTTTC
CDK6_F	TGTTTCAGCTTCTCCGAGGT
CDK6_R	CGTGACGACCACTGAGGTTA
cyclin D1_F	CAAATGTGTGCAGAAGGAGGT
cyclin D1_R	AGGAAGCGGTCCAGGTAGTT
cyclin D2_F	CTGGGGAAGTTGAAGTGGAA
cyclin D2_R	ATCATCGACGGTGGGTACAT
cyclin E1_F	AATGCGAGCAATTCTTCTGG
cyclin E1_R	CTGGTGCAACTTTGGAGGAT

primer	sequence 5'->3'
EGR3_F	CAACTGCCTGACAATCTGTACC
EGR3_R	AGTAGGTCACGGTCTTGTTGC
GAPDH_F	CGACCACTTTGTCAAGCTCA
GAPDH_R	AGGGGAGATTCAGTGTGGTG
IFNg_F	TTCAGCTCTGCATCGTTTTG
IFNg_R	TCTTTTGGATGCTCTGGTCA
KLF2_F	CTACACCAAGAGTTCGCATCTG
KLF2_R	AGTTGCAGTGGTAGGGCTTC
p27kip_F	CCGGCTAACTCTGAGGACAC
p27kip_R	AGAAGAATCGTCGGTTGCAG
SLFN11_F	CCTCCCCTTAGCAGACCAGT
SLFN11_R	TTCCCCGAAAGAAAGGTTG
SLFN12_F	CAAGCCAACCAGGAGAAAAG
SLFN12_R	AACCAACTCGGCATAATTCG
SLFN12_"B"_F	AACCGTGTGATGCAGTTGAC
SLFN12_"B"_R	TTCTGCAAAGGTTTTCTGGAG
SLFN12L_F	TTGACCGAGAAGGAATGGAT
SLFN12L_R	GCAGAAGGTTTTTGGAGCAC
SLFN13_F	GACGCAGATCCAGAGTTTCC
SLFN13_R	AAATGTCCTGGTGGAACTGG
SLFN14_F	TCAGTCAGCTCCTCCCAGTT
SLFN14_R	CAAGGATGTATCAGGGTCTTCA
SLFN5_F	AATTGCCCACAAGAGAATGG
SLFN5_R	AGCGTTTCTGCTGCTCTTTC

All primers were synthesized by Sigma-Aldrich (Steinheim, DE). Primer sequences for CD3E [94], CBL-b, EGR3 [95] and β2-microglobulin [96] have been described previously. Sequences for the IFN-γ primers were obtained from Dr. Peter Steinberger (Institute of Immunology). Another SLFN12 primer

used in some experiments was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

siRNA

target	sense strand of duplex
SLFN12	GCAAUUCUGGUCUUAAAUACAAUCUCC
SLFN12	CAUCACACGGUUAUCUUUCACAUGCCA
SLFN12	GUGAGCCUUCGACAAGAUUUAAACAUC

The SLFN12 Trilencer-27 siRNA and a Trilencer-27 Universal Scrambled Negative Control siRNA Duplex were purchased from Origene Technologies (Rockville, MD).

Buffers and media

LB medium: 25 mg Luria Broth Base per litre of distilled water; boil to dissolve and autoclave before use.

Heparin medium: 500 ml RPMI 1640 medium + FCS. Add 100 U/ml penicillin + 100 µg/ml streptamycin and 2mM L-glutamine (stored at –20°C, Sigma-Aldrich)). Add 10 U/ml Heparin (stock: 5000 U/ml, Baxter, Vienna).

Freezing medium: RPMI 1640 supplemented with 25% FCS and 10% DMSO
MACS-buffer (stored at 0°C): 1000 ml 1x PBS def. + 25 ml Human Serum Albumin (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0,5 M); sterile filtration.

10x PBS stock solution:
5.8 g KH_2PO_4
16.6 g Na_2HPO_4 $2H_2O$
72 g NaCl
Dissolve in aquabidest (=ddH_2O), fill up to 10 litres and adjust to pH 7.2

PBS/BSA stock solution (20%):
100 g BSA
10 g NaN_3

46

Dissolve in 500 ml PBS buffer. For the PBS/BSA wash buffer, prepare a 1:20 dilution with PBS buffer.

Solutions for western blot:

Running buffer (4x):
Tris 12 g
SDS 4 g
Glycin 57,6 g
Fill up to 1000 ml with ddH2O

Blotting Buffer:
250ml of 4x running buffer
200 ml methanol
ad ddH$_2$O to 1000 ml. Degas before use.

PBST (PBST 0.5%):
PBS with 0.5 % Tween 20

Dry milk solution
5% dry milk powder dissolved in PBST buffer.

List of reagents and chemicals

Reagent	final conc.	source
Beriglobin	20 mg/ml (in PBS/BSA)	Aventis Behring, Vienna, Austria
Glycogen	130 µg/ml	Fermentas, Burlington, Canada
GM-CSF	50 ng/ml	Novartis Research Institute, Vienna
IL-4	100U/ml	Novartis Research Institute, Vienna
Lipofectamine^{TM} 2000	1:150	Life technologies, Carlsbad, CA
methyl-3H-thymidine	1mCi/ml	Perkin Elmer, Waltham, MA
pegGOLD TriFast^{TM}	undiluted	PeqLab, Erlangen, DE
OPTI-MEM$^®$	undiluted	Life technologies, Carlsbad, CA

Cell lines

The following cell lines were used in this study: The Jurkat cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The SK-N-SH cell line was a gift from St. Anna Children's Cancer Research Institute, Vienna. Murine Bw 5417 cells were kindly provided by Dr. Peter Steinberger (Institute of Immunology, Medical University of Vienna). Phoenix-E cells were a gift from G. P. Nolan (Stanford University, Stanford, CA).

3.2. Cell isolation

Cell preparation

Peripheral blood mononuclear cells were separated from whole blood of healthy donors by density gradient centrifugation using Ficoll-Paque (Amersham, Little Chalfont, UK). Blood was diluted 1:2 to 1:3 with heparin-medium. For density gradient centrifugation 15 ml of Ficoll-Paque were prepared in 50 ml tubes and a layer of heparinized blood was carefully pipetted onto. The cells were spun 30 minutes at 900g without brake. After centrifugation granulocytes and erythrocytes gathered at the bottom of the tube, mononuclear cells in the interphase.

Magnetic activated cell sorting (MACS)

Magnetic activated cell sorting (MACS) is a method for the selective enrichment or depletion of cells which express a surface protein distinct for their cell type. Therefore cells can be labelled with antibodies directed against the specific molecule which have been coupled to biotin. These labelled cells can be targeted with magnetic beads that contain a secondary antibody targeted against the biotin residues on the primary antibody. Therefore, specific cells in a mixture can be selectively retained in a magnetic column. The mAbs we used were labelled with biotin. These biotinylated antibodies recognizing cell-type specific surface molecules were mixed with the cells to be separated. Then the cells were washed twice with MACS-buffer. In a second incubation step paramagnetic beads (50 nm in diameter) were coupled to the cells. The cells were applied onto a separation column placed in a strong permanent magnet. In this strong magnetic field the cells labelled with paramagnetic beads stack to

the iron mesh and were retained while non-labelled cells passed through. Retained cells were eluted by removing the column from the magnetic field.

Purification of T cells and monocytes

For the isolation of monocytes up to 1 x 10^9 PBMCs were incubated with 250µl of biotinylated CD14 (VIM13) and positively enriched (positive selection**)**, whereas T cells were isolated by collecting the flowthrough of PBMCs depleted (negative selection) by using an antibody cocktail containing anti-CD14 (monocytes), anti-CD16 (monocytes, NK–cells), anti-CD19 (B cells), anti-CD36 (monocytes, thrombocytes), anti-CD56 (NK-cells) and anti-CD123 (progenitor cells, megakaryocytes, granulocytes). Freshly isolated PBMCs were resuspended in 750 µl MACS buffer and incubated with 250 µl of biotinylated antibodies for 15 minutes at 4 °C. To remove unbound antibodies the cells were washed with MACS buffer and again resuspended in 750 µl buffer. Then 250 µl of anti-Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 minutes at 4°C. In the meantime a CS column (Miltenyi biotec) was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. Afterwards the labelled PBMCs were loaded onto the column and washed with 40 ml MACS buffer. The flow through was collected as the monocyte-negative fraction. The column was further washed 4 times with 10 ml MACS buffer and monocytes were collected by removal of the column from the magnet and aspiration of the retained cells from the side valve using a syringe. For T cells the flow-through was collected, washed with MACS buffer and counted. 20 µl of this solution were taken for cell counting with a Coulter Counter (Beckman Coulter, Miami, FL). Before measuring the cell number two drops of Zap-Oglobin® (Beckman Coulter) are added to the counting beaker to remove residual erythrocytes.

Freezing and thawing of cells

Mammalian cells can be stored in liquid nitrogen for prolonged periods of time with minimal loss of viability. For that purpose, cells were spun down, counted and resuspended in freezing medium to a concentration between 10^7 – 5 x 10^7 cells/ml. 1 ml aliquots were filled into cryotubes (Nalgene Nunc International,

Roskilde, Denmark) and kept overnight at - 80°C in a freezing box filled with isopropanol before being transferred to liquid nitrogen.

Isolation of naïve T cells from human cord blood

Naive T cells were isolated from cord blood as described above. Cord blood samples from healthy donors were collected during healthy full-term deliveries. Approval was obtained from the Medical University of Vienna institutional review board for these studies.

3.3. Cell culture

Cell Culture conditions

For general cell culture RPMI 1640 (NBK, Novartis Research Institute, Vienna) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine (NBK, Novartis Research Institute, Vienna), 100 U/ml penicillin and 100 µg/ml streptomycin (NBK, Novartis Research Institute, Vienna) was used. Phoenix-E cells were cultivated in IMDM medium supplemented with FCS, L-glutamine, penicillin, streptomycin and amphotericin. Cells were cultured at 37°C with 5% CO_2.

T cell activation/inhibition

For T cell activation/inhibition assays antibodies and Ig-fusion proteins were used. 96-well non-tissue culture plates (Nunc-immuno plate maxisorp, #439454, Nunc, Denmark) were coated with 3 µg/ml of Fc-specific goat anti-mouse IgG and goat anti-human IgG (Jackson Immunoresearch laboratories), overnight at 4°C, washed twice, and then incubated with 2 µg/ml of the respective fusion protein (ct-CD45-Fc and CTLA4-Fc) plus anti-CD3 (4 µg/ml) or the combination of anti CD3/CD28 or anti CD3/CD63 mAb (2 µg/ml of each antibody). After another washing step, T-cells ($1-1.5 \times 10^5$ cells/well) were added to the wells.

T cell restimulation

For restimulation T cells were activated in the presence of fusion proteins as indicated above and cultured for 4 days. Cells were then harvested, washed twice and rested in 24 well plates for 3 days. 96-well plates were pre-coated overnight at 4°C with 3 µg/ml of Fc-specific goat anti-mouse IgG, washed and

incubated with CD3/CD63 or CD3/CD28 (2 µg/ml for each antibody). Following washing, T-cells (1×10^5 cells/well) were added to the wells and cultured for 4 days. Proliferation was assessed as described below.

Cell proliferation assays

T cell proliferation was monitored in 96-well plates by activating 100.000 cells/well for 3 days as indicated above.

On day 3, 1.25 µCi of methyl-^{3}H-thymidine (Perkin Elmer, Waltham, MA) were added to each well followed by culturing for another 18 hours. Cells were then lysed by distilled water and harvested onto filter plates (Milipore Corp., Bedford, MA). Plates were dried for 1 hour at 37°C before addition of 25 µl/well of Microscint scintillation mix (Packard, Meriden, Connecticut). Incorporated radioactivity was determined using a microplate scintillation counter (Packard). All assays were performed in triplicate.

Generation of monocyte-derived DC

Monocyte-derived immature dendritic cells were generated by culturing peripheral blood CD14$^+$ monocytes for 7 days in the presence of 50 ng/ml GM-CSF (Novartis Research Institute, Vienna) and 100 U/ml IL-4 (Novartis Research Institute, Vienna).

LPS-challenge of monocyte-derived dendritic cells

1×10^6 cells in a 24-well plate were treated with 1 µg/ml lipopolysaccharide for up to 24 hours. Cells were then harvested and lysed for RNA isolation.

3.4. Generation of fusion proteins

Preparation of fusion protein-encoding plasmid vectors

Plasmids were prepared from glycerol stocks of E. coli cells transformed with a pEAK12 vector (Edge BioSystems, Gaithersburg, MD) encoding ct-CD45-Fc fusion proteins consisting either of D1, D2 or full length Fc fusion constructs.

For large scale preparation of plasmids, bacteria were inoculated in up to 500 ml of LB medium supplemented with ampicillin (70 µg/ml) to grow under shaking over night at 37°C. Plasmid maxipreparation was performed with the Qiagen Plasmid Maxi Kit, according to the manufacturer's protocol. Bacterial cells were

harvested by centrifugation at 5000 g (4000 rpm) for 15 minutes at 4°C. The pellet was resuspendend in 10 ml buffer P1 (Qiagen) before 10 ml of alkaline cell lysis buffer P2 (Qiagen) were added, the solution was gently mixed and incubated at room temperature for 5 minutes. In the next step 10ml of neutralization buffer P3 (Qiagen) were added to stop cell lysis.

Lysates were incubated on ice for 15 minutes followed by centrifugation at 5000 g (4000 rpm) for 30 minutes at 4°C to remove cell debris. The supernatant was filtered and transferred to a Qiagen tip 500 column, which had been equilibrated with 10 ml buffer QBT (Qiagen). After washing the column twice with 30 ml of buffer QC (Qiagen), DNA was eluted with 15 ml buffer QF (Qiagen) and precipitated by adding 10,5 ml isopropanol. The precipitated DNA was collected by centrifugation for 30 minutes at 4°C at 5000 g (4000 rpm). The DNA pellet was washed once with 70 % ethanol, dried and resuspendend in nuclease free water according to the pellet size. The DNA content was determined by measuring the OD at 260nm using a NanoDrop 2000 spectrophotometer (PeqLab, Erlangen, DE). Plasmid DNA was diluted to a final concentration of 1 µg/µl and stored at -20°C until use.

CaCl$_2$ Transfection of Phoenix cells

HBS-Buffer (pH 7,05):
140 mM NaCl
1,5 mM Na$_2$HPO$_4$
50 mM HEPES
2,5 M CaCl$_2$

24 h before transfection cells were harvested by trypsination and seeded in a concentration as indicated below. One hour before transfection fresh medium was applied. Plasmid DNA was diluted with sterile ddH$_2$O and CaCl$_2$ (see table below). The transfection mix was sterile filtered using a 0,22 µm filter and the required amount of 2 x HBS was added while vortexing on low level. Formation of DNA-calcium phosphate precipitates was allowed by incubation for exactly 1 minute at room temperature, before applying the mix drop wisely to the cells. After 16 - 24 h the medium was replaced. The supernatants were harvested 3

and 6 days after transfection. For generation of immunoglobulin fusion proteins 6 to 10 $\varnothing$10 cm dishes were transfected with the respective plasmid construct.

	Cell number/ml	DNA	ddH$_2$O	CaCl$_2$ 2,5 M *)	2 x HBS-Buffer
10cm plate	6 x 10^6	30 µg	ad 900 µl	100 µl	1000 µl
6-well	1 x 10^6	6 µg	ad 90 µl	10 µl	100 µl
24-well	0,3 x 10^6	3 µg	ad 45 µl	5 µl	50 µl
48-well	2 x 10^5	1 µg	ad 25 µl	2,5 µl	25 µl
optional: use 5 µg of a GFP-Plasmid to monitor transfection efficiency					
*) add shortly before transfection					

Purification of immunoglobulin fusionproteins

Buffers & solutions

All buffers were sterile filtered (0,22 µm filter) before use:

binding buffer (wash buffer): 20 mM sodium phosphat, pH7

elution buffer: 0,1 M sodium citrat, pH 3

Tris-HCl pH 9

PBS + 20 % EtOH

dialysis buffer : 150 mM NaCl, 50 mM HEPES, 4 mM DTT, 0,0035% Tween-20, pH 7.0

For protein purification of fusionprotein from cell-culture supernatants the HiTrap Protein A HP column (GE healthcare, UK) was used. The specifity of protein A is primarily for the Fc region of IgG.

The cell culture supernatants of Phoenix cells transfected with a vector encoding various ct-CD45-Fc fusion proteins were centrifuged at 2 200 rpm for 5 min to remove cell debris. Before applying to the column the sample was filtered using a 0,22 µm filter.

Using a constant flow rate of 1 ml/min the column was equilibrated with 5-10 ml binding buffer, 5-10 ml elution buffer and 5-10 ml binding buffer again. Then the sample was loaded to the column. Before elution the column was washed with

5-10 ml binding buffer and then 8 x 1 ml fractions were collected. 120 µl of Tris-HCl pH 9 were added so that the final pH of the sample would be neutral, to provide proper folding of the protein. The column was regenerated by washing with 5-10 ml binding buffer. For further use and storage (4°C) the column was washed with 20 % ethanol in PBS.

After purification the protein concentration of the eluted fractions was measured at OD_{280} (OD1 is equal to 0,7 µg/µl). Then all fractions which contained protein were pooled and dialysed o/n at 4°C against dialysis buffer under stirring. At the next day the protein concentration of the dialysed fractions was measured again. The Fc fusionproteins were aliquoted and stored at −80°C.

3.5. Flow cytometry

Principles

Flow cytometry can be used to examine diverse properties of cells including the relative size, relative granularity and relative fluorescence intensity. Cells are transported in a fluid stream to a laser beam. To accomplish that single cells are passing through the beam, a principle related to laminar flow is applied. The sample is injected into a stream of sheath fluid. Cells within this stream are accelerated and are focused to the center, a process called hydrodynamic focussing. The incident laser light is scattered by the cells and detected in different angles. Forward scatter light (FSC) is measured just of the axis of the incident beam by a photo diode and gives information about the size of a particle. Side scatter light (SSC) is collected by a photo-multiplier at approximately 90° of the laser beam axis and is proportional to the granularity. The argon ion laser used emits light at 488 nm, a wavelength matching with the absorption spectrum of a range of fluorescent dyes. These fluorochromes can be excited by the laser, which means that an electron is raised to a higher state of energy. After returning to ground state a photon is emitted and fluorescence can be detected after passing a system of lenses and filters. The intensities of different fluorochromes can be analysed at once, provided that their absorption maximae are not to close to each other.

