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Regulation of T cell activation by ct-CD45:
analysis of molecular and functional mechanisms

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Abstract

CD45 was one of the first signaling molecules identified on leukocytes and is used as a leukocyte marker molecule. It is the prototypic member of transmembrane receptors like protein tyrosine phosphatases (PTPs) and various forms of it are expressed on all nucleated hematopoetic cells. CD45 plays an essential role in immune functions by dephosphorylating different substrates. Recently an alternative function for the intracellular domain of CD45 (ct-CD45) was discovered by our group. It was shown that CD45 is cleaved and ct-CD45 is released during activation of human monocytes and granulocytes by fungal stimuli. Furthermore ct-CD45 was found to ct-CD45 act as a cytokine like factor which inhibits T cell proliferation induced by dendritic cells or CD3 antibodies. The cytoplasmatic tail of CD45 can thereby act as an intercellular regulator between the innate and the adaptive immune system. In this study the direct impact of ct-CD45 on the activation and function of T cells was further investigated in an antigen presenting cell free system. We found that ct-CD45 inhibited CD3 and CD3/CD63 but not CD3/CD28 induced proliferation in peripheral T cells and that it did not inhibit the proliferation of cord blood T cells which were stimulated the same way. Surprisingly the production of IL-2, IL-4, IL-10, IL-13, IL-17 and IFN gamma was strongly reduced even in those T cells whose proliferation was not inhibited. The only exception was IL-4 which showed an increased production in cord blood T cells upon stimulation in the presence of ct-CD45. In line with the downregulation of cytokine production the expression of de novo synthesized activation markers on the cell surface of T-cells like CD25, CD97 and MHCII and the surface expression of CD69, which originate in part from preformed intracellular pools, was down regulated. Furthermore we found out that these ct-CD45 treated T cells show no proliferation upon restimulation without ct-CD45

and that this effect can be only partly reversed by addition of exogenous IL-2.

In addition to that we were trying to find a possible receptor candidate for ct-CD45 on activated T cells. It was shown that this protein binds specifically to activated T cells and that this binding can be blocked by mAb. Binding assays with a cDNA library for activated T cells gave evidence for interaction of ct-CD45 and a protein associated with Toll-like receptor 4 (PRAT4A). PRAT4A was recently discovered as an ER-resident chaperon which is indispensable for the trafficking of most TLRs. Even though this protein is described as ER resident binding assays showed that PRAT4A is also expressed on the cell surface of activated T cells.

In this study we showed that PRAT4A is a possible receptor candidate for ct-CD45 and that the binding of this cytoplasmatic part of CD45 to activated T cells lead to an anergy like hyporesponsive state that can only be partly reversed by the addition of exogenous IL-2.

Zusammenfassung

CD45 war eines der ersten signaling Moleküle die auf Leukozyten identifiziert worden sind und wird als Leukozyt Markermolekül verwendet. Es ist der Prototyp von Transmembranrezeptor ähnlichen Protein Tyrosin Phosphatasen (PTPs) und es werden verschiedene Isoformen davon auf allen kernhaltigen hematopoetischen Zellen exprimiert. CD45 spielt eine essenzielle rolle im Immunsystem indem es verschiedene Substrate dephosphoryliert. Vor kurzem wurde, von unserer Gruppe, eine alternative Funktion für die intrazelluläre Domäne dieses Proteins (ct-CD45) gefunden. Es wurde gezeigt, dass wenn humane Monozyten und Granulozyten mit Pilzstimuli aktiviert werden CD45 gespalten und ct-CD45 freigesetzt wird. Dieses ct-CD45 agiert als zytokinähnlicher Faktor, der die T Zell Proliferation hemmt wenn diese mit dendritischen Zellen oder CD3 Antikörper aktiviert werden. Der zytoplasmatische Teil von CD45 kann dabei als interzellulärer Regulator zwischen dem angeborenen und dem adaptiven Immunsystem wirken. In dieser Studie wurde die direkte Wirkung von ct-CD45 auf die Aktivierung und die Funktion von T Zellen in einem APC freien System weiter untersucht. Wir haben entdeckt, dass ct-CD45 die Proliferation von peripheren T Zellen inhibiert wenn diese über CD3 oder CD3/CD63 aktiviert werden aber nicht wenn sie mit CD3/CD28 stimuliert werden. Im Gegensatz dazu hatte ct-CD45 keinen Einfluss auf die Proliferation von Nabelschnurblut T Zellen, die auf die gleiche Weise aktiviert worden sind. Überraschender Weise wurde dennoch die Produktion von IL-2, IL-4, IL-10, IL-13, IL-17 und IFN gamma stark reduziert, auch in den T Zellen bei denen die Proliferation nicht gehemmt worden ist. Die einzige Ausnahme war IL-4, welches eine erhöhte Produktion in Nebelschnur Blut T Zellen in der Anwesenheit von ct-CD45 zeigte. Genauso wie die Zytokine wurde auch die Expression von neu

synthetisierten Aktivierungsmarkern wie CD25, CD97 und MHCII und auch die Oberflächenexpression von CD69, das zum Teil schon in intrazellulären Pools gespeichert wird und dann von dort aus an die Zelloberfläche gebracht wird, herunter reguliert. Darüber hinaus haben wir entdeckt, dass diese mit ct-CD45 behandelten T Zellen bei Restimulierung ohne ct-CD45 keine Proliferation zeigen und dass dieser Effekt nur zum Teil durch Zugabe von IL-2 rückgängig gemacht werden kann.