Membrane staining with unconjugated mAb

Unspecific binding of mAbs to Fcγ-receptors was blocked by incubation of cells with human immunoglobin Beriglobin (Aventis Behring GmbH, Vienna). As an isotype-matched control a polyclonal rabbit anti-human KLF2 antibody was applied. A phycoerythrin (PE)-conjugated goat anti-rabbit IgG was used for secondary labelling.

Firstly, the cell suspension ($2-5 \times 10^5$ /assay) was centrifuged for 5 minutes at 300g and the pellet was resuspended with 50 µl Beriglobin/assay (20 mg/ml in PBS/BSA 1%) and kept 10 minutes on ice. Then 20 µl of the antibody (20 µg/ml in PBS/BSA 1%) were prepared in Micronic tubes and 50 µl of the cell suspension was added, mixed and incubated 30 minutes at 4°C. Each assay was washed twice with PBS/BSA 1% followed each time by centrifugation at 300g, at 4°C. 20 µl of PE-conjugated antibody (20 µg/ml) were added to the cells and again incubated for 30 minutes at 4°C. Each assay was washed twice with PBS/BSA 1% resuspending the cells in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA)

Binding studies with fusion proteins

Binding studies with fusion proteins were performed analogous to staining with unconjugated antibodies. As described above, unspecific binding of fusion proteins or antibodies to Fcγ-receptors was blocked via human Beriglobin (Aventis Behring GmbH, Vienna). CTLA-4 Fc was used as a non-binding control fusion protein. A phycoerythrin (PE)-conjugated goat anti-human IgG (Fcγ-specific) antibody was used for secondary labelling. For blocking of ct-CD45 binding to PRAT4A a polyclonal PRAT4A antiserum (kindly provided by Dr. Kensuke Miyake, University of Tokyo) at a 1:100 dilution in PBS/BSA 1% was incubated for 20 minutes before addition of fusion protein. Fusion proteins and secondary antibodies were used at a concentration of ~20 µg/ml.

Binding was analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson).

3.6. mRNA quantitation

RNA-Isolation and cDNA preparation

Total RNA was isolated using peqGOLD TriFast^{TM} reagent (peqLab, Erlangen, DE) For isolation 1-2x10^6 cells/ml were resuspended in 500 µl of TriFast^{TM} reagent and incubated for 5 minutes (optional: -20°C until need) at room temperature. To separate RNA from cellular protein and DNA 100 µl of chloroform were added, mixed by vortexing and incubated again for 5 minutes at room temperature. After centrifugation for 15 minutes at 13 000 rpm at 4°C the aqueous phase was transferred into a fresh tube and 300 µl of isopropanol as well as 4 µl of a 20 mg/ml solution of glycogen serving as an inert co-precipitant were added. The contents were mixed by inversion, incubated for 5 minutes at RT and centrifuged again at 13 000 rpm at 4°C for 10 minutes. The pellet was washed in 1 ml of 75 % ethanol. The dried pellet was resuspended in distilled water (12 µl) and the concentration was measured using a NanoDrop 2000 spectrophotometer(PeqLab, Erlangen, DE). RNA of an OD260/280 nm >1.6 was used for reverse transcription. Total RNA was reverse transcribed with MuLV-RT (Fermentas, Burlington, Canada) using Oligo (dT)18 primers, according to the manufacturer's protocol. 1-2 µg RNA were mixed with 0,5 µg Oligo (dT)18 primers and distilled water was added up to 11 µl, incubated for 5 minutes at 70°C and chilled on ice. To each reaction 4 µl of 5x M-MuLV reverse transcriptase buffer, 1 mM dNTP mix, 20 U ribonuclease inhibitor, 200 U of RevertAid H Minus M-MuLV reverse transcriptase (all Fermentas) and distilled water to a final volume of 21 µl were added. The mixture was incubated for 5 minutes at 37°C, followed by incubation for 60 minutes at 42°C and 10 minutes at 70°C. Finally cDNA was diluted 1:2 prior to qPCR. cDNA was stored at -20°C until use.

Microarray analysis

Microarray analysis was performed by extracting total RNA from T cells activated via plate-bound CD3/CD63 or CD3/CD28 antibodies in the presence and absence of ct-CD45Fc fusion proteins, respectively. T cell activation and cDNA preparation were performed as described above. cDNA from five human donors was pooled for each condition and subjected to microarray analysis

using an Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The analysis was performed in close collaboration with Novo Nordisk A/S (Bagsvaerd, Denmark).

Quantitative real-time PCR (qPCR)

Real-time PCR is a sensitive tool for the quantification of mRNA expression in different samples. Amplification is monitored via emission of a fluorescence signal corresponding to the increase of the PCR product. Detection is performed during the logarithmic phase of the PCR as soon as the fluorescence signal generated during the reaction significantly exceeds background noise [79]. Common detection methods used include, for example, SYBR Green and TaqMan detection. SYBR Green is a non-specific DNA-binding dye which only emits fluorescence when intercalating into DNA double-strands and thus corresponds to the increase of the (double-stranded) amplicon of the gene of interest. However, this method of detection also yields signals when binding to primer dimers and other non-specific PCR products. TaqMan probes are specific for the cDNA to be detected and are located within the sequence amplified by the primer pair. Probes harbour both a reporter dye and a quenching dye located at the 5' and 3' end of the probe, respectively. During the amplification polymerase 5' exonuclease activity displaces the fluorescent dye from the quenching dye thus resulting in the release of a fluorescent signal [79].

Although absolute quantification using DNA standards encoding the sequence of interest would be possible expression differences are usually determined in a relative way. Housekeeping genes serve as internal standard to account for variations during RNA isolation and reverse transcription [84]. This way of quantification requires stable expression of this reference which must not be altered during any treatment of the cells during cell culture. However, many classical housekeeping genes have been shown to be actively regulated, especially in lymphocytes upon activation [85]. As regulation of housekeeping genes can vary considerably depending on the experimental setting it is crucial to validate several housekeeping genes for their suitability in the respective assay [86]. Thus, we analyzed four genes regarding stability of expression in our T cell activation/inhibition assays using ct-CD45 48 hours post activation.

Among the genes tested, a distinct bias was detected for GAPDH which shows reduced upregulation in T cell activated in the presence of ct-CD45 compared to controls. The most stable genes in our assays were found to be CD3 epsilon (CD3E) and β2-microglobulin which were barely altered by ct-CD45 treatment and were thus chosen as references for experiments.
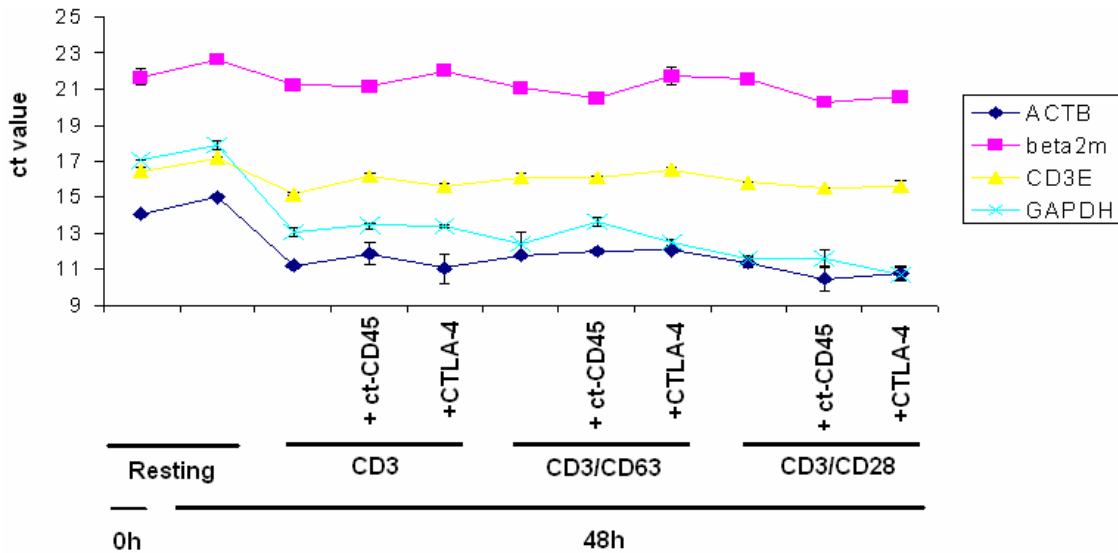
Fig. 8: Validation of various housekeeping genes for normalization of real-time PCR experiments with ct-CD45-treated samples. 96-well plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3, anti-CD3/CD63 or anti-CD3/CD28 monoclonal antibodies in the presence or absence of ct-CD45/CTLA-4-Fc. T cells were plated and cultured for the indicated time. Total RNA was extracted, reverse transcribed using oligo-dT primers and quantitative realtime PCR was performed using intron-spanning primers. Mean values and standard deviation of duplicate determination is shown. Ct….crossing point (i.e. first significant increase above background fluorescence).

Quantitative real–time PCR (qPCR) was performed using the CFX 96 realtime-PCR detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I (Bio-Rad). Detection was performed according to the manufacturer's protocol. In all assays, cDNA was amplified using a standard program (2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 sec at 95°C / 15 sec at 60°C / 45 sec at 72°C).

3.7. RNA interference

RNA interference is a cell intrinsic mechanism serving the degradation of foreign double-stranded RNA molecules such as found in many viruses.

Double-stranded RNA entering a cell is bound by protein complex with nuclease activity (Dicer) which cleaves the double-strand to fragments of 23 nucleotides which are termed short-interfering RNAs (siRNAs). siRNAs are bound by the RISC complex which separates double-strands to base pair them with other double-stranded RNA molecules containing these sequences. As a result, dsRNA is effectively degraded within a cell. The mechanism of RNA interference can be experimentally harnessed for the specific silencing of a gene of interest by introducing siRNA containing the desired sequence into a cell [48].

We transfected a mix of three different siRNA each of them targeted against SLFN12 and a control siRNA (Origene Technologies, Rockville, MD) at a final concentration of 10 nmol each into T cells.

The general transfection procedure was performed according to the manufaturer's protocol. Amounts given are indicated for transfection in 96-well plates. Briefly, Lipofectamine 2000 (1µl per well) was diluted into 25 µl of OPTI-MEM® medium (Life technologies, Carlsbad, CA) and incubated for 5 minutes at room temperature before it was combined with equal volumes of siRNA duplex diluted in OPTI-MEM. The mixture was incubated for 20 minutes at room temperature to allow complex formation. Complexes were then added to the cells, mixed by gently rocking the plate and incubated at 37°C, 5% CO_2.

T cells were transfected 3 days after activation in 96-well plates coated with CD3/CD63 antibodies as described above. Cells were harvested 24 hours after transfection, washed and were allowed to rest for two days before restimulation with CD3/CD63 in the presence of fusion proteins for 3 days before measuring proliferation.

3.8. SDS-PAGE and Western Blot

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) is a useful system to separate proteins according to their size. SDS is a strong detergent, having a hydrophobic tail (the lipid-like dodecyl part) and a negatively charged head group (the sulphate group). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on

interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures, transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups masking the actual charge of proteins. Thus, after boiling in the presence of SDS proteins are unfolded and are separated according to their size. The reason why β-mercaptoethanol is usually included in the sample buffer is to reduce disulfide bonds within or between molecules, allowing molecules to adopt an extended monomeric form.

Laemmli sample buffer (Biorad, Richmond, CA) containing 5% ß-mercaptoethanol (Biorad) was used for preparation of SLFN12 fusion proteins and lysis of cells. Samples were boiled at 95 °C for 5 min before loading. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels using the Hoefer Mighty Small system (Amersham, Little Chalfont, UK). As a running buffer 25 mM Tris base, 192 mM glycine, 0,1% SDS was used. A SeeBlue Plus2 prestained marker (Invitrogen) was used as a size marker.

Polyacrylamide gel composition

Reagent	10% separation gel	2% stacking gel
30% Acrylamide-solution	4 ml	440 µl
H_2O	4,8 ml	2 ml
1,5 M Tris HCl pH 8,8	3 ml	-
0,5 M Tris HCl pH 6,8	-	840 µl
10% SDS-solution	100 µl	35 µl
Ammoniumpersulfate (10%)	100 µl	35 µl
TEMED	8 µl	4 µl

Western Blotting

Western Blotting allows determining relative amounts of the protein present in different samples via the use of specific antibodies. Separated proteins are transferred to a membrane for detection. The membrane is incubated with a generic protein to bind to any remaining sticky places on the membrane and

therefore blocking unspecific antibody adherence on the membrane itself. A primary antibody is then added to the solution which is able to bind to its specific protein. A secondary antibody-enzyme conjugate, recognizing the primary antibody is added to bind and detect bound primary antibody.

Proteins that were separated by SDS-PAGE were blotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA) using the Hoefer Semiphor TE77 system (Amersham, Buckinghamshire, UK) for 1 hour at 15 V. As a blotting buffer 25 mM Tris base, 192 mM glycine, 5% Methanol was used. Membranes were blocked with 5% dry milk / 0,05% Tween20 and incubated with primary antibody (1 µg/ml) in the same solution. Bound Abs were detected using HRP-conjugated goat antibodies to rabbit IgG (used at 1:2500; Dako) and chemiluminescence detection (SuperSignal West Femto Substrate, Thermo Fisher Scientific, Waltham, MA). Blots were developed on Kodak Biomax XAR films (Sigma-Aldrich).

4. Results

4.1. ct-CD45 reduces cluster and blast formation of activated T cells

The cytoplasmic tail of CD45 was shown to be a potent inhibitor of T cells activated via CD3 or CD3/CD63 but not via CD3/CD28 [76, 77]. We found that this inhibition can even be achieved during strong activation as indicated by high levels of ^{3}H-methyl thymidine uptake by T cells (Fig. 9) emphasizing the potent inhibitory action of ct-CD45.
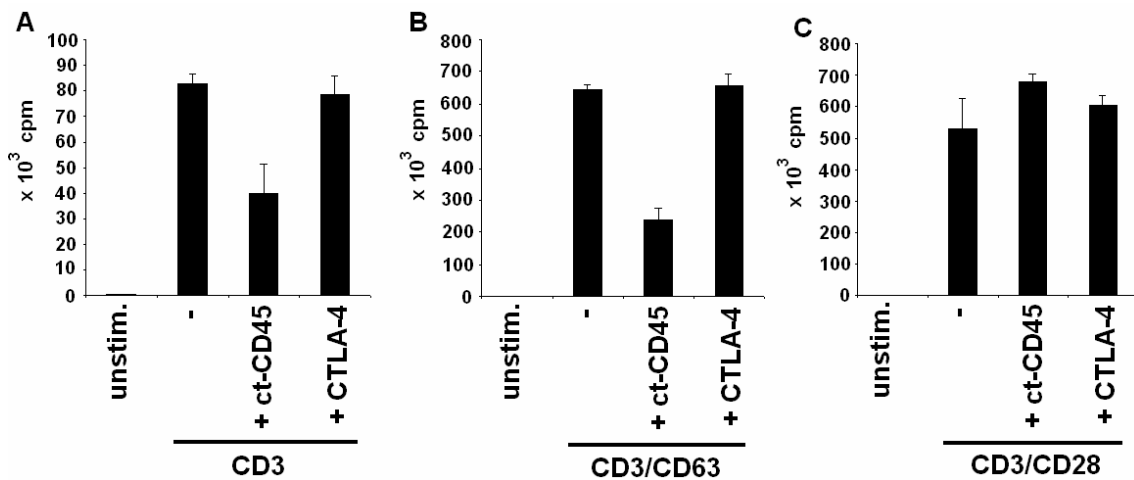
Fig. 9: Inhibition of proliferation by ct-CD45 during strong T cell activation. 96-well plates were coated with anti-human and anti-mouse IgG antibodies, washed and incubated with antibodies targeted against (A) CD3, (B) CD3/CD63 and (C) CD3/CD28 in the presence or absence of ct-CD45. Proliferation was measured via ^{3}H-methyl thymidine incorporation on day 3 of activation. Data is displayed as counts per minute (cpm). Mean values +/- standard deviation (SD) of triplicate determination are indicated. One representative out of 4 independent experiments is shown.

The marked reduction in T cell proliferation exerted by the action of ct-CD45 is accompanied by distinct morphological differences between proliferating and growth-inhibited cells. Two main parameters characterizing T cell morphology upon activation *in vitro* were found to be drastically reduced. One typical feature most commonly observed is the formation of T cell blasts which is characterized by a profound increase in size of activated cells. As a second parameter clustering of neighbouring cells may occur depending on cell density as well as on the strength of activation.
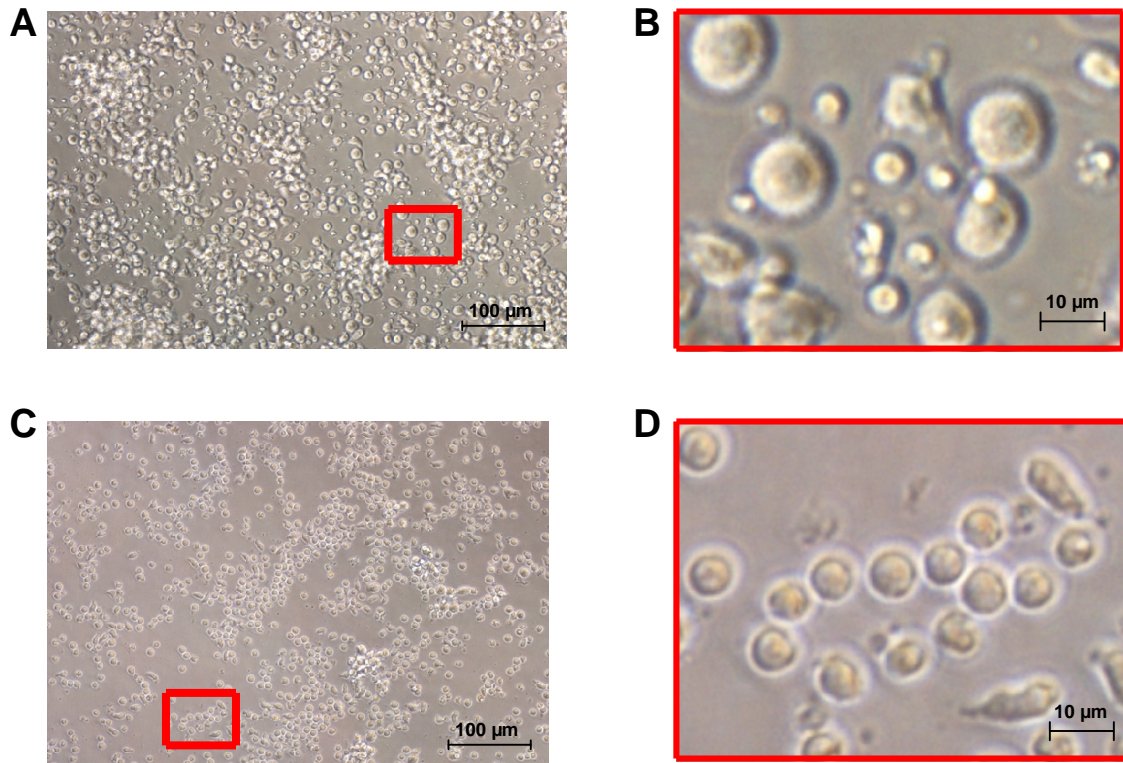
Fig. 10: Changes of cell morphology caused by ct-CD45 during T cell activation. Plates were coated with anti-human and anti-mouse IgG antibodies, washed and incubated with anti-CD3/CD63 in the presence or absence of ct-CD45. Activated T cells were observed via phase contrast microscopy on day 3 of activation. (A) T cells activated via CD3/CD63, (B) activated cells at 10-times higher magnification as indicated by red panels (C) T cells activated in the presence of ct-CD45 (D) Inhibited cells at higher magnification (10x).