Außerdem haben wir versucht einen möglichen Rezeptorkandidaten für ct-CD45 auf aktivierten T Zellen zu finden. Es wurde gezeigt, dass dieses Protein spezifisch an aktivierte T Zellen bindet und dass diese Bindung mit mAb blockiert werden kann. Bindungsassays mit einer cDNA Bibliothek für aktivierte T Zellen haben eine Bindung zwischen ct-CD45 und einem Protein (PRAT4A), das mit dem Toll-like Rezeptor 4 assoziiert ist, gezeigt. PRAT4A wurde vor kurzem entdeckt und als ER-residentes Chaperon beschrieben, das unentbehrlich für das Trafficking der meisten TLRs ist. Obwohl dieses Protein als ER-resident beschrieben worden ist ist es uns gelungen es auch auf der Zelloberfläche von aktivierten T Zellen nachzuweisen.

In dieser Diplomarbeit haben wir gezeigt, dass PRAT4A ein potentieller Rezeptorkandidat für ct-CD45 ist und dass die Bindung dieses cytoplasmatischen Teiles von CD45 an aktivierte T Zellen zu einem Anergie ähnlichen Stadium führt, das nur zum Teil durch Zugabe von IL-2 umgekehrt werden kann.

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Introduction

Innate and Adaptive Immunity

The defense against pathogens is mediated by the innate immunity and the adaptive immunity. Innate immunity provides the early line of defense against microbes and consists of cellular and biochemical defense mechanisms that are in place even before infections. These mechanisms only react to microbes and not to noninfectious substances, and they respond in essentially the same way to repeated infections. They are specific for structures that are common throughout microbial groups like LPS or flagelin. The innate immune system consists of various components: physical and chemical barriers, like epithelia and the antimicrobial substances, which are produced at epithelial surfaces; phagocytical cells like neutrophils and macrophages; natural killer cells; blood proteins like the complement system proteins and other mediators of inflammation and cytokines which regulate many of the activities of the innate and the adaptive immunity.

The adaptive immunity, in contrast to innate immunity, develops as a response to infection and adepts to the infection. The characteristics of adaptive immunity are specificity for distinct molecules and ability to remember and respond stronger and faster to repeated exposure to the same pathogen.

There are two types of adaptive immune responses, called humoral immunity and cell mediated immunity. Those types of immune responses are mediated by different components of the immune system and function to eliminate different types of microbes.

Humoral immunity is mediated by antibodies, which are produced by B lymphocytes (B cells). Antibodies are molecules in the blood and mucosal secretions. They are the principal defense mechanism against extracellular microbes and toxins because secreted antibodies can bind to those

microbes and toxins. They can promote the phagocytosis of the bound microbes and toxins, can neutralize them or can trigger the release of inflammatory mediators from leukocytes.

Cell-mediated immunity is mediated by T lymphocytes (T cells). Intracellular microbes, like viruses, survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defense against such infections is a function of cell mediated immunity, which promotes the destruction of microbes residing in phagocytes or killing of infected cells to eliminate reservoirs of infection.

The adaptive and the innate immunsystem can not be seen as separated systems because they work closely together to defend the organism against infectious diseases and they need each other to function properly. Interferon gamma for example is produced by CD4 pos T cells, which are cells of the adaptive immune system and activates macrophages, which are cells of the innate immune system.

T cells also need cells of the innate immunity for activation because they can not bind directly to an antigen. They can only bind to an antigen when it is presented to them by an antigen presenting cell (APC) like macrophages or dendritic cells.

There are numerous other examples for the collaboration of the adaptive and innate immunity.[1]

T cells

The precursors of T lymphocytes (Tcells), which are the mediators of cellular immunity, arise in the bone marrow but then migrate to and mature in the thymus.

During their maturation in the thymus these cells are positive and negative selected. Weak interaction between T cell antigen receptors on double pos thymocytes and complexes of self peptide and major histokompatibility

complex on cortical thymic epithelial cells induce positive selection and prevent death by neglect.[2-6] Positive selection ensures that TCRs are restricted by the self MHC molecules present in the thymus. If the double positive T cell progenitors bind too strong to the self peptide MHC complex the cells die by apoptosis to ensure that no auto reactive T cells are produced.[1,4-6]

T cells recognize the antigens of intracellular microbes and function to destroy these microbes or the infected cells. They consist of functionally distinct populations, which are helper T cells and cytolytic or cytotoxic T lymphocytes (CTLs).

T cells do not produce antibody molecules. Their antigen receptors are membrane molecules different from but structurally related to antibodies. T cells do not recognize whole antigens like soluble proteins. Their receptors can only bind peptide antigens which are bound to host proteins. These host proteins are encoded by genes in the major histocompatibility complex (MHC) and that are expressed on the surface of other cells. As a result T cells recognize and respond only to cell surface-associated but not soluble antigens.

T cells are specific for amino acid sequences of peptides, because the antigen receptor of T cells recognizes very few amino acid residues within a single peptide, and different T cells can distinguish peptides that differ even at single amino acid residues.

T cells from any one individual recognize foreign peptide antigen only when these peptides are displayed by the MHC molecules of that individual. This phenomenon is called self MHC restriction.

Helper T cells recognize only peptides bound to class II MHC molecules. These peptides are mainly obtained from extracellular proteins that are internalized into the vesicles of antigen presenting cells.