The most dramatic effects caused by treatment with ct-CD45 on cell morphology were observed for T cells activated via antibodies against CD3 and CD63. Typically, cells receiving this type of stimulation show very little changes in cell morphology during the first 24 hours which may be due to a delayed onset of proliferation since CD63 expression is low on resting cells but is induced upon TCR triggering [37]. Usually, after 48-72 hours cells show dramatic increases in size (Fig. 10B). However, we found that activation in the presence of ct-CD45 most potently inhibits the formation of these T cell blasts (Fig. 10D). Additionally, CD3/CD63-activated T cells form clusters by day 3 (Fig. 10A). Ct-CD45 treatment nearly seems to abrogate this effect (Fig. 10C). These distinct changes in morphology perfectly match the dramatic alterations in cytokine and cell surface activation marker expression reported previously [77].

The effects caused by ct-CD45 can also be readily visualized using flow cytometric analysis (Fig. 11). Both, forward scatter and side scatter referring to cellular size and intracellular complexity, respectively, are drastically reduced in CD3/CD63 stimulated T cells treated with ct-CD45 while T cells activated via CD3/CD28 are not susceptible towards this inhibitory signal.
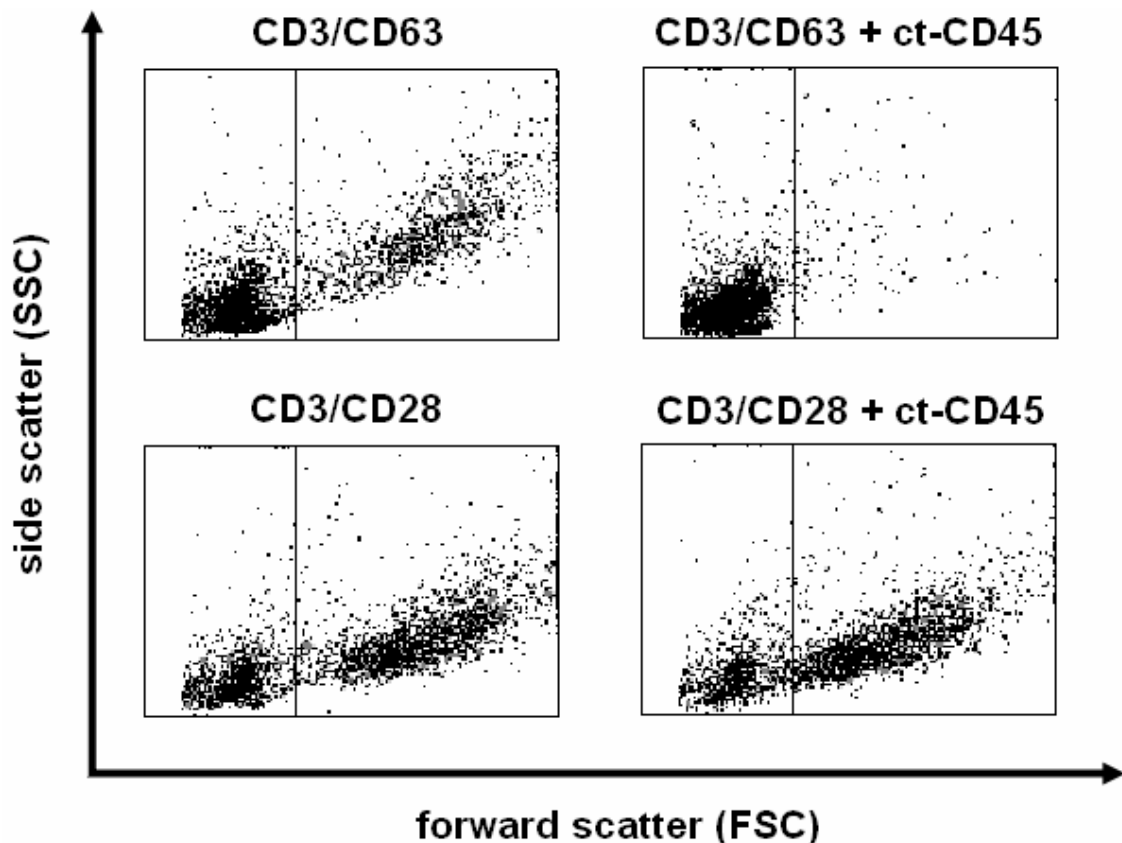

Fig. 11: ct-CD45 reduces activation-induced increase in cellular size and complexity. T cells were activated via plate-bound CD3 and CD63 antibodies in the presence/absence of ct-CD45 fusion proteins for four days. Cellular morphology was then analysed via flow cytometry. One representative out of at least four independent experiments is shown.

4.2. T cell anergy induced by ct-CD45 can be partly reversed by IL-2 and by IL-7

IL-2 has been described as a classical cytokine for the reversal of clonal T cell anergy [45]. Using this approach, IL-2 has been previously used to show that T cells rendered hyporesponsive by ct-CD45 can be partly restimulated in the presence of this growth factor [77]. IL-7 is a cytokine implicated in the homeostatic proliferation of naïve T cells and in memory T cell survival [80]. However, recently it was shown that IL-7 can be superior to IL-2 for the

expansion of tumour-specific CD4^{+} T cells *ex vivo* [81]. Thus, we investigated whether IL-7 can be used for a complete reversal of ct-CD45-induced T cell anergy.

Using a restimulation assay we activated T cells for 4 days via CD3/CD63 and CD3/CD28 in the presence and absence of ct-CD45 using CTLA-4 as a control fusion protein. The cells were then profusely washed and rested in fresh medium for 3 days before they were restimulated in the absence of fusion proteins but in the presence of either IL-2, IL-7 or medium control.
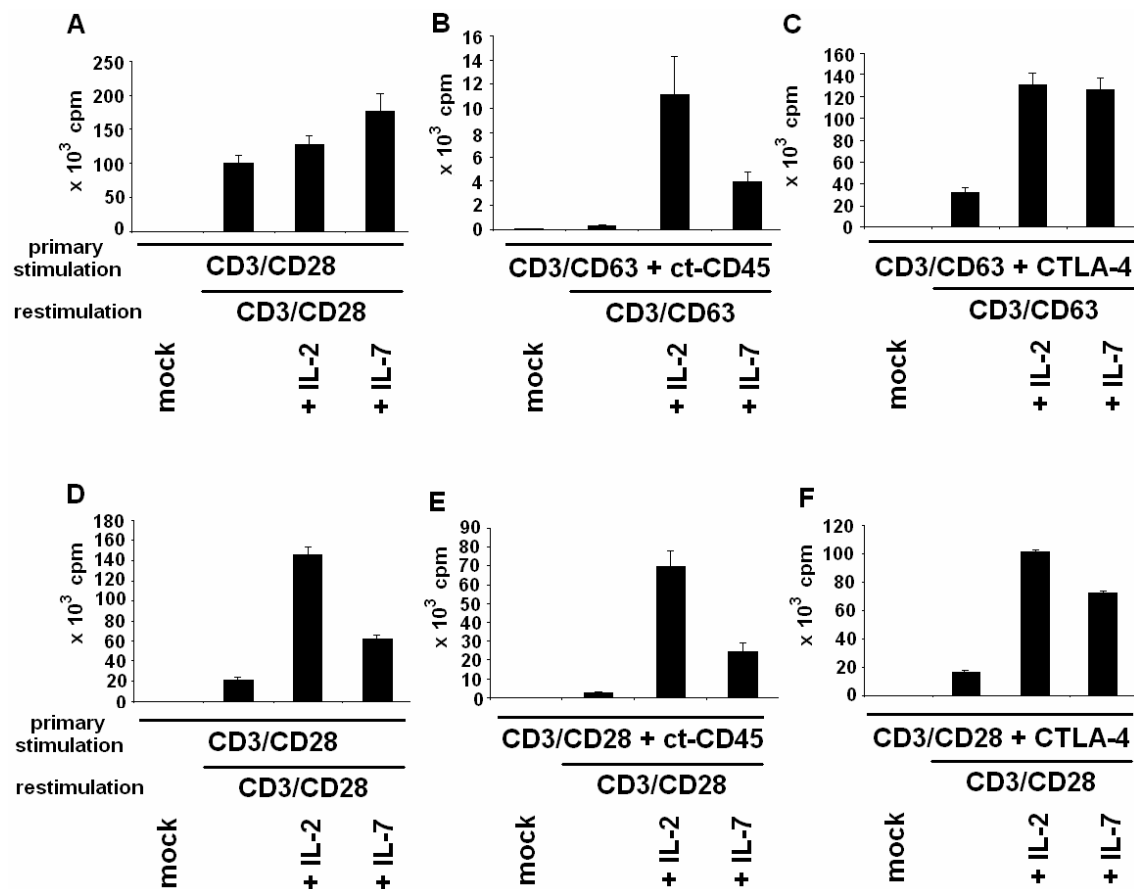
Fig. 12: ct-CD45-induced anergy of T cells can be partly reversed by both, IL-2 and IL-7. T cells primarily activated for 4 days via (A) CD3/CD63, (B) CD3/CD63 in the presence of ct-CD45, (C) CD3/CD63 and CTLA-4 were restimulated in the absence of fusion proteins for 4 days using CD3/63 supplemented with 10 U/ml IL-2, 10 ng/ml IL-7 or medium only. The same experimental procedure was performed for cells initially activated via (D) CD3/CD28, (E) CD3/CD28 and ct-CD45 and (F) CD3/CD28 including CTLA-4 as a control except using CD3/CD28 for restimulation. Proliferation was measured by the uptake of ^{3}H-methyl thymidine which was added to the culture on day 3. Data is displayed as counts per minute (cpm). Mean values +/- standard deviation (SD) of triplicate determination are indicated. One representative experiment out of four is shown.

We found that IL-7 is capable of inducing proliferation in cells otherwise hyporesponsive to restimulation (Fig. 12B). It shows comparable effects to IL-2 in enhancing the proliferation of control cells activated via CD3/CD63 (Fig 12A and 12C) but has a weaker effect on ^{3}H-methyl thymidine incorporation of anergic T cells (Fig 12B and 12E) and cells restimulated via CD3/CD28 (Fig. 12D and 12F). We also tested whether IL-2 and IL-7 might act synergistically in reversing the anergic state but could not find improved cell division compared to IL-2 and IL-7 alone (data not shown).

4.3. Molecular factors induced in ct-CD45-treated T cells are actively regulated during human T cell activation

The reduced T cell proliferation accompanied by reversible hyporesponsiveness to restimulation suggested an anergic state of ct-CD45 treated T cells. Aiming to identify molecular factors involved we performed microarray analysis of a pool of five donors comparing T cells activated via CD3/CD63 or CD3/CD28 antibodies to T cells activated with the stimuli in the presence of plate-bound ct-CD45. Surprisingly, we did not find elevated gene expression of anergy factors like diacylglycerol kinases, E3 ubiquitin ligases or transcription factors like EGR3 or Ikaros. Among the genes which were most strongly induced on the microarray were *krüppel-like factor 2* and *Schlafen family member 12* (*SLFN12*) (data not shown). While KLF2 is a well established T cell quiescence factor [58], the function of SLFN12 in T cells has not been investigated before.

Since KLF2 is downregulated during T cell activation [58] we speculated that SLFN12 is similarly regulated. To test this hypothesis, we stimulated T cells with plate bound anti-CD3, anti-CD3 in the presence anti-CD28 or anti-CD3 and CD63 antibodies for 6 hours, 12 hours, 24 hours and 48 hours. Total RNA was extracted from the cells at the indicated times, subjected to reverse transcription and mRNA expression levels were assessed using quantitative realtime PCR.

Upon activation SLFN12 was rapidly downregulated compared to unstimulated controls, remained low for up to 24 hours but was increasing again after 48 hours (Fig. 13A). Compared to the classical T cell quiescence factor KLF2 (Fig 13B) loss of SLFN12 after activation appears to be less pronounced. After 6 hours expression levels are reduced by 50-65% while KLF2 mRNA shows

reduction between 80% and 90%. In contrast to SLFN12, KLF2 mRNA does not seem to increase again during a 48 hour time course.
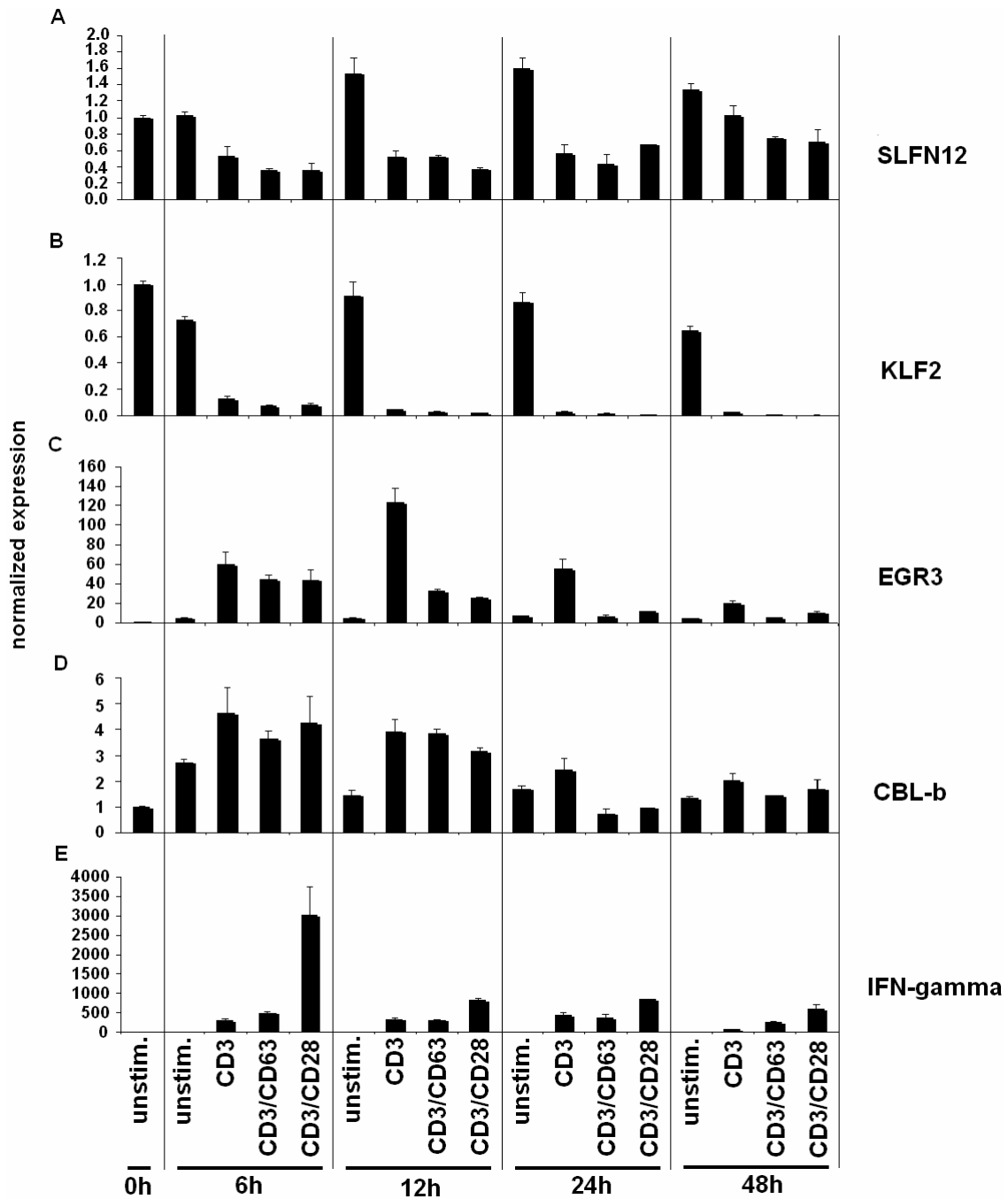
Fig. 13: Regulation of SLFN12, KLF2 and classical anergy factors upon Tcell activation in the presence or absence of costimulation. T cells were activated via plate-bound antibodies for 6 hours, 12 hours, 24 hours and 48 hours. Total RNA was extracted, reverse transcribed using oligo-dT primers and quantitative realtime PCR was performed using mRNA-specific primers. (A) SLFN12, (B) KLF2, (C) EGR3 and (D) CBl-b mRNA. IFN-γ (E) was used as a control for T cell activation. Data was normalized against untreated cells. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene. One representative experiment out of two is shown.

Since both genes were indicated to be regulated in ct-CD45-induced T cell anergy we compared their expression to those of canonical anergy factors.

Both, EGR3 (Fig. 13C) and CBL-b (Fig. 13D) appear to be upregulated in the presence of an activation stimulus and thus show inverse regulation to quiescence genes. As reported before [50], EGR3 was strongly transcribed very early in T cell activation and shows elevated expression levels for CD3 stimulation in the absence of costimulation (Fig 13C) which is a classical way to induce anergy in T cells [46]. Compared to EGR3, CBL-b mRNA shows only low upregulation upon T cell activation. CBL-b levels were only found to be significantly increased in CD3 stimulated cells after 24 hours (Fig. 13D). However, it was shown that CBL-b is proteolytically degraded upon T cell activation in a PKCθ-dependent manner [83] suggesting that its regulation might be more prominent on the protein level.

CD3 stimulation without costimulation did not seem to trigger significant induction of both SLFN12 and KLF2 indicating that both factors are presumably not involved in canonical T cell anergy.

4.4. SLFN12 is not regulated during the activation of monocyte-derived dendritic cells via LPS

Since the down-regulation of SLFN12 during the activation of human T cells represents a novel finding we investigated whether this factor was regulated in a similar manner by the activation of other immune cells. Recently, it was reported that SLFN12 was upregulated during the activation of human monocyte-derived macrophages via LPS [70]. Since this mechanism might be similar in other immune cells challenged with LPS the expression of SLFN12 and of other members of the human SLFN family was tested in monocyte-derived dendritic cells (mDCs).

Reproducible expression of all SLFN family members except SLFN14 (data not shown) was obtained for this cell type in vitro. Upon LPS challenge two SLFN family members were found to be substantially regulated in mDCs. SLFN5 was strongly upregulated 2h after addition of LPS to the culture medium reaching its maximum induction after 5 hours to decline again after 24 hours (Fig. 14A). Conversely, SLFN11 was downregulated by about 60% 2 hours after LPS challenge and remained at this level 22 hours later (Fig. 14B). We did not find

substantial changes in the expression of SLFN12 (Fig. 14C), SLFN12L (Fig. 14D) and SLFN13 (Fig. 14E) mRNA expression suggesting that these Schlafens are presumably not regulated by LPS-mediated TLR4 signalling. These results also indicate that the regulation of SLFN12 via LPS as reported by van Zuylen et al. [70] is probably cell type-specific and does not represent a universal mechanism within the human immune system.
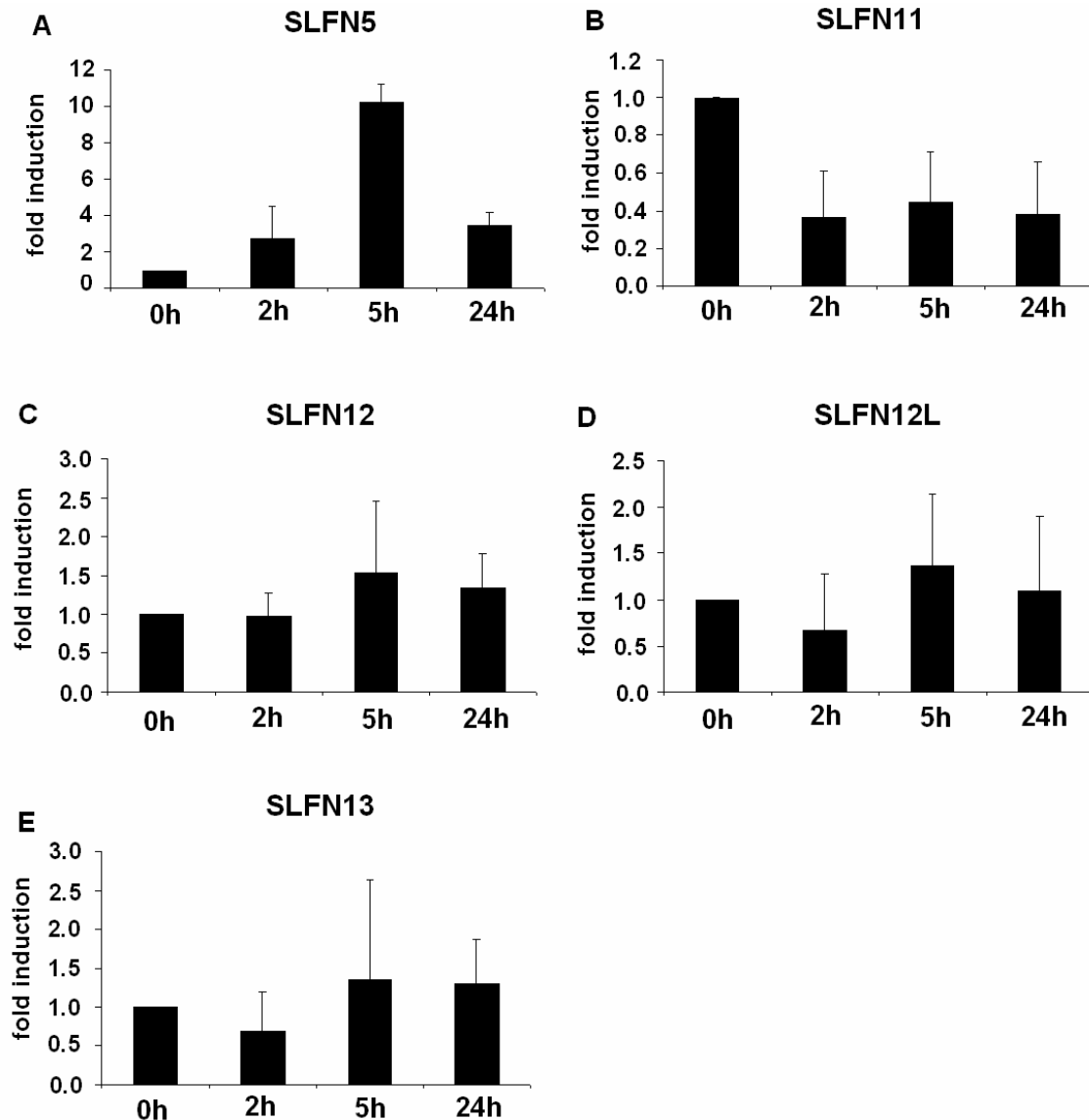
Fig. 14: SLFN5 and SLFN11 but not other SLFNs are regulated by LPS challenge of mDCs. Monocytes were differentiated via IL-4 and GM-CSF to dendritic cells. mDCs were stimulated on day 7 of differentiation with 1 µg/ml of LPS. RNA was extracted from the cells at the indicated times and reverse transcribed. (A) SLFN5, (B) SLFN11, (C) SLFN12, (D) SLFN12L, (E) SLFN13 mRNA expression was quantified via qPCR. Mean values +/- standard deviation of two independent experiments are shown.

4.5. Expression of Schlafen family members in human T cells and in human cell lines

The Schlafen gene family has been implicated in cellular growth control by several studies carried out in the murine system [61, 62, 65, 66]. Thus, we asked ourselves whether human Schlafens might exert a similar role in immune cells as well as in other tissues. In order to obtain a first indication for their involvement in cellular growth we compared SLFN gene expression in naïve T cells to continuously dividing cells lines. The human T cell leukaemia line Jurkat [87] and SK-N-SH, a human neuroblastoma cell line [88] were chosen as models for this purpose. In addition to the six members of the human SLFN family we also tested for KLF2 and Cyclin E1 expression serving as controls for highly and lowly expressed genes in resting T cells, respectively.

While cyclin E1 is roughly 5 to 8 fold increased in the cell lines compared to naïve T cells, KLF2 is considerably higher expressed in T cells compared to both cell lines (Fig. 15). The same holds true for most Schlafen family members. Expression differences observed for T cells vs. the neuroblastoma cell line SK-N-SH range from about $2x10^1$ to 10^4, with the least differences observed for SLFN12 and the highest for SLFN5 and SLFN12-like (SLFN12L). The expression profile for T cells vs. Jurkat cells looked slightly different. While the biggest differences were observed for SLFN12L (~10^4-fold), SLFN11 was even found to be slightly increased in Jurkat cells questioning a possible role for this Schlafen family member in T cell growth control. SLFN12 was observed to be robustly expressed in naïve T cells with about 350-fold higher expression in these quiescent cells relative to proliferating Jurkat cells.

4.6. SLFN12 and KLF2 mRNA are induced by ct-CD45 in T cells

Microarray analysis is valuable tool for genome wide screens of gene expression. However, its accuracy and reproducibility is limited, especially for low abundance transcripts [92]. Thus, it was essential to validate our results obtained when analysing gene expression of T cells activated in the presence of ct-CD45. Testing SLFN12 and KLF2 expression 48 hours after activation we indeed found upregulation of both mRNAs in peripheral T cells which were anergized by ct-CD45. Regulation of SLFN12 closely correlated with the degree of inhibition observed for T cells activated with three different stimuli. Its

strongest induction was observed for T cells activated via CD3 and CD63 monoclonal antibodies (Fig. 16B), followed by CD3 stimulation alone (Fig. 16A). Substantial upregulation of SLFN12 was also obtained for CD3/CD28 stimulation (Fig. 16C) even though in our hands proliferation of T cells receiving these stimuli was never found to be impaired.

Regulation of KLF2 was more subtle with less strong induction (Fig 16D-F). However, we also obtained slightly increased KLF2 expression for ct-CD45 treated T cells in the context of a CD3/CD28 stimulus (Fig. 16F) indicating an involvement of this gene in the anergic state induced.
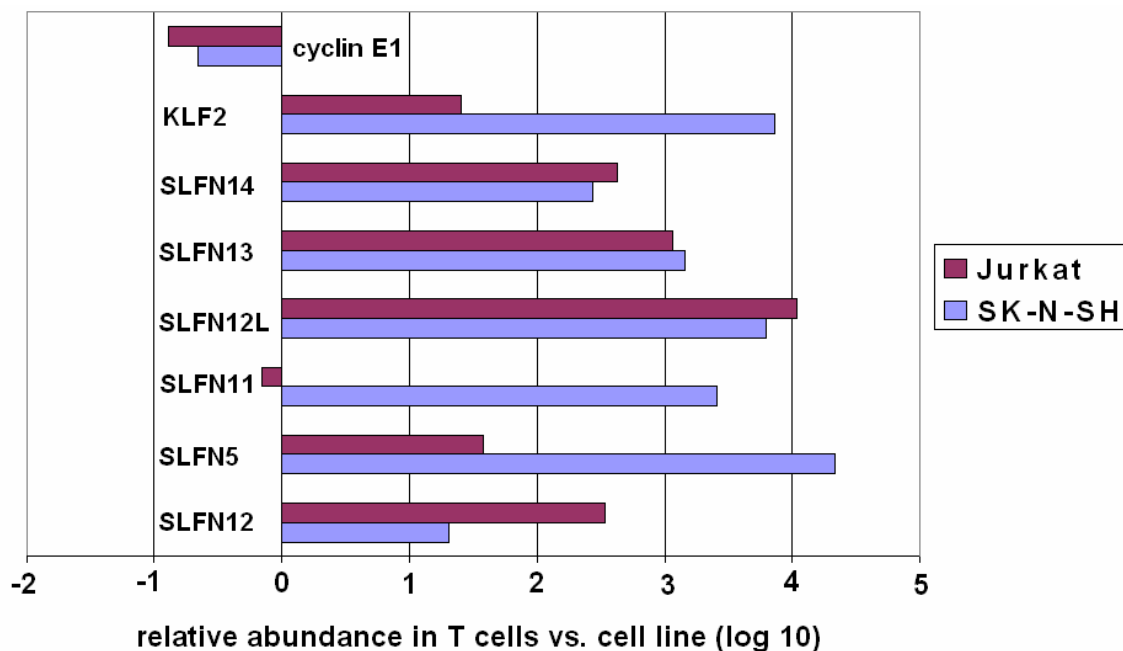
Fig. 15: Expression profiles of Schlafen family members in T cells relative to continuously dividing cell lines. Cryopreserved naïve human T cells were thawed from liquid nitrogen stocks and were lysed without prior culturing. Cell lines were harvested the day after medium renewal to ensure logarithmic growth. Adherent cells (SK-N-SH) were directly lysed in the tissue culture plate without prior detachment with trypsin. Total RNA was isolated and mRNA was reverse transcribed using oligo-dTTT primers. Quantitative realtime-PCR was performed using specific intron-spanning primers for the genes indicated. Gene expression was calculated using CD3E (T cells vs. Jurkat cells) and beta-Actin (T cells vs. SK-N-SH) as reference genes. Relative expression was normalized to the respective cell line.

SLFN12 mRNA

KLF2 mRNA
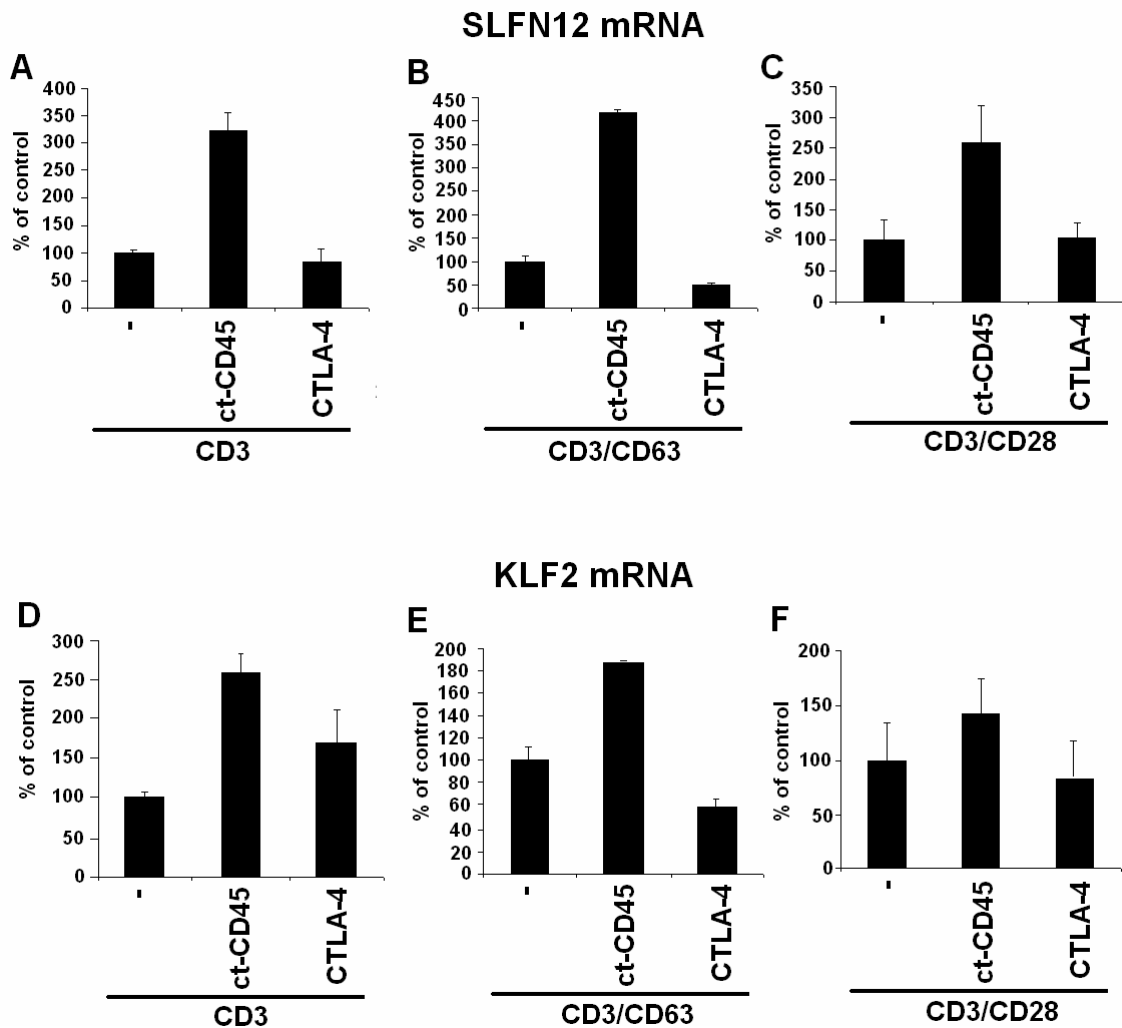
Fig. 16: SLFN12 and KLF2 are both induced in T cells activated in the presence of ct-CD45. 96-well plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3, anti-CD3/CD63 or anti-CD3/CD28 monoclonal antibodies in the presence or absence of ct-CD45. CTLA-4-Fc was used as a control fusion protein. Total RNA was isolated, reverse transcribed using oligo-dTTT primers and subjected to quantitative realtime PCR. SLFN12 regulation in the presence/absence of ct-CD45 and CTLA-4 for (A) CD3, (B) CD3/CD63, (C) CD3/CD28 stimulation. KLF2 regulation towards the same stimuli for (D) CD3, (E) CD3/CD63, (F) CD3/CD28. Beta2-microglobulin was used for reference. Data is displayed as percent of stimulation without fusion protein +/- standard deviation of duplicate measurement of the reference gene. One representative experiment out of two is shown.

4.7. Confirmation of SLFN12 expression on the protein level – lack of reliable antibodies

In order to have another line of evidence for SLFN12 expression we tried to detect the mature protein in cell lysates. We purchased two polyclonal antibodies specific for the N-terminus and C-terminus of SLFN12 as indicated in Fig. 17A. Using a recombinant SLFN12-GST fusion protein as a positive control

we found strong reaction of both antibodies with the fusion protein. As the latter represents a truncated form of SLFN12 which lacks the N-terminal end of the native protein this result is somewhat surprising. When probing cell lysates of T cells and the SK-N-SH cell line both antibodies reacted distinctly with both cell types. However, when probing BW 5417, a murine thymoma cell line, which should serve as a negative control strong bands were obtained with both antibodies at approximately the same migration distance as observed for T cells and SK-N-SH (Fig. 17A). We performed quantitative real-time PCR testing SLFN12 expression in the respective cell types and could detect SLFN12 mRNA in both T cells and the SK-N-SH cell lines but not in BW 5417 (Fig. 17B). Thus, we have to conclude that bands observed for cell lysates do not correspond to SLFN12 but are rather unspecific signals. Concentration of our protein of interest and/or antibody affinity might be too low to enable detection.

4.8. Kinetics of SLFN12 induction in peripheral blood T cells

Since inhibition of T cells by ct-CD45 might occur from the beginning, we investigated the regulation of SLFN12 and of KLF2 during the first 24 hours post activation in the presence of the respective fusion protein. Using CD3/CD63 antibodies as a stimulus we activated human T cells for 6 hours, 12 hours and 24 hours and isolated RNA at the given times.