CTLs recognize only peptides derived from cytosolic, usually endogenously synthesized, proteins, which are bound to class I MHC molecules. They

kill cells that produce and present foreign antigens on their MHC class I receptor, such as cells infected by viruses and other intracellular microbes. Some T lymphocytes, which are called regulatory T cells, function mainly to inhibit immune responses

In response to antigenic stimulation, helper T cells secrete proteins called cytokines. The function of the cytokines is to stimulate the proliferation of the T cells themselves, and other cells like B cells, macrophages and other leukocytes.

The initiation and development of adaptive immune responses require that antigens are captured and displayed to specific lymphocytes. The cells that serve this role are called antigen-presenting cells (APCs). The most highly specialized APCs are dendritic cells, which capture microbial antigens that enter from the external environment, transport these antigens to naïve T cells to initiate immune responses.

In cell mediated immunity, $CD4^+$ T cells activate macrophages to destroy phagocytosed microbes, in humoral immunity, $CD4^+$ helper T cells interact with B lymphocytes and stimulate the proliferation and differentiation of these B cells. Both the induction phase and the effector phase of T cell responses are triggered by the specific recognition of antigen.[1]

T cell subsets

CD4 positive T cells ($CD4^+$ T cells)

The antigen-driven differentiation of naive T cells into effector cells is central to adaptive immunity. After activation, naïve $CD4^+$ T cells differentiate into two subsets. Those two subsets are called T helper type 1 (Th1) and T helper type 2 (Th2) cells. $CD4^+$ T cells have been assigned to the Th1 or Th2 lineage based on their cytokine profiles.[7-9] These $CD4^+$ helper T cells recognize peptid antigens, which are presented by class II MHC molecules.

Th1 cells evolved to enhance clearance of intracellular pathogens and are defined on the basis of their production of interferon-gamma (IFN-gamma). Th2 cells are critical for the control of certain parasitic infections through the production of the clustered group of cytokines interleukin 4 (IL-4), IL-5 and IL-13. Both lineages have been associated with immune pathogenesis in the setting of dysregulated or unchecked activation. Th1 cells have been linked to many chronic autoinflammatory disorders, whereas Th2 cells are linked to atopy and asthma.[9]

Substantial advances have been made in understanding the developmental pathways that give rise to Th1 and Th2 cells.[9-13] The decision to develop into Th1 or Th2 effector cells is dependant on a large extent on cytokines.[14] Th1 cell development is coupled to the sequential involvement of cell-extrinsic and cell-intrinsic factors, including signal transducer and transcription activator 1 (STAT1), the transcription factor T-bet, IL-12 and STAT4, whereas TH2 cell development is coupled to IL-4, STAT6 and the transcription factor GATA-3. Cytokines, which are produced by mature effector cells of each lineage, can reinforce their own developmental program through positive and negative feedback acting on both naive T cells and innate immune cells. IFN gamma produced by mature Th1 cells or innate immune cells, and IL-27, an IL-12 family member produced by innate immune cells, induce STAT1 signaling and T-bet expression in antigen-activated, naive $CD4^+$ T cells, leading to upregulation of the IL-12 receptor (IL-12R) on developing Th1 cells and suppression of GATA-3. [9,15-17] Similarly, IL-4 produced by mature Th2 cells initiates Th2 cell development through its upregulation of GATA-3 via STAT6 and suppresses Th1 cell development by blocking IL-12R expression.[9,18] The ability of GATA-3 to promote its own transcription through a cell-intrinsic, positive feedback loop represents a potent mechanism for rapidly stabilizing Th2 cell development.[9,19] As a result of these robust counter-regulatory pathways, Th1 and Th2 cell development diverges rapidly after antigen priming to pro-

duce mature effector cells with stable, mutually exclusive expression of IFN gamma and IL-4.[9]

CD8 positive T cells ($CD8^+$ T cells)

Naïve $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs). Those effector $CD8^+$ T cells recognize and kill target cells presenting foreign peptide antigens bound to class I MHC molecules. $CD8^+$ T cells mediate their effector functions through production of cytokines such as IFN gamma and tumor necrosis factor (TNF) alpha and/or by cytolytic mechanisms. Such responses are important in preventing or maintaining control against disease in a variety of intracellular infections and perhaps also against certain tumors.[20]

Cytotoxic T lymphocytes (CTLs) are effector lymphocytes that have cytotoxic pathways that are necessary for defence against virus-infected or transformed cells. Those cells kill their cellular targets by either of two mechanisms that require direct contact between the effector and target cells. In the first pathway, cytoplasmic granule toxins, primarily a membrane-disrupting protein known as perforin, and a family of structurally related serine proteases (granzymes) with various substrate specificities are secreted by exocytosis and induce apoptosis of the target cell.[21,22] The granule-exocytosis pathway activates cell-death pathways that operate through the activation of apoptotic cysteine proteases (caspases), but it also leads to cell death in the absence of activated caspases.[22-24] The second pathway involves the engagement and aggregation of target-cell death receptors, such as FAS (CD95), by their ligands, such as FAS ligand (FASL), on the killer-cell membrane, which results in classical caspase-dependent apoptosis.[22,25]

Cord blood T cells

It is now well established that CB lymphocytes are more naive than adult lymphocytes. Although there is much evidence for this, the most common description of CB naivete´ is the observation that the majority of CB lymphocytes are CD45RA, whereas the majority of adult lymphocytes are CD45RO.[26-29]

A good indication of how a cell is functioning is by the cytokines it produces.[29] In the presence of endogenous APCs, human cord-blood T cells proliferate poorly and are poor producers of cytokines, including interleukin 2 (IL-2), interferon gamma, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5, when stimulated with soluble anti-CD3 or anti-CD2.[30-32] Similarly, stimulation with anti- CD3 crosslinked via a plastic-immobilized bridging antibody generally results in poor IL-2[32,33] and IFN gamma[32,34] production, although exceptions have been reported.[32,35] By contrast, T-cell receptor (TCR)-independent stimulation promotes equivalent IL-2 production and proliferation from cord-blood and adult T cells. These results indicate that neonatal T cells respond weakly to physiologically relevant stimuli that is, activation via cell surface molecules, notably the TCR-associated CD3 molecules, in an APC dependent [soluble monoclonal antibody (mAb)] system. Nonetheless, they are capable of adult-level IL-2 production and proliferation when the TCR is bypassed.[32]

T cell activation

An antigen-specific immune response is initiated by the accumulation of T cells in specialized lymphoid regions, such as lymph nodes or inflamed peripheral tissues, by a phase of physical contact between T lymphocytes and APCs, which move between and within tissues for immune surveillance and defense against bacteria, viruses and damage.[36,37]

In order to be activated, T cells must recognize peptide fragments that are bound to MHC molecules. This alone is not sufficient. The delivery of a costimuatory signal presented by specialized antigen presenting cells is also necessary. Dendritic cells, macrophages and B cells are capable of delivering both the MHC/antigen complex and the costimulatory signal to activate naïve T cells.[1,38]

The ability of naive T cells to clonally expand and acquire effector functions depends on the strength of signals received by the T-cell receptor (TCR) and by an array of co-stimulatory receptors.[39]

If T cells bind to the MHC/antigen complex in the absence of costimulatory signals, they fail to respond efficiently to antigenic stimulation and are rendered anergenic. The interaction between T-cells and the APC during antigen recognition creates the immunological synapse. In this immunological synapse specific ligands and costimulator molecules trigger and sustain T cell activation.[40]

The activation of T cell requires immunogenic and tolerogenic stimuli[41] that can be divided into two main categories. The first signal (Signal 1) is relayed through the ligation of the T-cell receptor (TCR) by peptide/MHC complexes, which ensures that the ensuing immune response is specific and is essential for removing T cells from quiescence (G0 phase) and preparing them for proliferation and differentiation. The second subset of stimuli (Signal 2), generally referred to as co-stimuli, ensure that immune responses are induced when they are needed and not against harmless substances or self antigens. They are conferred by counter-receptors expressed by APCs or by soluble factors such as cytokines and chemokines. Costimuli might or might not coincide spatially and temporally with the peptide/MHC stimulus and contribute to upregulated synthesis of macromolecules and energy metabolism, to promote cell-cycle progression, protect from or enhance cell death and regulate cell differentiation.[39]

The most prominent and best described of the costimulatory receptors is CD28 and its binding partners on the APC side B7.1 (CD80) and B7.2 (CD86). CD28 potently enhances T-cell receptor induced proliferation and differentiation of naive T cells, especially at low TCR occupancy, making it responsible for the signal two predicted by the two-signal hypothesis of lymphocyte activation. CD28 favors T-cell survival by inducing nuclear factor-κB (NF-κB)-dependent expression of the anti-apoptotic protein BCL-X_L and the development of T helper 2 (Th2) cells by promoting the expression of a 'second wave' of co-stimulatory receptors (such as CD40 ligand, OX40 and inducible co-stimulatory molecule (ICOS).[39]

CD63 is a lysosomal-associated membrane protein (LAMP-3). It is not expressed on the surface of resting T cells but is upregulated upon T cell stimulation.[42] Stimulation of the T cell receptor together with CD63 leads to a stong T cell activation.

T cell anergy

Central tolerance

The thymus is an organ that supports the differentiation and selection of T cells.[43-46] The thymic development of T cells consists of several processes that require the dynamic relocation of developing lymphocytes into, within and out of the multiple environments of the thymus. These processes include: first, the entry of lymphoid progenitor cells into the thymus; second, the generation of $CD4^+CD8^+$ double-positive (DP) thymocytes at the outer cortex of the thymus; third, the positive and negative selection of DP thymocytes in the cortex; fourth, the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete thymocyte development and ensure central tolerance; and last, the export of mature T cells from the thymus.[47-51,46]

The immature repertoire of T lymphocytes consists of cells whose receptors may recognize any peptide/MHC molecule complex (self or

foreign), or no peptide/MHC complex at all. The only useful T cells are the ones that are specific for foreign peptides and self MHC molecules. T cells that recognize self antigen are dangerous because such recognition may trigger autoimmunity. The selection process ensures that only the useful T cells complete the maturation.

When double positive thymocytes first express αβ TCRs, they encounter self peptides presented by self MHC molecules. Positive selection is the process in which thymocytes which bind the self peptide/self MHC complex with low avidity are stimulated to survive. Thymocytes which do not recognize self MHC molecules die by apoptosis. This ensures that the T cells that mature are self MHC restricted. Positive selection also ensures that $CD8^+$ T cells are specific for peptides presented by class I MHC molecules and $CD4^+$ T cells for MHC class II associated peptides.[1,2,52,53]

Negative selection is the process in which thymocytes whose TCR bind strongly to the self peptide/ self MHC complex are deleted. This is necessary to ensure that no autoreactive T cells leave the thymus.[53,54]

The result of the positive and the negative selection process is the generation of self MHC restricted and self tolerant T cells.