We found that SLFN12 seems to be induced very early after activation in the presence of ct-CD45 (Fig. 18A) although we cannot exclude that this elevated expression is due to mRNA stabilization rather than enhanced transcription. However, although SLFN12 levels in ct-CD45 treated cells seem to decrease compared to controls 12 hours post activation, they seem to recover after 24 hours suggesting an oscillating expression pattern. Interestingly, we did not observe KLF2 induction for peripheral T cells treated with ct-CD45 during the first 24 hours of induction (Fig. 18B) indicating that its induction might only occur after this period.

The transcription factor EGR3 (Fig. 18C) was slightly induced by ct-CD45 treatment but only after 12 hours of activation. CBL-b (Fig. 18D) was barely affected throughout the time course showing only weak induction after 24 hours.
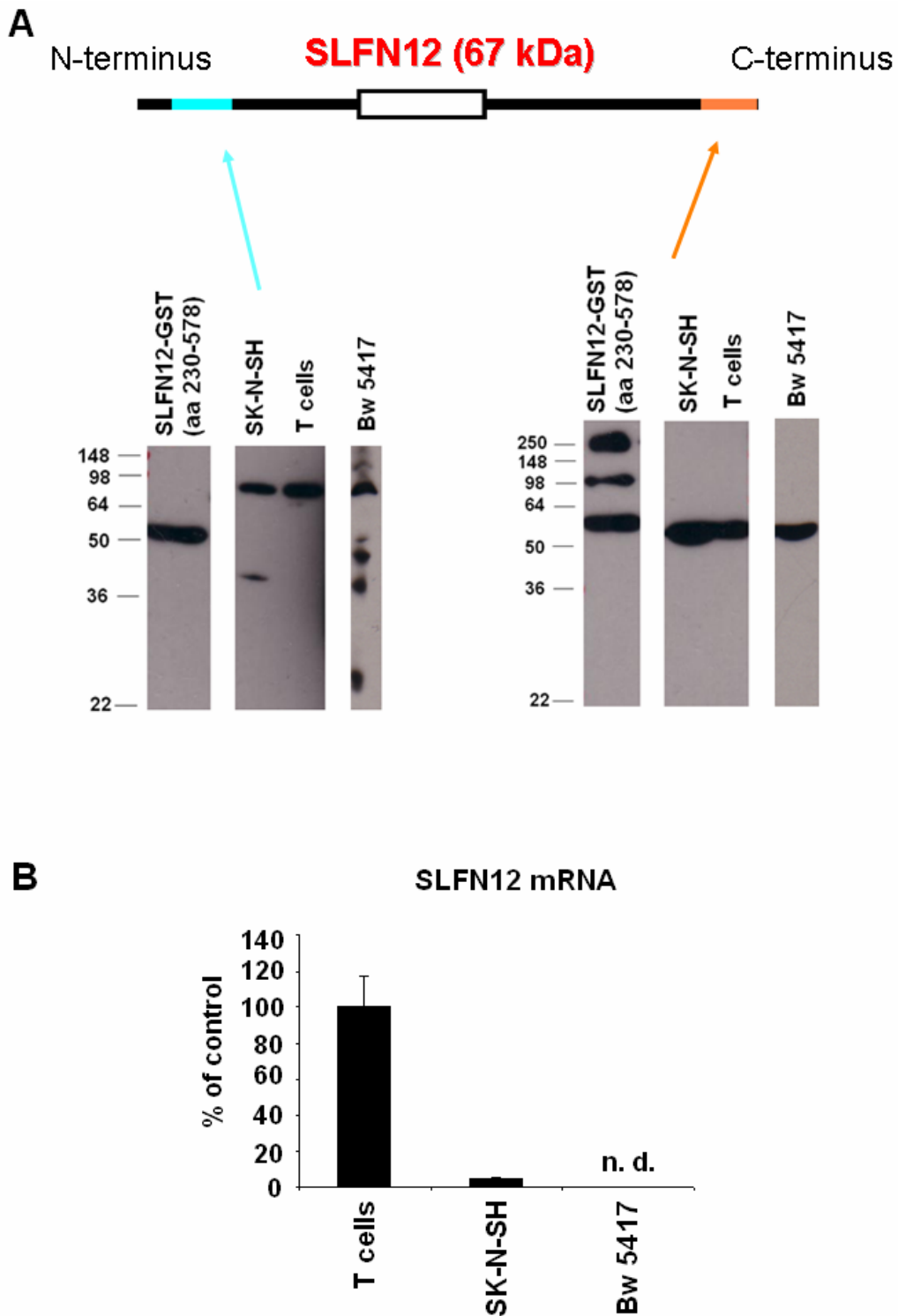
Fig. 17: Reaction of two polyclonal SLFN12 antibodies with a SLFN12 fusion protein and cell lysates. (A) Western Blotting was performed using an N-terminal and a C-terminal SLFN12 antibody. The SLFN12-GST fusion protein corresponds to amino acids 230-578 of the full length (578 aa, 67kDa) protein. 2µg of fusion protein and total protein of 500 000 cells were loaded per lane. Arrows and marked regions show the specificity of both antibodies within full-length SLFN12. (B) SLFN12 expression as determined by qPCR. One representative experiment out of three is shown.

## 4.9. Kinetics of SLFN family regulation by ct-CD45 in peripheral T cells

All proteins of the Schlafen family share common sequence motifs [65-67] suggesting potential redundancy in molecular function. Thus, we speculated that other Schlafens might be similarly regulated upon ct-CD45 binding to human T cells. Addressing this question, we analyzed the expression of other SLFN family members comparing their induction over a 24 hour timecourse to that observed for SLFN12. Indeed, we found intriguing similarities in the regulation of most human SLFN family members in response to ct-CD45. SLFN12-like (Fig. 19C) shares the strongest resemblance to SLFN12 (Fig. 18A) as we found strong induction after 6 hours, accompanied by a decline by hour 12 to be reinduced after 24 hours. This expression pattern mirrors regulation of SLFN12 over this time course (Fig. 18A). Other SLFNs including SLFN5 (Fig. 19A) and SLFN13 (Fig. 19D) were induced by ct-CD45 after 6 hours to decline after 12 hours. However, unlike SLFN12 and SLFN12-like they were not found to be reexpressed after 24 hours.

SLFN11 (Fig. 19B) and SLFN14 (Fig. 19E) were barely affected by ct-CD45 treatment although we could find substantial downregulation of the latter 24 hours after activation compared to non-activated T cells. We found little regulation of SLFN11 and SLFN13 by activating stimuli, whereas SLFN5 and SLFN12L were downregulated by 24 hours.

4.10. Exogenous IL-2 reduces SLFN12 mRNA levels in ct-CD45 treated T cells

Based on the observation that IL-2 partly reverses the ct-CD45-induced hyporesponsive state in human T cells it was investigated whether exogenous IL-2 alters expression SLFN12 and KLF2 in the induction phase of this state.

Indeed, we found that addition of IL-2 has a dramatic effect on SLFN12 expression. SLFN12 was drastically reduced regardless of the nature of the activating stimulus leading to a decrease even below the levels of the untreated controls (Fig. 20A-C). We did not obtain the same results for KLF2. Although KLF2 was reduced by CD3 stimulation alone (Fig 20D), a reduction of mRNA levels was not obtained neither for CD63 (Fig. 20E) nor for CD28 (Fig. 20F) costimulation.
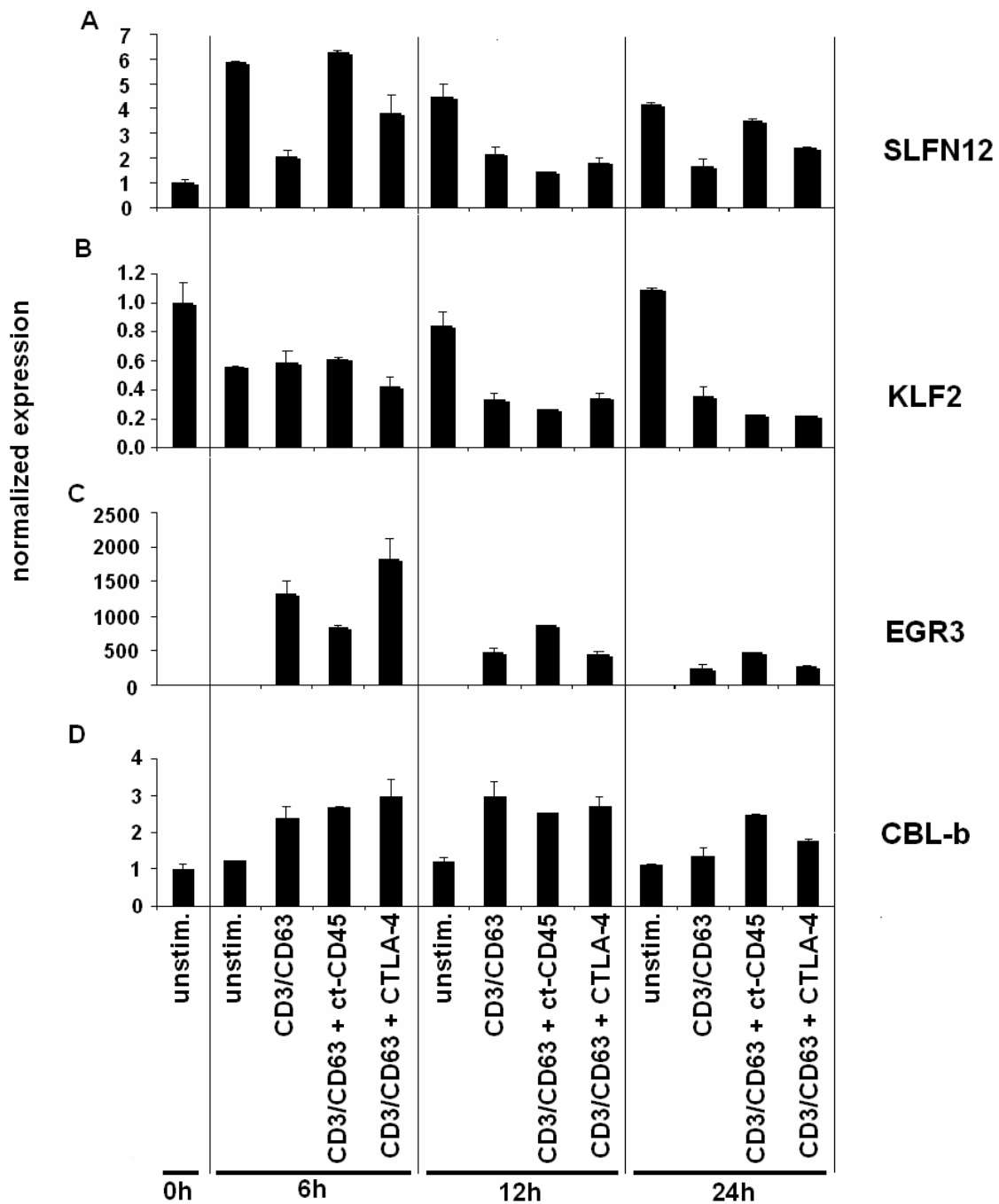
Fig. 18: Kinetics of SLFN12 induction in human peripheral blood T cells. 96-well plates were coated with anti-human and anti-mouse IgG, washed and incubated with anti-CD3/CD63 in the presence or absence of ct-CD45 and CTLA-4 or were left untreated. T cells were activated for 6 hours, 12 hours and 24 hours. Total RNA was extracted and was reverse transcribed using oligo-dTTT primers. Regulation of (A) SLFN12, (B) KLF2, (C) EGR3, and (D) CBL-b was determined using quantitative realtime PCR. Data was normalized against untreated T cells. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene. One representative out of two independent experiments is shown.
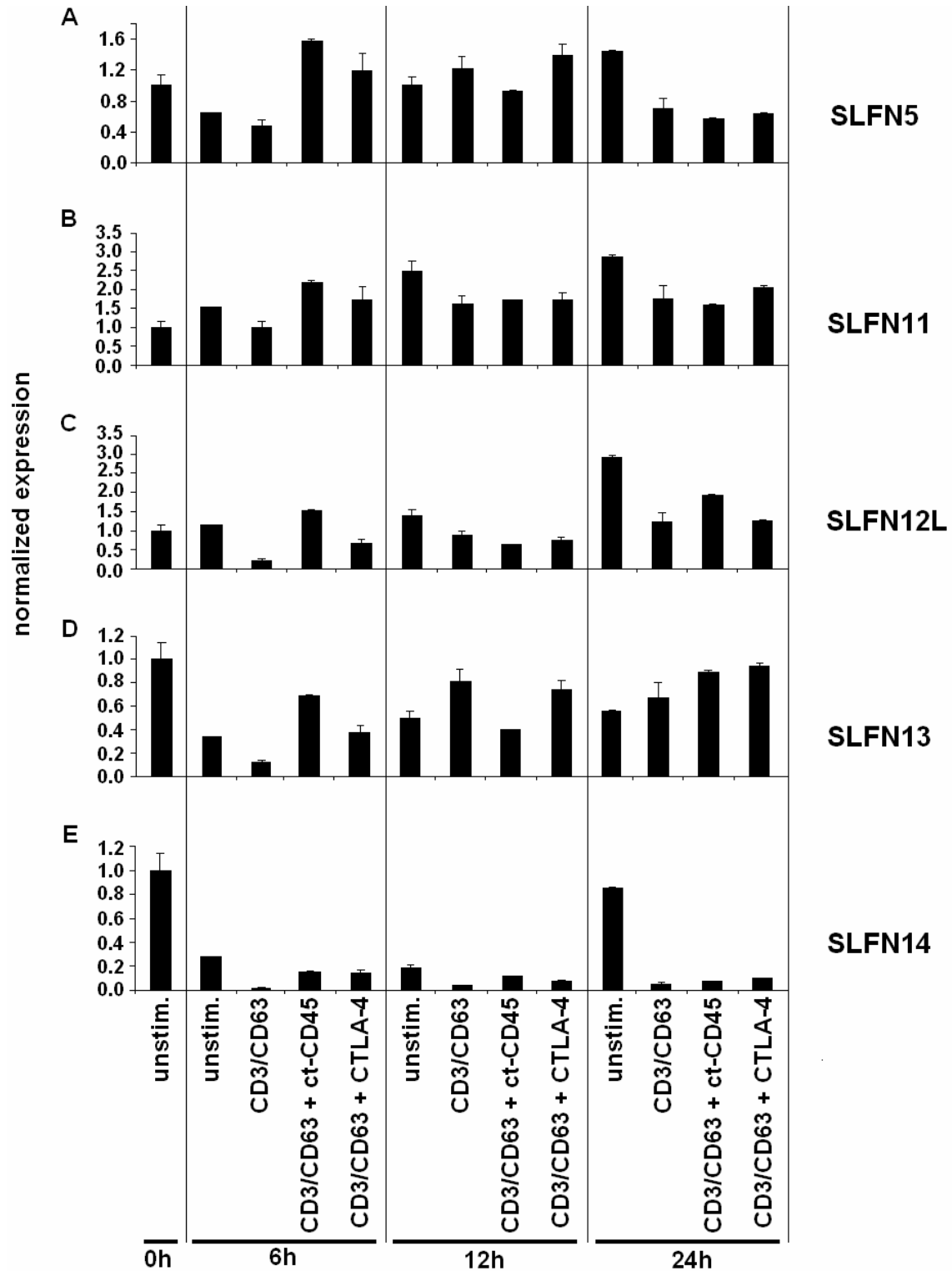
Fig. 19: Regulation of other SLFN family members by ct-CD45. T cells were activated for 24 hours via CD3/63 in the presence or absence of ct-CD45/CTLA-4. Harvests were performed after 6 hours, 12 hours and 24 hours. Total RNA was extracted and was reverse transcribed using oligo-dTTT primers. Regulation of (A) SLFN5, (B) SLFN11, (C) SLFN12-like, (D) SLFN13 and (E) SLFN14 was determined using quantitative realtime PCR. Data was normalized against untreated T cells. Mean values +/- standard deviation (SD) of duplicate determination of the

reference gene are indicated. CD3E was used as a housekeeping gene. One out of two independent experiments is shown.

SLFN12 mRNA
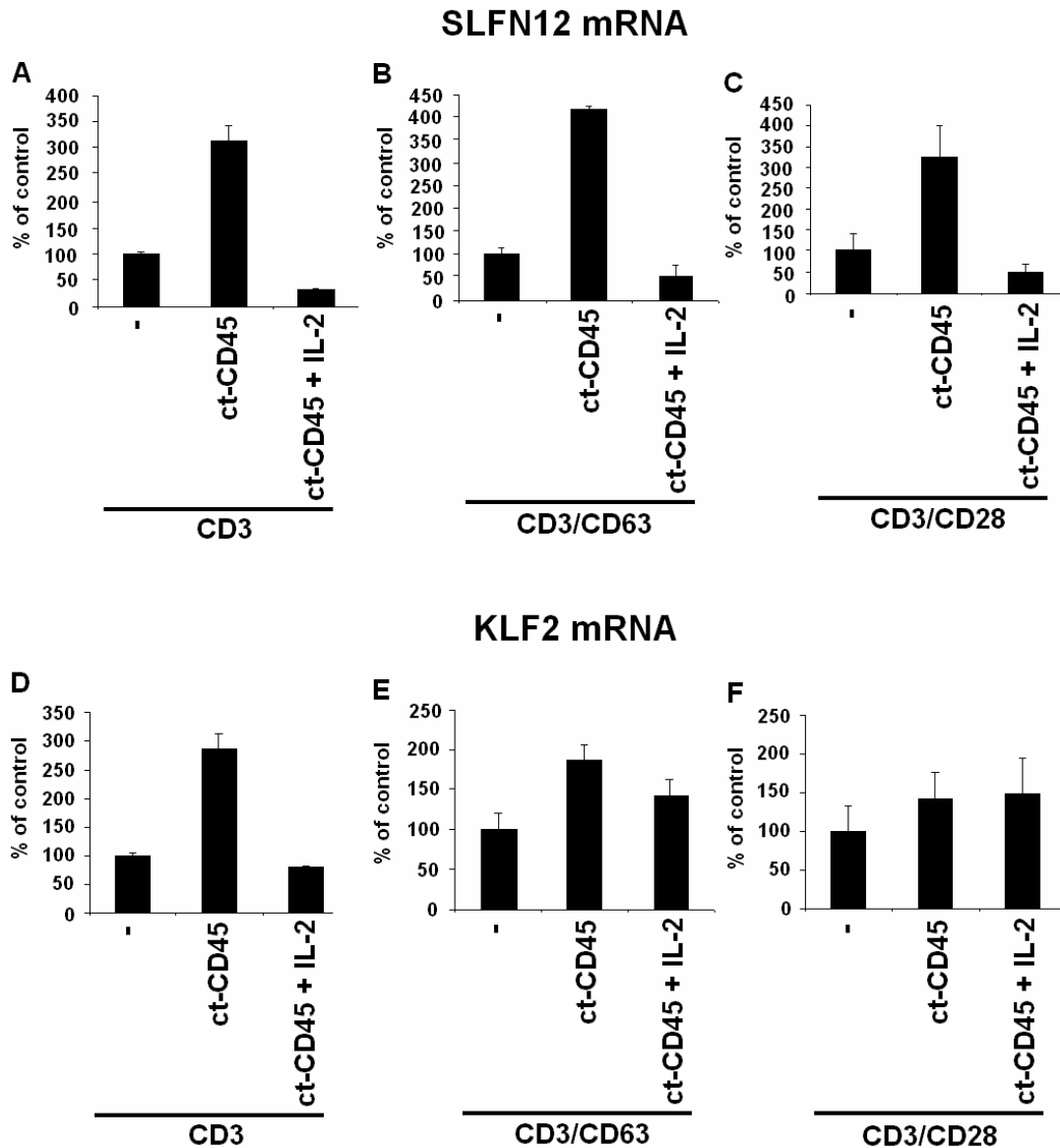
KLF2 mRNA

Fig. 20: Exogenous IL-2 reduces levels of SLFN12 but not of KLF2 mRNA in T cells treated with ct-CD45. 96 well plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3, anti-CD3/CD63 or anti-CD3/CD28 monoclonal antibodies in the presence or absence of ct-CD45 or in the presence of ct-CD45 including 10 U/ml of IL-2. Total RNA was isolated, reverse transcribed using oligo-dTTT primers. Quantitative realtime PCR was performed using primers specific for SLFN12 and KLF2. (A-C) SLFN12 regulation in the presence of ct-CD45 and IL-2 for different activation stimuli. (D-F) KLF2 regulation upon addition of IL-2. Beta-2-microglobulin was used as a reference. Data is displayed as percent of stimulation without fusion protein +/- standard deviation of duplicate measurement of the reference gene. One representative experiment out of two is shown.

4.11. Kinetics of SLFN12 induction in Cord Blood T cells

Since human cord blood T cells could not be inhibited in the presence of ct-CD45 we investigated whether differences in the induction of SLFN12 might provide an explanation for this phenomenon. As done before with peripheral blood T cells we determined the kinetics within a 24 hour time course using CD3/CD63 stimulation as a model.

As observed before, SLFN12 was downregulated upon T cell activation already after six hours, remained low after 12 hours and seemed to increase again in after 24 hours (Fig. 21A). Treatment with ct-CD45 does not seem to alter SLFN12 expression during the first hours but then rapidly peaks after 12 hours to reach expression levels of unstimulated (i.e. resting) T cells. By 24 hours SLFN12 expression declines again nearly reaching the levels of activated controls.

KLF2 seems to be induced in a similar manner. However, KLF2 mRNA levels upon ct-CD45 treatment are already higher after 6 hours (Fig. 21B) but also peak after 12 hours to decrease again by 24 hours.

As observed for peripheral T cells, EGR3 (Fig. 21C) and CBL-b (Fig. 21D) are not strongly regulated by treatment with ct-CD45. EGR3 appears to be slightly increased after 24 hours but was not found to be regulated before this time. No significant expression changes throughout the observed time course were detected for CBl-b.

4.12. Expression kinetics of other SLFN family members induced by ct-CD45

Since other members of the human SLFN family were partly regulated in a similar way to SLFN12 in T cells treated with ct-CD45 we also tested their expression kinetics during a 24 hour time course in cord blood T cells.

Again, striking similarities were found. Generally, there was a transient downregulation of SLFN mRNA by 12 hours which nearly returned to the level of the unstimulated control by 24 hours except for SLFN5 which remained low. In the presence of ct-CD45 there was no significant difference to activated controls after 6 hours. Similar to SLFN12 (Fig. 21A) other Schlafen family members including SLFN5 (Fig. 22A), SLFN11 (Fig. 22B), SLFN12-like (Fig. 22C) and SLFN13 (Fig. 22D) were induced after 12 hours but decreased by 24

hours. While SLFN11 expression only subtly changed during T cell activation as well by ct-CD45 treatment SLFN14 (Fig. 22E) was not inducible at all by ct-CD45 in cord blood T cells. Although all Schlafen family members were decreased during the first hours of culture regardless of stimulus SLFN14 and SLFN13 were most severely affected and never returned to their initial levels.
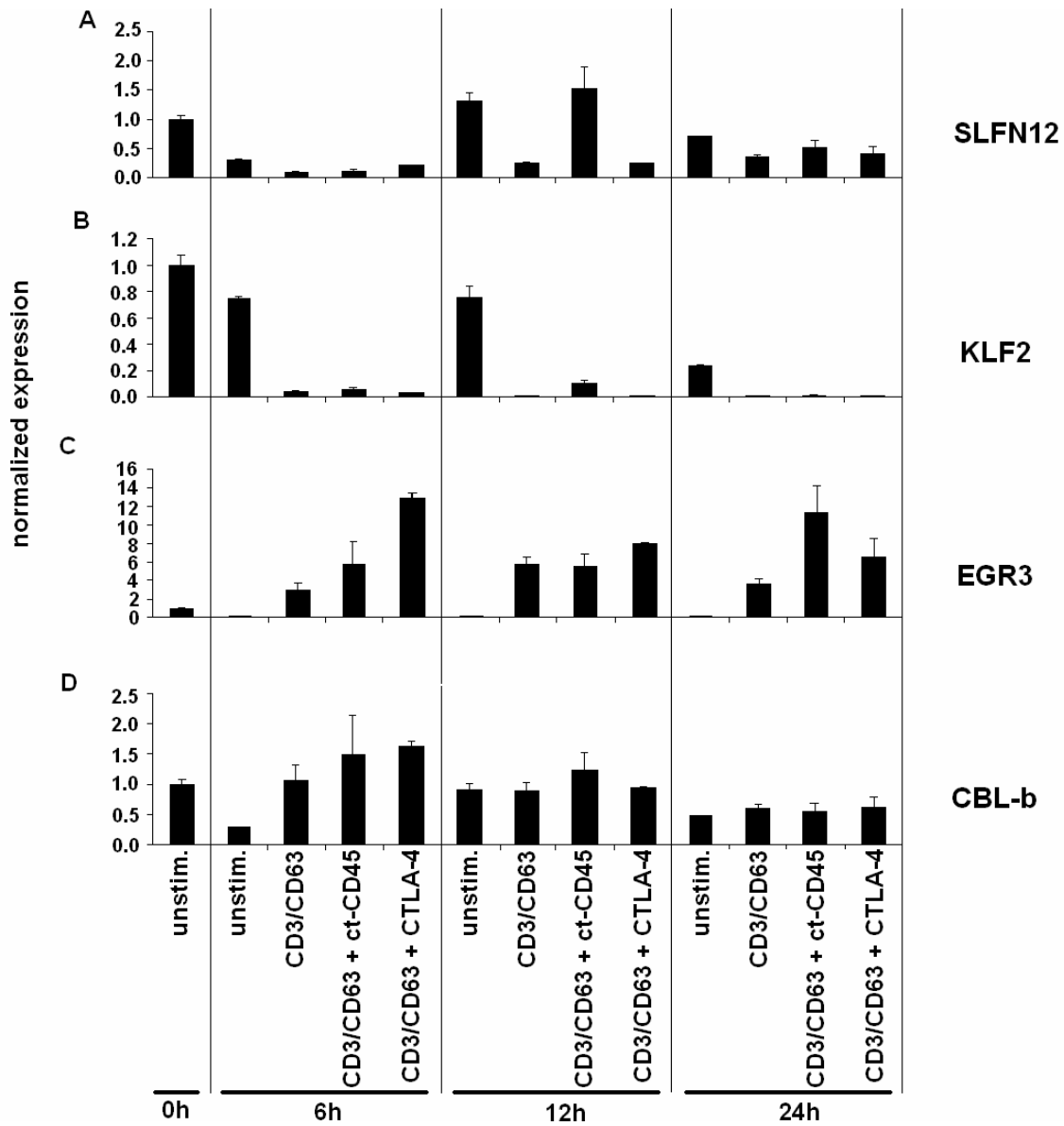
Fig. 21: Kinetics of SLFN12 induction in human cord blood T cells. Plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3/CD63 in the presence or absence of ct-CD45 and CTLA-4 or were mock treated. Coord blood T cells were activated for 6 hours, 12 hours and 24 hours. Total RNA was extracted reverse transcribing mRNA into cDNA. Regulation of (A) SLFN12, (B) KLF2, (C) EGR3, and (D) CBL-b was determined using quantitative realtime PCR. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene.
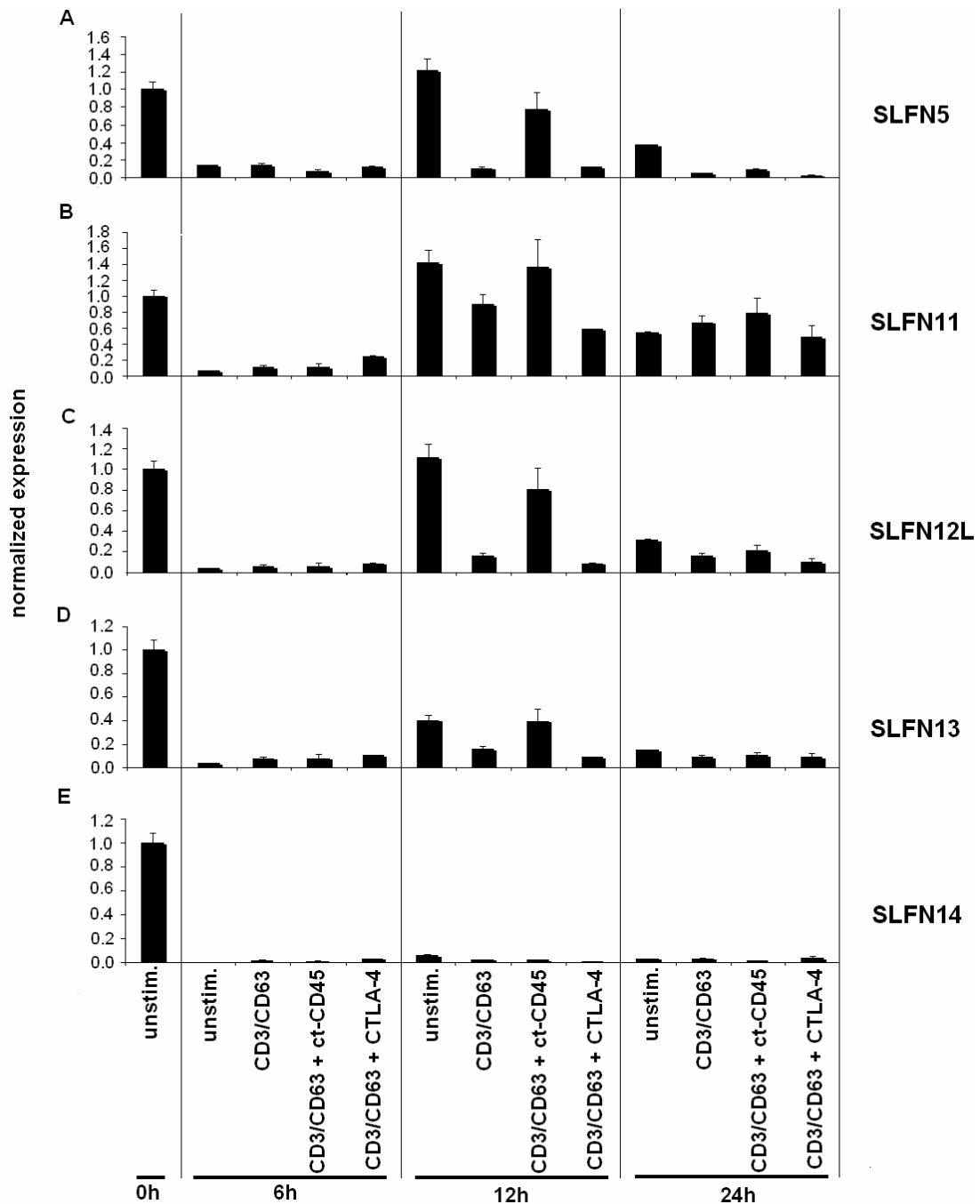
Fig. 22: Expression of other human SLFN family members during activation of human cord blood T cells. Plates were coated with anti-human and anti-mouse IgG antibodies, washed incubated with anti-CD3/CD63 in the presence or absence of ct-CD45 and CTLA-4 or were mock treated. Coord blood T cells were activated for 6 hours, 12 hours and 24 hours. Total RNA was extracted and reverse transcribed into cDNA. Regulation of (A) SLFN5, (B) SLFN11, (C) SLFN12-like, (D) SLFN13, (E) SLFN14 was analysed using quantitative realtime PCR. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene.

4.13. Inhibition by ct-CD45 is accompanied by a reduction in cyclin D1 mRNA levels

Growth regulation by some members of the Schlafen family in the mouse has been shown to occur via specific inhibition of cyclin D1 [62] although these findings have not been left unchallenged [63]. Hypothesizing that SLFN12 might exert its action via a similar mechanism we investigated the expression of selected D- and E-type cyclins and their respective kinases.

Again we relied on CD3/CD63 activation over a 24 hour course as a model. We did not find expression of cyclin-dependent kinases (CDK) 2 and 4 (Fig. 23A-B) impaired in T cells provided with ct-CD45 during activation indicating that kinase activity rather than kinase protein levels must be affected. However, when looking at cyclin expression patterns we found substantial inhibition of cyclin D1 (Fig. 23C) expression although this decrease in mRNA levels was only detectable after 24 hours. Detection of cyclin D1 mRNA in human T lymphocytes is of special interest since it was reported not to be expressed in this cell type [89]. A plausible explanation for these diverging results might lie in the advances of PCR technology since the publication of the prior finding.

We did not obtain reproducible inhibition of cyclin D2 mRNA (Fig. 23D) although this cyclin is induced early after T cell activation [89]. A similar result was obtained for the E-type cyclin E1 (Fig. 23E).

p27^{kip1} has been described as specific inhibitor of CDK2 activity and as a potential anergy factor [55]. Thus, we investigated whether it acts in a similar manner in ct-CD45-induced T cell anergy. However, we could not find prolonged and substantial induction of p27^{kip1} (Fig. 23F) during the time course indicating that it might not be causative factor for the distinct hyporesponsive state of T cells anergized via ct-CD45.

4.14. Cyclin gene expression is also reduced in cord blood T cells

We investigated whether induction of cyclins is also impaired in cord blood T cells, despite lack of reproducible inhibition of proliferation [77]. However, cytokine levels are similarly reduced as in peripheral T cells suggesting some effect of ct-CD45 binding [77].
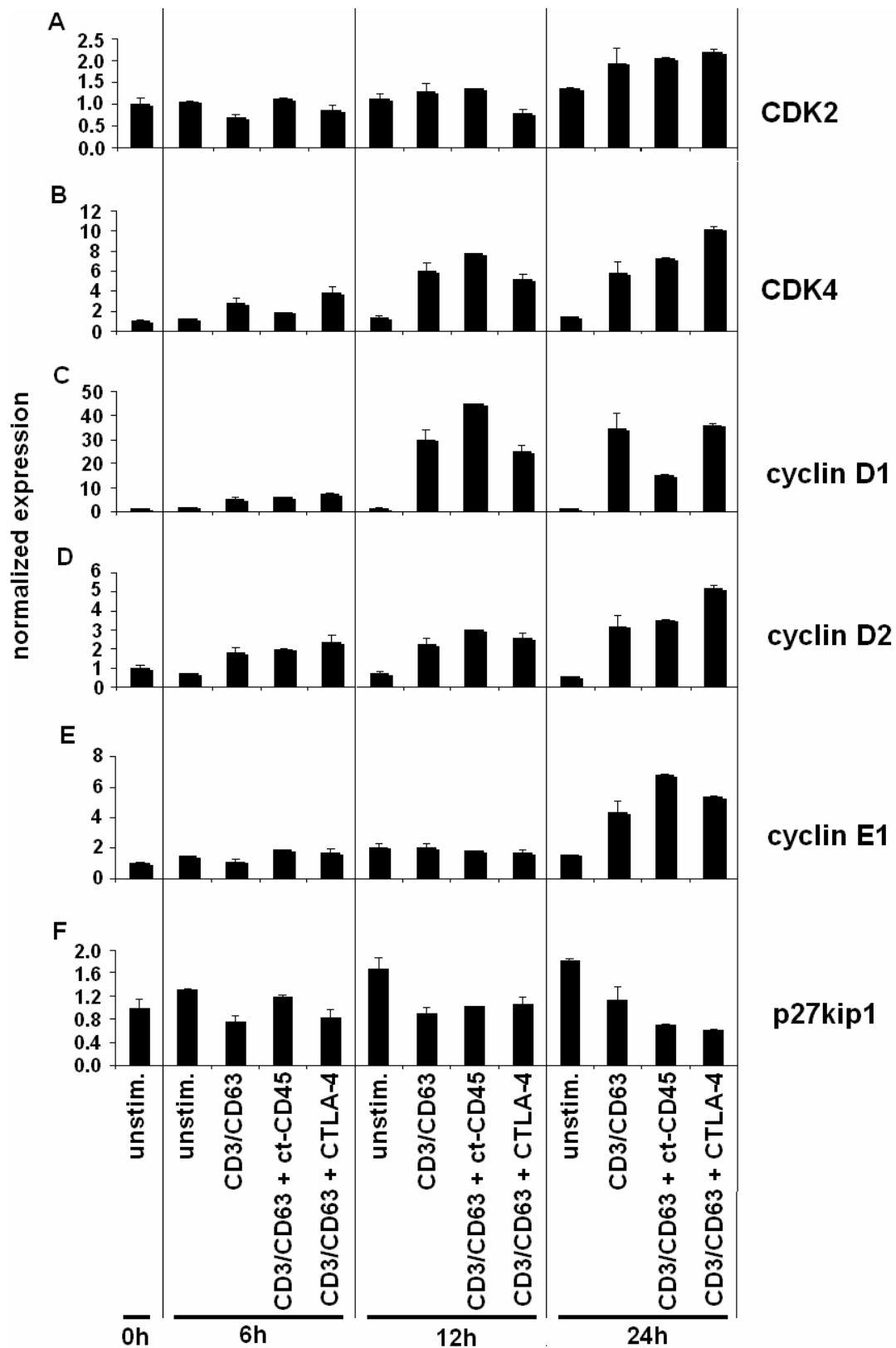
Fig. 23: Regulation of cyclins and cyclin-dependent kinases by ct-CD45. Plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3/CD63 in the presence or absence of ct-CD45 and CTLA-4 or were mock treated. Peripheral T cells were activated for 6 hours, 12 hours and 24 hours. Total RNA was extracted reverse transcribing mRNA into cDNA. Regulation of (A) CDK2, (B) CDK4, (C) Cyclin D1, (D) Cyclin D2