Peripheral tolerance

Whereas high-avidity recognition of peptide-MHC complexes by developing T cells in the thymus results in deletion and promotes self-tolerance, such recognition by mature T cells in the periphery results in activation and clonal expansion.[55] Since it is possible that auto reactive T cells escape the central tolerance mechanisms and are not completely eliminated, the immune system had to develop mechanisms to protect the organism against this threat. Peripheral tolerance is the mechanism by which mature T cells that recognize self antigens in peripheral tissues become incapable of responding to these antigens.[2,55]

T cell anergy is a tolerance mechanism in which the T cells are functionally inactivated following an antigen encounter, but remain alive for an extended period of time in a hyporesponsive state. Anergy is induced, when T cells bind to an antigen/MHC complex without costimulationor or with inhibitory signals.[56] This can be done by stimulating T cells with only anti CD3 or anti TCR antibodies. Those T cells which were stimulated this way became anergic. The stimulation of T cells with anti CD3 or anti TCR antibodies together with anti CD28 antibodies lead to full T cell activation.[57-59]

The decision between anergy and productive T cell activation is made by a series of intracellular proteins that oppose the induction of the interleukin 2 (IL-2) gene but are inactivated by signals from CD38 or the IL-2R. These factors are called Ikaros, Tob NFAT1 and $p27^{kip1}$. They are present in naive T cells and are required for the induction of anergy. In the absence of Tob or Ikaros, quiescent T cells are able to produce IL-2 and differentiate in the absence of a costimulatory signal.[60]

In response to T cell signaling alone, NFAT1 induces an array of anergy associated genes like Cbl-b, Itch, GRAIL, DGKalpha, Casp3, Egr3 and CREM. Each of those proteins belongs to the category of an "anergy factor". The se proteins are induced under anergic conditions, where they are required to execute the anergic program by targeting critical factors involved in signal transduction or gene transcription.[60] Clonal T cell anergy was found not to be a terminal fate, as the addition of exogenuos IL-2 during restimulation reverses the anergy.[61]

Regulatory T cells (Treg cells) have emerged as important means of enforcing peripheral self tolerance too.[62] Treg cells are characterized by the expression of the transcription factor Foxp3 and play a key role in immune homeostasis. Foxp3 is a specialized anergy factor that also acts as a sensor of mitogenic signals. It responds to antigenic signaling by binding to and silencing proinflammatory cytokine genes.[60] Treg cells

maintain tolerance to self and control autoimmune deviation prevent runaway responses to pathogens or allergens, help maintain a balance with obligate microbial flora, and faciliate tumor escape from immune monitoring. Treg cells were initially identified in the mouse as $CD4^+$ $CD25^+$ Tcells.[63] Studies in the past years have demonstrated that $CD4^+$ $CD25^+$ T cells are the products of a unique lineage of T cells developing in the thymus.[62,64,65] In addition to those naturally occurring $CD4^+$ $CD25^+$ T cell population, it is possible to generate in the periphery a distinct population of regulatory T cells.[62,66] In general, these regulatory T cells are antigen-specific populations that are derived from conventional $CD4^+$ $CD25^-$ naive precursors following exposure to antigen under conditions of limiting costimulation. Regulatory T cells block the activation and functions of effector T cells. The mechanism of action of regulatory T cells is not completely understood. One of the best known means of regulatory T cells is the secretion of immunosuppressive cytokines such as IL-10 or transforming growth factor-β (TGF-β).[62,67,68] TGF-β inhibits B and T cell proliferation and IL-10 supresses macrophage activation and antagonizes IFN gamma.[1,69,70]

Cytokines

Cytokine is a term derived from Greek roots meaning “to set cells in motion”. Cytokines are intercellular signaling peptides (usually between 8 and 30 kDa in mass) that can act at any range (autocrine, paracrine, endocrine).[71] They are secreted by the cells of innate and adaptive immunity that mediate many of the functions of these cells. Cytokines are produced in response to microbes and other antigens, and different cytokines stimulate diverse responses of cells involved in immunity and inflammation. In the activation phase of adaptive immune responses, cytokines stimulate the growth and differentiation of lymphocytes and in the effector phase of

innate and adaptive immunity. They activate different effector cells to eliminate microbes and other antigens.[1]

Cytokine activity in any clinical or biological context is a complex issue because of the variety and multiple activities of cytokines. Furthermore, one cytokine can radically alter (even reverse) the activity of another cytokine on a target cell. As a result, it is sometimes useful to consider cytokines in functional groups. For example IL-2, granulocyte- macrophage colony stimulating factor, and interferon-γ promote cytotoxicity, whereas IL-4 and -13 promote antibodymediated immunity.[71]

Cytokines are usually not stored as preformed molecules, and their synthesis is initiated by new gene transcription as a result of cellular activation. Such transcriptional activation is transient, and the messenger RNAs encoding most cytokines are unstable. The production of some cytokines additionally is controlled by RNA processing and by posttranscriptional mechanisms. Once synthesized, cytokines are rapidly secreted, resulting in a burst of release as needed.[1]

IL-2

Interleukin 2 (IL-2) was the first interleukin that was discovered and originally was called T cell growth factor because it is responsible for clonal T cell expansion after stimulation with antigen. IL-2 is produced by activated CD4 pos and CD8 pos T cells and acts mainly on the cells that produce it.[1,72]