and (E) Cyclin E1 and (F) p27^{kip1} was determined using quantitative realtime PCR. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene. One representative out of two experiments performed is shown.
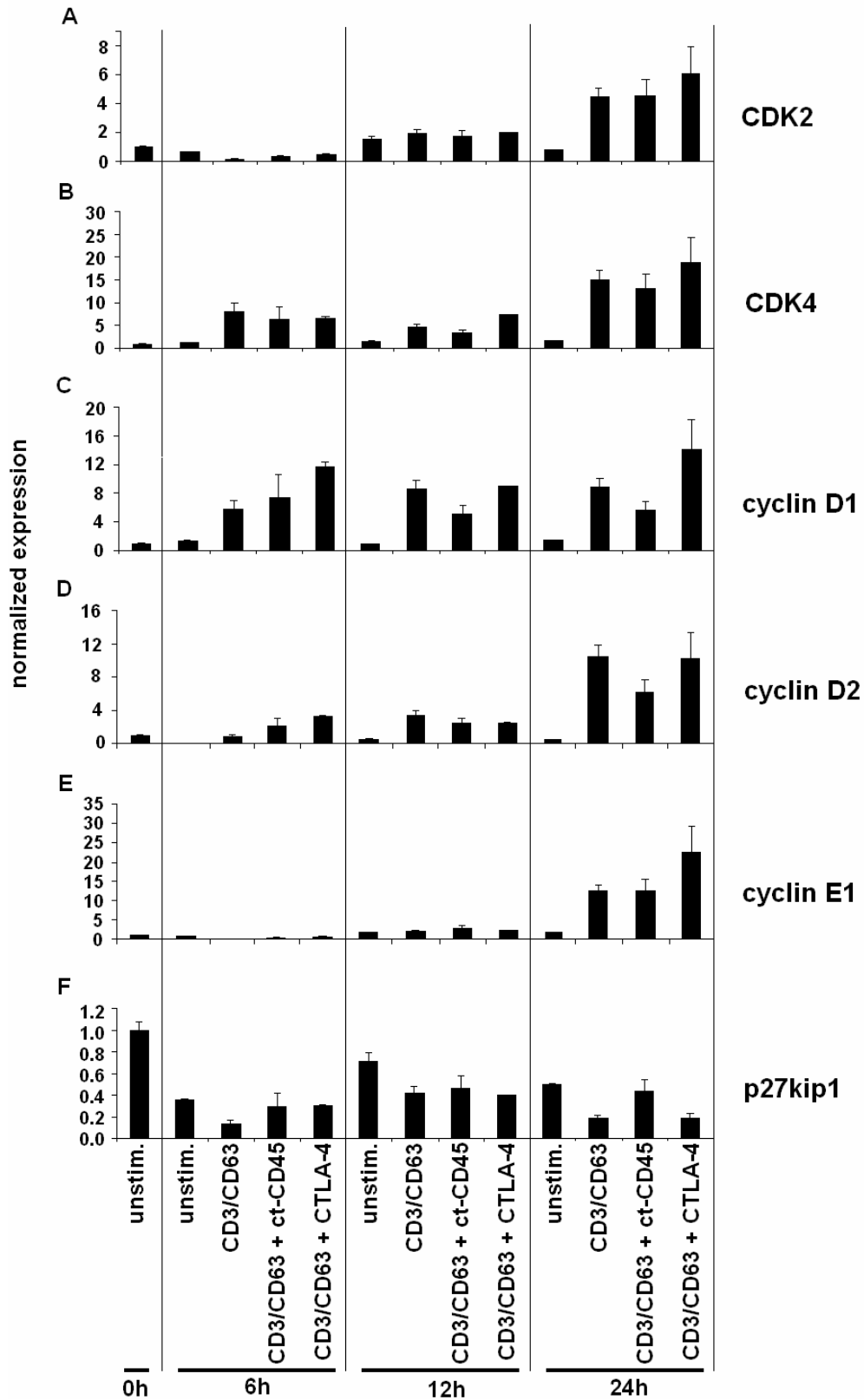
Fig. 24: Regulation of cyclins and cyclin-dependent kinases in cord blood T cells. Plates were coated with anti-human and anti-mouse IgG antibodies, washed, incubated with anti-CD3/CD63 in the presence or absence of ct-CD45 and CTLA-4 or were mock treated. T cells from human umbilical cord blood were activated for 6 hours, 12 hours and 24 hours. Total RNA was extracted reverse transcribing mRNA into cDNA. Regulation of (A) CDK2, (B) CDK4, (C) Cyclin D1, (D) Cyclin D2 and (D) Cyclin E1 and p27^{kip1} was determined using quantitative realtime PCR. Mean values +/- standard deviation (SD) of duplicate determination of the reference gene are indicated. CD3E was used as a housekeeping gene.

Nevertheless, we did not expect to see effects on cyclin levels which should be associated with altered CDK activity and subsequently impaired cell cycle progression. We were quite surprised to find that even cord blood T cells display reduction in cyclin D1 (Fig. 24C) as well as in cyclin D2 expression (Fig. 24D). As observed before for peripheral T cells, induction of cyclin E1 was unaltered (Fig. 24E). Similarly, expression of cyclin-dependent kinases CDK2 (Fig. 24A) and CDK4 (Fig. 24B) was barely affected. The CDK inhibitor p27^{kip1} was not found to be regulated by ct-CD45 during the first 12 hours (Fig. 24F). However, unlike in peripheral T cells a slight increase in p27^{kip1}expression was obtained after 24 hours.

4.15. Transfection of a SLFN12 siRNA reduces inhibition of T cell proliferation

As our results strongly suggested a role for SLFN12 in the inhibition of peripheral T cell proliferation we investigated whether loss of SLFN12 might prevent this inhibition observed. Applying a siRNA-mediated knockdown approach, we hoped to achieve sufficient reduction of SLFN12 mRNA to obtain a phenotype. The major obstacle for such an approach lies in the lack of efficient non-viral transfection protocols [90]. Using Lipofectamine^{TM} 2000 which is a cationic lipid-based transfection reagent [91] we transfected human T cells which were pre-activated via plate-bound CD3 and CD63 antibodies for three days with a mix of three different SLFN12 siRNAs at a concentration of 10 nmol per duplex. Transfected T cells were harvested 24 hours after transfection and rested for 2 days in fresh medium until they were restimulated with CD3/CD63 in the presence or absence of ct-CD45 fusion proteins. Proliferation of re-stimulated T cells was tested using ^{3}H-methyl thymidine incorporation as a read-out. Slightly increased proliferation was observed for ct-CD45-treated T

cells after transfection with the SLFN12 siRNA mix compared to scrambled and transfection reagent controls (Fig. 25A).
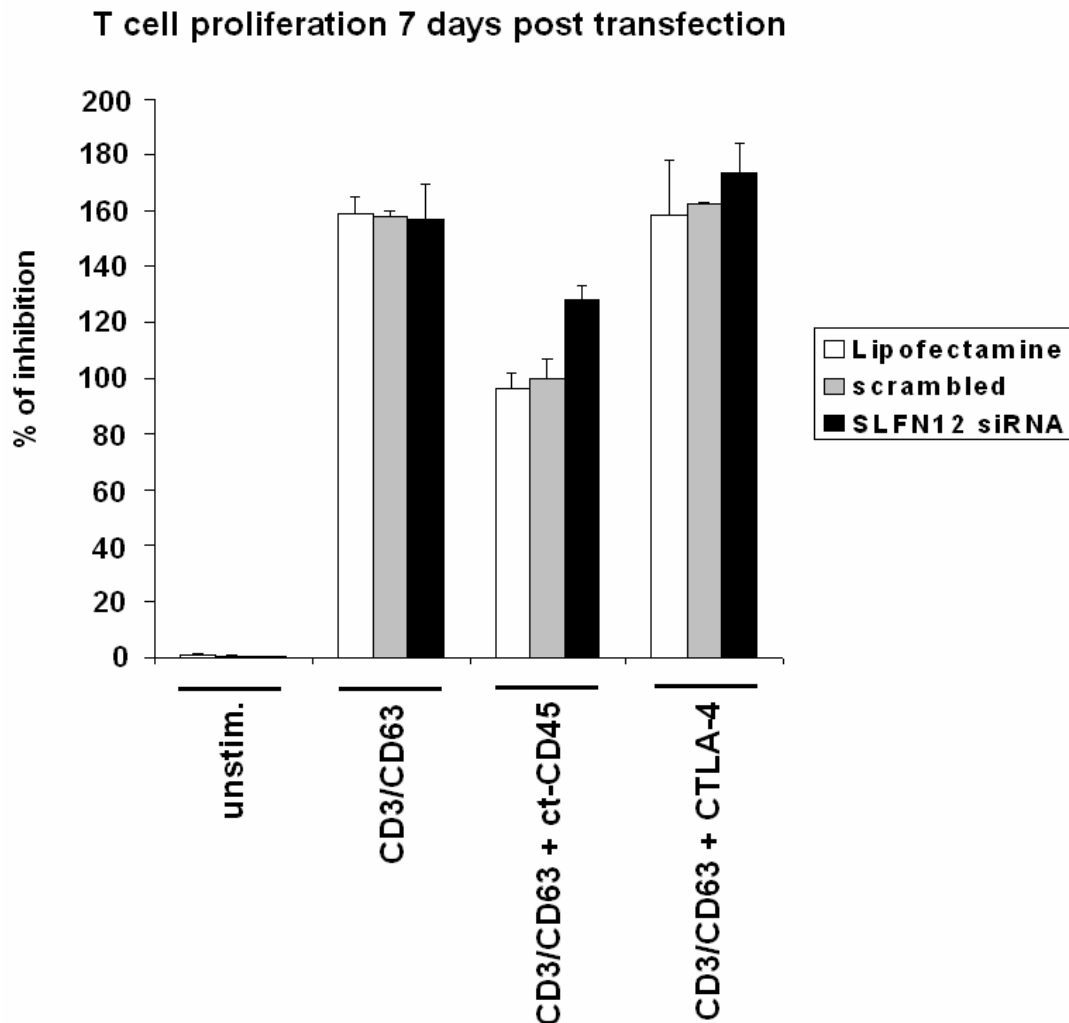
Fig. 25: Transfection of a SLFN12 siRNA mix enhances proliferation of ct-CD45 treated T cells. T cells were activated via plate-bound CD3/CD63 antibodies for four days in a 96-well plate. Transfection of scrambled control and a mix of three SLFN12 siRNAs (10nmol each) were transfected using Lipofectamine^{TM} 2000 on day 3. Cells were harvested on day four, washed and let rest for two days until they were restimulated with CD3/CD63 and plate-bound fusion proteins. ^{3}H-methyl thymidine was added on day 3 of restimulation and incorporation was measured 18 hours later. Proliferation of transfected T cells after restimulation is displayed relative (%) to inhibition. Data shown are mean values +/- SD of triplicates.

4.16. ct-CD45 shows blockable binding to a Bw clone expressing PRAT4A

A screen of a human T cell cDNA library expressed in the Bw 5417 cell line revealed the protein associated with Toll-like receptor 4 A (PRAT4A) as a potential receptor candidate for ct-CD45 [77]. Although a siRNA knock-down of

PRAT4A in the PRAT4A-expressing BW clone 1C8 could reduce binding of ct-CD45 by about 50%, a direct blockade of ct-CD45 via antibodies could not be shown [77]. Initially, PRAT4A was described as a protein regulating the cell surface expression of TLR4 [78] as well as of other TLRs which resides in the endoplasmic reticulum [82]. However, PRAT4A seems to be expressed at low levels at the cell surface of activated T cells [77].

We tested whether binding of ct-CD45 can be blocked by a PRAT4A antibody. Bw clone 1C8 was preincubated with a polyclonal rabbit anti-PRAT4A antibody or mock-treated before addition of ct-CD45 fusion proteins. Flow cytometric analysis revealed markedly reduced binding of ct-CD45 to the cell line upon PRAT4A blockade suggesting interaction between the two molecules (Fig. 26).
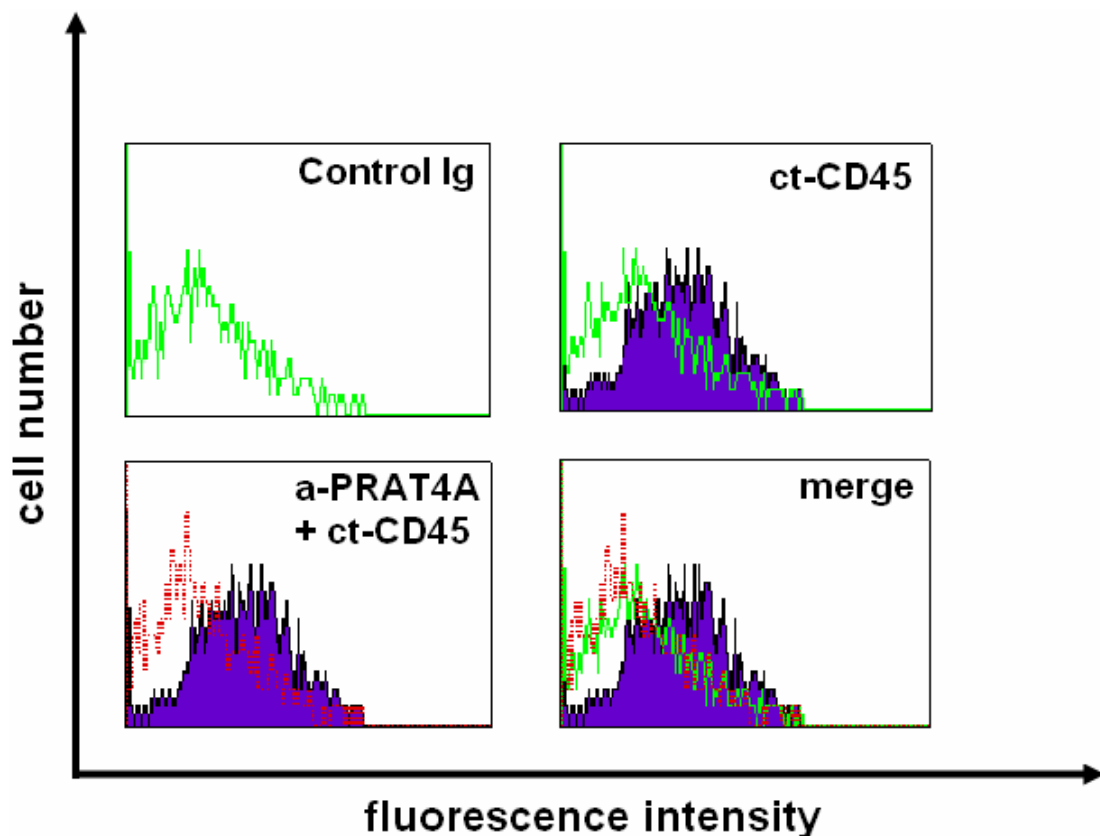
Fig. 26: ct-CD45 binding to a PRAT4A-overexpressing BW clone can be blocked by a PRAT4A antibody. The BW clone 1C8 was stained with a ct-CD45-Fc fusion protein with or without prior incubation with a polyclonal rabbit anti-murine PRAT4A antibody. CTLA-4-Fc was used as a non-binding control. A phycoerythrin-labelled antibody specific for the Fc fragment of human IgG was used for detection via flow cytometry. The shaded histogram represents ct-CD45 binding to the cell line; dotted lines stand for preincubation with a PRAT4A antibody prior to addition of ct-CD45. Full lines are the non-binding control.

5. Discussion

The study by Kirchberger et al. [76] suggested that the alternative cleavage of the well characterized tyrosine phosphatase CD45 [71] gives rise to a product negatively regulating the proliferation of human T cells. However, the distinct molecular pattern governing this state remained unidentified. It was already known that this reduction in proliferation was not due to cell death as affected cells were still alive. As demonstrated in the present work, one remarkable feature of ct-CD45-inhibited T cells is the inability of lymphoblast formation, characterized by increase in cellular size (Fig. 10). Instead, the cells remained relatively small which was accompanied by a lack of T cell activation markers as shown before [77]. Another finding was that T cells treated with ct-CD45 are hyporesponsive to restimulation but can be partly restimulated in the presence of exogenous IL-2 [77]. Since microarray analysis indicated downregulation of the IL-2 receptor in ct-CD45-treated lymphocytes, we concluded that restimulation via this classical growth factor might probably be inefficient due to a lack of IL-2 binding. Thus, it was further investigated whether alternative factors reported for their cabability of promoting T cell expansion in vitro might better serve our purpose. IL-7 is a cytokine which was originally implicated in maintenance of memory cell homeostasis [80] However, a recent study showed that IL-7 was more efficient than IL-2 in ex vivo expansion of tumour-specific CD4$^+$ T cells [81]. Indeed, it was found that IL-7 aids in restimulation of T cells anergized via ct-CD45 (Fig. 12). Nevertheless, it displayed lower efficiency than IL-2 indicating that IL-7 cannot act as a full growth factor in this distinct anergic state.

To our surprise, the microarray data did not show induction of established molecules involved in anergy. Instead, SLFN12 and KLF2 were most strongly transcribed which was confirmed via quantitative real-time PCR (Fig. 16). While KLF2 represents a well characterized factor involved in T cell quiescence [60] little was known to date about SLFN12.