IL-2 is also important for the development of regulatory T cells. The IL-2/IL-2 receptor pathway is clearly important in the development and expansion of $CD4^+CD25^+$ cells in vivo as IL-2 and IL-2 receptor deficient mice all die early in life of severe lymphoproliferation and autoimmune disease.[73-76]

Although IL-2 and its receptor play a critical role in the homeostasis of $CD4^+CD25^+$ T cells in vivo, the contribution of IL-2 to their suppressive

function is poorly understood. Indeed, the molecular basis for suppression of T cell activation by $CD4^+CD25^+$ T cells in vitro is inhibition of IL-2 gene transcription in the $CD4^+CD25^-$ responder T cells. Furthermore, addition of IL-2 or the enhancement of costimulation by the addition of anti CD28 are thought to break the anergic state of the $CD4^+CD25^+$ T cells and abrogate their suppressive function.[77] [76]

IL-4

Interleukin 4 (IL-4) is a member of the four-alpha-helical cytokine family. The principal cellular sources of IL-4 are $CD4^+$ T cells of the TH2 subset, activated mast cells and basophils.

IL-4 stimulates the development of Th2 cells from naïve $CD4^+$ T cells and functions as an autocrine growth factor for differentiated Th2 cells. In addition to that it is the main cytokine that stimulates B cell Ig heavy chain class switching to the IgE isotype and is therefore the major stimulus for the production of IgE antibodies and for the development of Th2 cells.[1]

IL-10

Interleukin 10 (IL-10) is an important regulatory cytokine whose involvement extends into diverse areas of the human immune system.[78] It has a four-alpha-helical structure and binds to type II cytokine receptor. IL-10 is mainly produced by activated macrophages. It is a good example of a negative feedback regulator, because it inhibits macrophage functions.[1]

It inhibits the production of IL-12 by activated macrophages and dendritic cells. Because IL-12 is a stimulus for IFN-gamma secretion and is an inducer of innate and cell mediated immune reactions, IL-10 functions to down-regulate all such reactions.[1]

IL-10 inhibits the expression of costimulators and class II MHC molecules on macrophages and dendritic cells. So IL-10 serves to inhibit T cell activation and terminate cell mediated immune reactions.[1]

IL-13

Interleukin 13 (IL-13) is structurally related to IL-4 and is produced by Th2 CD4 pos T cells and by some epithelial cells. The IL-13 receptor is found on nonlymphoid cells, like macrophages, and can be activated by IL-13 and IL-4.[1]

Even though IL-13 was thought to be functionally redundant with IL-4, studies conducted with knockout mice, neutralizing antibodies, and novel antagonists demonstrate that IL-13 possesses several unique effector functions that distinguish it from IL-4.[79]

It mimics the effects of IL-4 on nonlymphoid cells like macrophages but seems to have less effect on T or B cells than IL-4. The major function of IL-13 on macrophages is to inhibit their activation and to antagonize IFN-gamma. IL-13 stimulates mucus production by lung epithelial cells and it is possible that it plays a role in asthma.[1]

IL-17

More recently, T cells were shown to produce cytokines that could not be classified according to the Th1–Th2 scheme. Interleukin-17 (IL-17) was among these cytokines[80,81] and the T cells that preferentially produce IL-17, but not interferon-γ or interleukin-4, were named Th17 cells.[9,81,82] Like Th1 and Th2 cells, Th17 cells produce a group of distinctive cytokines: Interleukin-17 (also called interleukin-17A), interleukin-17F, interleukin-22, and interleukin-21. All of those cytokines participate in orchestrating a specific kind of inflammatory response.[81]

Accordingly the biological actions of IL-17 are quite proinflammatory in character. It increases the local production of chemokines such as IL-8[83-85], monocyte chemoattractant protein-1 (MCP-1)[85-87], and Gro-alpha[85,88], thereby promoting the recruitment of monocytes and neutrophils[85,89-91]. Further, it stimulates the production of the hematopoietic cytokines G-CSF

and granulocyte macrophage (GM)-CSF that promote the expansion of these myeloid lineages.[85,92-94] Other actions such as the stimulation of IL-6 and PGE2 production enhance the local inflammatory environment.[85,95-98]
In addition, IL-17 also drives T-cell responses, notably through the induction of the costimulatory molecule intercellular adhesion molecule (ICAM).[85,99-101]

Interferon gamma

Interferon-gamma (IFN gamma), also called type II interferon, is the principal macrophage activating cytokine and therefore is crucial for immunity against intracellular pathogens and for tumor control.[1,102]
It is produced in response to viral or intracellular bacterial infection by NK cells, $CD4^+$ Th1 cells, and $CD8^+$ T cells. Its function is to activate macrophages, to increase expression of major histocompatibility complex molecules and to exert direct antiviral activity on infected cells.[1,103]
IFN gamma promotes the differentiation of naïve $CD4^+$ T cells to the Th1 subset, which then produce IFN gamma in a positive feedback loop. IFN gamma inhibits the proliferation of Th2 cells and acts on B cells to promote isotype switching to certain IgG subclasses and inhibits the isotype switching to IL-4 dependend isotypes (IgE).[1]

CD45

CD45 (PTPRC, leukocyte common antigen, B220, T200) was one of the first signaling molecules identified on leukocytes and is used as a leukocyte marker molecule.[104-114] It is the prototypic member of transmembrane receptors like protein tyrosine phosphatases (PRTPs) and various forms of it are expressed on all nucleated hematopoetic cells.[111-114]