Most research related to the Schlafen gene family has been carried out in the murine system. Initial studies associated this family with the regulation of lymphocyte development and growth control [61]. The inhibitory roles of Schlafen family members appeared to occur very early in the cell cycle before

G1 to S phase transition [61] and was molecularly characterized to occur at the transcriptional level via inhibition of cyclin D1 promoter activity at least in the case of prototypic Slfn1 [62]. The most striking finding was by Berger et al. [66] demonstrating a non-redundant function of murine Slfn2 in lymphocyte quiescence, again indirectly providing evidence for a growth regulatory role of SLFNs. The human SLFN family bears little orthology to the murine slfns. SLFN12 is exclusively expressed in primate species [99]. Compared to SLFN12, Slfn2 [66] and Slfn3 are mostly similar. However, a BLAST search using SLFN12 mRNA as a query (NM_018042.3) indicates low overall similarity implying some distinctiveness in function. Nevertheless, our results with human T cells inhibited by ct-CD45 suggests a role for SLFN12 in the quiescent state and in T cell anergy similar to other factors exerting dual roles such as Tob or Ikaros [51]. KLF2 is another classical quiescence factor [60] but has hitherto never been reported to be highly expressed in anergic T cells. While an anti-proliferative role for KLF2 has been established via overexpression experiments in the T cell leukaemia line Jurkat [93] such a role has still to be shown for SLFN12. Nonetheless, the expression of these quiescence factors in activated T cells retaining their small cellular size while being hyporesponsive to restimulation suggests the repertoire of functional plasticity of T cell differentiation states extended. It is tempting to speculate that these combined features represent another functional state for T cells - "quiescent anergy". Quiescent-anergic lymphocytes share features with anergic T cells regarding their hyporesponsiveness to activation while retaining small cellular size similar to cellular quiescence (Table 2).

Expression profiling of naive T cells versus Jurkat cells and the neuroblastoma cell line SK-N-SH showed considerably lower expression not only of SLFN12 and KLF2 but of most other SLFN family members in both cell lines (Fig. 15). This indicates that high expression of these genes would not be beneficial for survival or growth of continuously dividing cell lines. We also assessed SLFN12 and KLF2 regulation during the first few hours upon T cell activation showing that both factors are downregulated already after 6 hours (Fig. 13). However, while KLF2 transcription is sustainably decreased, SLFN12 downregulation seems to be transient. After 24 hours SLFN12 mRNA levels start to rise in activated cells and have nearly reached the levels of unstimulated controls after

48 hours. This pattern raises a question whether a factor only transiently lost upon activation can have an essential role in proliferation. Nevertheless, this supports the role of SLFN12 during earlier stages of T cell activation probably negatively regulating cell cycle entry from the G_0 phase.

We did not find any evidence for an involvement of SLFN12 or KLF2 in the induction of canonical T cell anergy. Altered transcription of any of these two factors was not detected for TCR stimulation in the absence of costimulation whereas EGR3 was found to be substantially upregulated.

phenotype	quiescent lymphocytes	activated lymphocytes	anergic lymphocytes	„quiescent-anergic" lymphocytes
cell size	small	large	large	small
rate of protein synthesis	low	high	low	low
response to activation	proliferation	expansion or apoptosis	none or apoptosis	none

Table 2: T cell differentiation states extended. The morphological and functional features as well their gene expression profile suggest a novel functional state for T cells binding ct-CD45 sharing features of both, quiescent and anergic lymphocytes Table adapted and modified from Yusuf and Fruman [58].

Additionally, it was investigated whether SLFN12 is regulated in other immune cells upon activation. Based on the observation of van Zuylen et al. [70] showing upregulation of SLFN12 during LPS stimulation of human monocyte-derived macrophages we tested the expression of SLFN12 and other SLFN family members during the LPS challenge of human monocyte-derived dendritic cells. While little to no regulation was observed for SLFN12, SLFN12L, SLFN13 and SLFN14, SLFN5 was found to be substantially increased peaking 5 hours after LPS challenge. Conversely, SLFN11 was sustainably downregulated during a 24 hour time course (Fig. 14). These results suggest a possible role for these two SLFNs but not of other SLFN family members during the LPS-induced maturation of human dendritic cells. The regulation of SLFN12 as shown for macrophages might possibly be specific for this cell-type.

Having confirmed SLFN12 and KLF2 expression via qPCR 48 hours post activation we also hoped to confirm this expression on the protein level.

Unfortunately reliable antibodies serving this purpose are not available. Although reactivity with a SLFN12 fusion protein and human cell lysates could be shown, substantial differences in the molecular weights of detected bands were found for two different SLFN12 antibodies. Both antibodies also displayed strong reaction with cell lysates of the murine Bw 5417 cell line (Fig. 17). The latter observation suggests that the bands observed might be cross-reactions to other ubiquitously expressed proteins. As SLFN12 mRNA appears to be expressed at moderate to low levels it has to be concluded that the protein amount in the cell lysates is presumably below the limit of the detection for a western blot.

The kinetics of SLFN12 induction were observed over a shorter 24-hour time course and it was found that SLFN12 is induced very early in ct-CD45-treated T cells reaching its maximum after 6 hours (Fig. 18). We cannot exclude that this early upregulation is a result of mRNA stabilization rather than being an active transcriptional process. However, SLFN12 seems to be re-expressed 24 hours post activation suggesting an oscillating expression pattern. Surprisingly, elevated expression of KLF2 within the first 24 hours could not be shown, posing the question whether KLF2 might be important for the induction phase of anergy at all. Expression at a later time might just reflect a reduction in cell cycle progression. During the observed time course slight induction of EGR3 and of CBL-b was obtained after 12 hours and 24 hours, respectively. Since molecular targets of SLFN12 are unknown it cannot be excluded that both genes are transcribed as a result of SLFN12 expression. However, this upregulation appears to be moderate and cannot be compared in magnitude to the classical mode of anergy induction via TCR triggered in the absence of costimulation.

When looking at the expression pattern of other SLFN family members we found to our surprise that nearly all SLFNs display upregulation at early time points upon activation of T lymphocytes in the presence of ct-CD45 similar to SLFN12 (Fig. 19). Only SLFN11 and SLFN14 do not seem to be affected by ct-CD45 treatment at all. These results indicate that a certain degree of redundancy might exist within the human SLFN family which makes it difficult to exclude that the concerted action of SLFN family members might be required for the ct-CD45-induced hyporesponsive state of human T cells.

As IL-2 could partly reverse the anergic state imposed by ct-CD45 on T cells it was tested whether addition of exogenous IL-2 to the culture medium alters the expression of SLFN12 and KLF2. Indeed, it could be shown that IL-2 strongly promotes the downregulation of SLFN12 mRNA in T cells treated with ct-CD45 whereas KLF2 was only found to be altered in the absence of costimulation (Fig. 20). These results again suggest that SLFN12 rather than KLF2 might play the prominent role in this form of T cell anergy.
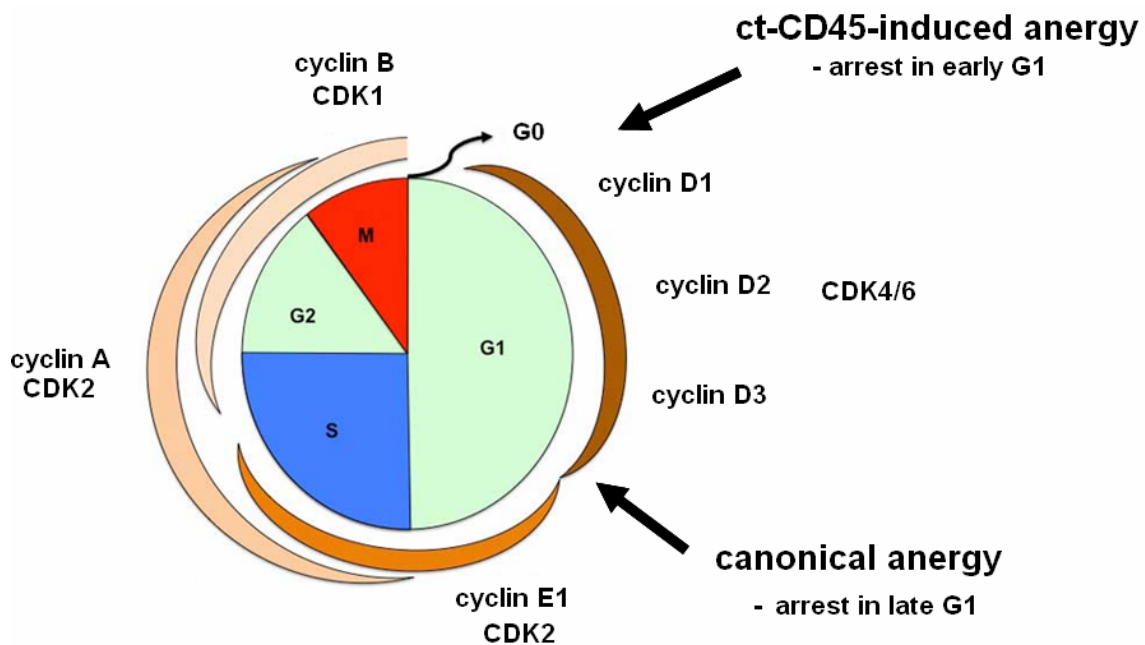

Fig. 27: ct-CD45-induced anergy is a non-canonical form of anergy. Impairment of cyclin D1 induction in T cell inhibited by ct-CD45 suggests an earlier inhibition than in canonical anergy which typically involves impairment of cyclin E1 expression [47]. Figure was adapted from [100] and modified with additional information from [47] and [53].

Although ct-CD45 has a potent action on human peripheral T cells, inhibition of proliferation was never observed for T cells from umbilical cord blood [77]. Thus, it was investigated whether SLFN12 and KLF2 are induced in these cells. Having determined the expression pattern again over a 24 hour time course, both factors were found to be expressed in cord blood T cells although the induction was observed to occur at a later stage than in mature T cells. SLFN12 upregulation in ct-CD45-treated T cells was found only 12 hours post activation and coincided with a slight upregulation of KLF2 (Fig. 21). This was different to peripheral T cells where we could not find differences in KLF2 expression within the first 24 hours. However, unlike SLFN12, KLF2 mRNA levels never reached

the levels of naïve T cells. Somewhat similar to mature T cells, a slight but delayed induction of EGR3 mRNA was observed. Looking at the expression pattern of other members of the human SLFN family suggested a potential redundancy as SLFN5, SLFN12L and SLFN13 were induced at the very same time as SLFN12 (Fig. 22). The differential induction of SLFN genes for mature and naïve T lymphocytes provides a possible explanation for the lack of overt inhibition of the latter.

As Slfn1 has been implicated in the regulation of cyclin D1 expression [62] we also investigated the expression of G_1 phase cyclins as well as of their respective cyclin-dependent kinases. Indeed, substantial downregulation of cyclin D1 in ct-CD45 treated peripheral T cells (Fig. 23) was obtained. Interestingly, this observation was made only after 24 hours of activation suggesting the need for another factor to be synthesized to enable the subsequent inhibition of cyclin D1. Inhibition of cyclin D2 or the E-type cyclin E1 was not detected nor was CDK2 and CDK4 induction found to be impaired. Since many reports have suggested the CDK2 inhibitor p27^{kip1} as an anergy factor [47, 55] we tested its expression in our system but did not obtain any evidence for its involvement. As done before for SLFN gene expression cyclin mRNA levels were also determined in human cord blood T cells. Reduced expression of cyclin D1 and cyclin D2 was even detected in these cells whereas expression of cyclin E1 and CDKs was unaltered (Fig. 24). p27^{kip1} appeared to be slightly upregulated 24 hours after activation of cord blood T cells, but we lack further evidence for a major involvement of this factor. In conclusion, this data indicates that impairment of T cell proliferation is achieved via specific inhibition in the early G1 phase of the cell cycle which presumably results in diminished expression of IL-2 subsequently promoting the induction of anergy.

In order to prove the involvement of SLFN12 in the action of ct-CD45 siRNA-mediated knockdown of SLFN12 was performed showing slight enhancement of T cell proliferation in ct-CD45-treated T cells compared to controls (Fig. 25). The subtle phenotype obtained requires further investigation to conclusively characterize SLFN12 as the major factor in the ct-CD45-induced anergic state. Since primary human T cells are hard to transfect a more efficient transfection protocol needs to be established.

A protein associated with TLR4 (PRAT4A) had been identified as a potential receptor candidate by our group [77] but a physical blockade via antibodies could never been shown. Thus, blocking experiments were performed using a polyclonal antibody against PRAT4A. Preincubation with this antibody strongly reduced ct-CD45 binding to a PRAT4A overexpressing cell line thereby providing another piece of evidence for a direct interaction between these two molecules (Fig. 26).
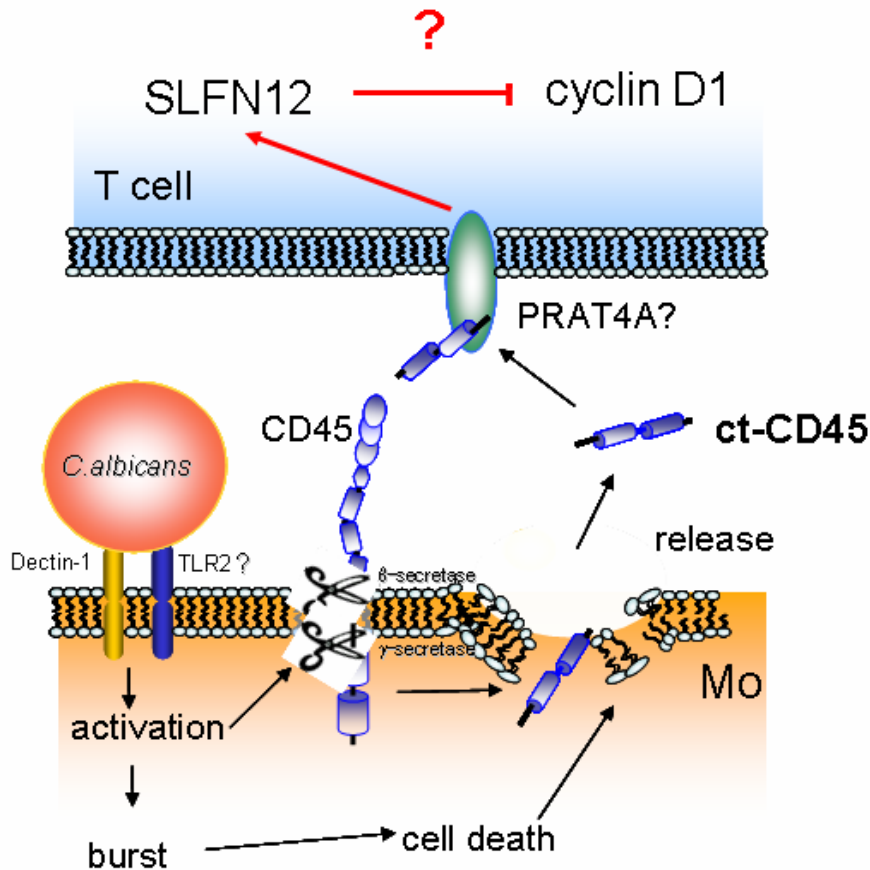
Fig 28: Current model for the molecular mechanisms behind ct-CD45 generation and subsequent inhibition of T cell proliferation and anergy induction.

To conclude, binding of ct-CD45 to PRAT4A on activated T cells leads to the upregulation of SLFN12 and KLF2. The results obtained during this study especially demonstrate a role for SLFN12 as an anergy factor since induction of this factor occurs early and is impaired upon addition of exogenous IL-2. Furthermore, partial restoration of T cell proliferation after transfection of a SLFN12 siRNA provides further support for its involvement in the anergic state induced.

Regulation of cell cycle-associated factors in T cells activated in the presence of ct-CD45 appears to be different to canonical anergy that is characterized by a block in the late G1 phase [47]. The impairment of cyclin D1 mRNA induction in ct-CD45-challenged T cells suggests an earlier inhibition taking place already at the entry stage from the quiescent G_0 to the G_1 phase (Fig. 27). Since this inhibition follows SLFN12 induction it is tempting to conclude a direct effect of SLFN12 on cyclin D1 regulation (Fig. 28).

Taken together, this work gives insight into the molecular mechanisms of T cell anergy induced via ct-CD45. The biological relevance of this state possibly lies in the dampening of exaggerated adaptive immune responses against ubiquitous but mostly harmless fungal agents. However, this mechanism might be exploited in immunocompromised individuals during systemic fungal infections where excessive generation of ct-CD45 would promote a state of severe immunosuppression.

Future studies will try to unravel these relations by trying to establish more efficient ways of SLFN12 knock-down in primary human T cells and by analysing the effects of SLFN12 overexpression on the activity of the cyclin D1 promoter.

6. Abbreviations

Abbreviation	meaning
AAA	ATPase family associated with various cellular activities
ACTB	actin beta (β-Actin)
AP-1	activator protein 1
APC	antigen-presenting cell
β2-m	β2-microglobulin
CBL-b	Casitas B cell lymphoma B
CD	cluster of differentiation
CD3E	CD3 epsilon (CD3ε)
CDK	cyclin-dependent kinase
ct-CD45	cytoplasmic tail of CD45
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
D1 / D2	domain 1 / domain 2 of CD45
DGK	diacylglycerol kinase
DMSO	dimethyl sulfoxide
EGR	early growth response protein
ER	endoplasmic reticulum
FCS	fetal calf serum
FOXO	forkhead box O
FOXP3	forkhead box protein 3
GAPDH	glycerinaldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
GRAIL	gene related to anergy in lymphocytes
GST	glutathione S-transferase
HRP	horseraddish peroxidase
IFN	interferon
Ig	immunoglobulin
IκB	Inhibitor of NFκB
IL	interleukin
KLF	Krüppel-like factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
mDC	monocyte-derived dendritic cell
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells

NFκB	nuclear factor κB
PE	phycoerythrin
PRAT4A	protein associated with Toll-like receptor 4
PTP	protein tyrosine phosphatase
qPCR	quantitative (real-time) PCR
siRNA	short interfering RNA
SLFN	Schlafen (family member)
SLFN12L	SLFN12-like
TCR	T cell receptor
TLR	Toll-like receptor

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