CD45 plays an essential role in immune functions. Humans with certain mutations in CD45 develop a severe-combined immunodeficiency phenotype, systemic lupus, multiple sclerosis and other diseases.[111,112,115-118]

It is generally accepted today that CD45 sets threshold of positive and negative signaling events in leukocytes.[111-114] It dephosphorylates and activates the src-family kinases Lck, Fyn and Lyn. That way CD45 serves as a positive regulator for signaling via the T-cell and B-cell receptor.[119-121] On the other hand, through dephosphorylation of JAK, CD45 negatively regulates cytokine receptor signaling and promotes viral infection. In addition CD45 has been shown to be involved in regulating development, adhesion and apoptosis in leukocytes.[122]

CD45 is a large cell surface glycoprotein of 180-220 kDa. It covers up to 10% of the leukocyte surface area.[123] The CD45 family consists of multiple members that are all products of a single complex gene. This gene contains 34 exons and the primary RNA transcript of three of the exons (4,5 and 6) are alternatively spliced to generate up to 8 different mRNAs and 8 different protein products.[112] The protein products contain external domains of variable lengths which are heavily glycosylated. All isoforms of CD45, however, share the 707 amino acid long (95 kDa) cytoplasmatic domain, which is one of the largest identified among membrane proteins.[124-128]

The cytoplasmatic part of CD45 (ct-CD45) is composed of a juxtamembrane spacer segment, two tandemly duplicated protein tyrosine phosphatase domains (PTP), the active domain 1 (D1) and the catalytically impaired domain 2 (D2), which are divided by a short interdomain linker, and a COOH-terminal tail.[129-133]

CD45 D1 (37 kDa) is 273 amino acids long (608-890) and the catalytic cysteine residues are at the amino acid 828. D1 is responsible for the dephosphorylation of the different substrates, for example the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3zeta.[129-133]

CD45 D2 (42 kDa) is 310 amino acids long (895-1205) and the catalytic cysteine residues are at the amino acid 1144. The strong structural conservation of D2 implies that it serves a cellular function, but it is still of major mystery what that function may be. Inspection of the D2 structure suggests, that it plays a role in substrate recruitment and it may contribute to stabilize D1.[129-133]

PTPs are now known to undergo posttranslational proteolytic processing.[134-138] Cleavage of the intercellular parts of type 1 cell surface receptors by presenilin/gamma-secretase is typically executed after the receptors have undergone ectodomain-shedding.[134,135,137,138] This proteolysis frequently generates fragments of cell surface receptors with new biologic functions.

Our group has shown an alternative function for the intracellular domain of CD45. We discovered that CD45 is sequentially cleaved by serine/metalloproteases and gamma-secretases during activation of human phagocytes by fungal stimuli or phorbol 12-myristate 13-acetate (PMA). Stimulation with other microbial stimuli did not lead to this cleavage of CD45. Proteolytic processing of CD45 occurred upon activation of monocytes or granulocytes but not of T-cells, B-cells, or dendritic cells and resulted in a 95 kDa fragment of the cytoplasmatic tail of CD45 (ct-CD45).[139]

The finding that those ct-CD45 molecules are created even though the ribosomal translation is blocked, the apearence of ct-CD45 within one hour and that ct-CD45 could not be found after induction of celldeath, suggests that this protein is not created from newly synthesized different splice variants of CD45.[139]

Since ct-CD45 could only be generated in monocytes and granulocytes, which are capable of of producing a strong respiratory burst, but not in T cells, B cells or dendritic cells, and the observation that the generation of ct-CD45 could be blocked by inhibiting the NADPH-oxidase, it was specu-

lated that the formation of an oxidative burst is an important factor in the generation of ct-CD45.[139]

Binding studies with this protein revealed a counter receptor on preactivated T-cells. Moreover, T-cell proliferation induced by dendritic cells or anti-CD3 antibodies was inhibited in the presence of ct-CD45.[139]

Aim of the study

As mentioned before, our group observed that the cytoplasmatic tail of CD45 molecules (ct-CD45) is cleaved and released upon activation of monocytes and neutrophil granulocytes and that it acts as a cytokine-like factor, which inhibits T cell proliferation (Figure1).[139]

The exact nature of this ct-CD45 induced inhibition effect is unknown. Since the specific binding of this protein to preactivated T cells and the fact that this binding is saturable and can be blocked by mAb it is very likely that there is a specific receptor for ct-CD45[139]. This receptor has jet to be found though.

Therefore, the first aim of this study was to further investigate the function of ct-CD45 and its effect on T cell proliferation and polarisation and if the inhibition of T cell proliferation is due to anergy. The second aim was to find a possible receptor candidate for ct-CD45.

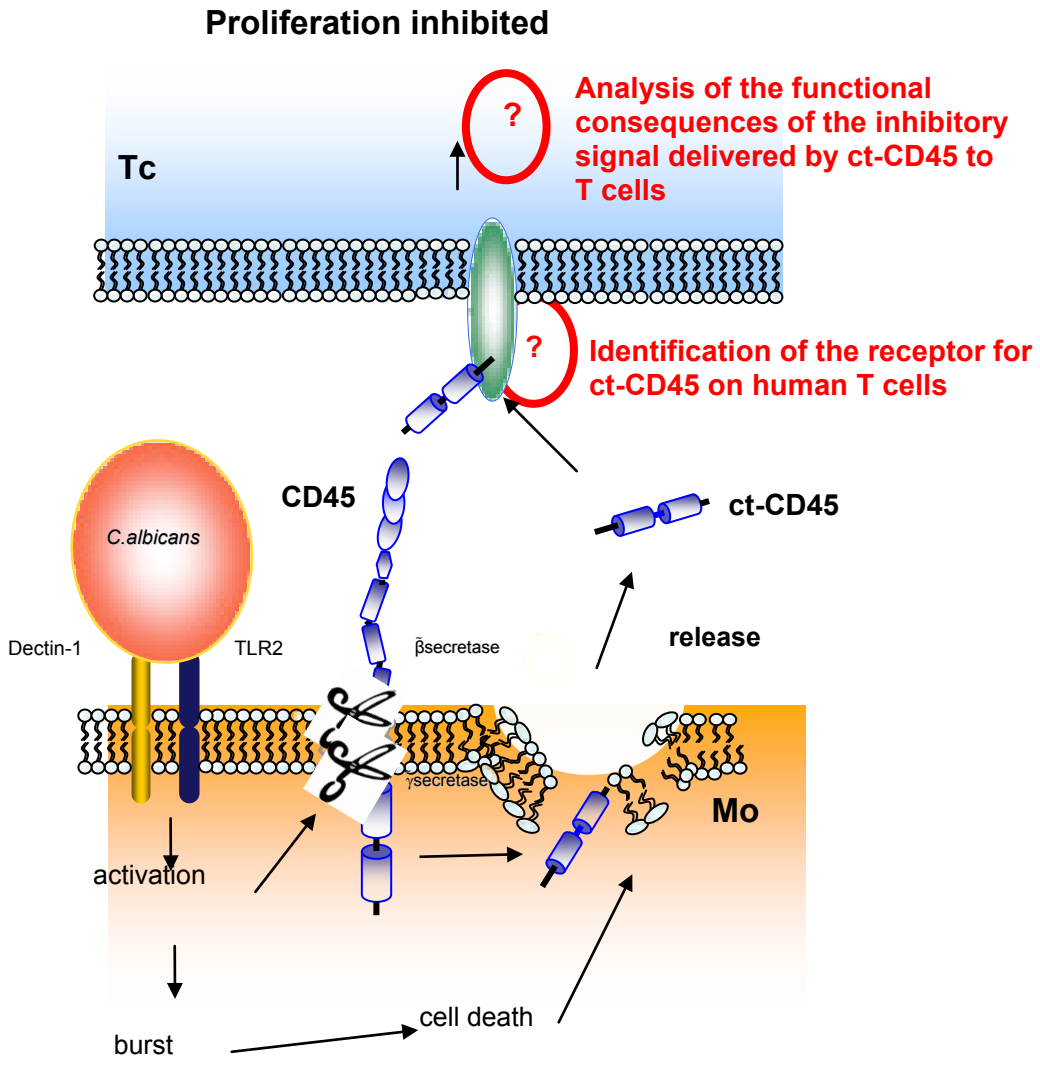


Figure 1: The binding of fungal stimuli like C. albicans to monocytes leads to activation and to the formation of an oxidative burst. The activation of monocytes also seems to lead to the cleavage of CD45 by gamma-secretases. The oxidative burst then leads to celldeath. Upon celldeath ct-CD45 is released from the cell and then binds to a yet un-known receptor on T cells. This binding of ct-CD45 triggers a signalling cascade which inhibits T cell proliferation.

Abbreviations

APC	antigen presenting cell
BSA	bovine serum albumin
CD	cluster of differentiation
cpm	counts per minute
ct-CD45	cytoplasmatic part of CD45
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
ER	endoplasmatic reticulum
FITC	fluorescin-5-isothiocyanate
ICOS-L	ICOS ligand
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MNC	mononuclear cells
NK	natural killer cells
PBMNC	peripheral blood mononuclear cells
PE	phycoerythin
PBS	phosphate buffered saline
PRAT4A	protein associated with toll like receptor 4
TCR	T cell receptor
TGF	transforming growth factor
Th	T herlper cell

TLR	toll like receptor
TNF	tumor necrosis factor
Treg	rerulatory T cell

Materials and Methods

Antibodies

specifity	clone	isotype	source
T cell activation			
CD3	OKT3	IgG2a	Jansen-Cilag, Vienna
CD28	15E8	IgG1	Caltag Laboratories, Burlingame, CA
CD63	CD63-11C9	IgG3	Otto Majdic, Institute of Immunology, Vienna
staining			
VIAP	VIAP-2D5	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD25-PE	CD25-3G10	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD69-FITC	FN50	IgG1	BD PharMingen, San Diego, CA
MHCII	L243	IgG2a	ATCC
IL-4-PE	MP4-25D2	IgG1	BD PharMingen, San Diego, CA
CD97	VIM3b	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD27	VIT14a	IgG2a	Otto Majdic, Institute of Immunology, Vienna
CD54	CD54-6F2	IgG2a	Otto Majdic, Institute of Immunology, Vienna
PRAT4A	polyclonal		Santa Cruz Biotechnology

Tabel 1: Antibodies

Fusionproteins

fusionproteins	source
ctCD45Fc	Stefan Hopf, Institute of Immunology, Vienna
CTLA4Fc	Bristol-Meyers Squibb, NY
ICOS-L-Fc	Peter Steinberger, Institute of Immunology, Vienna
CD80-Fc	Peter Steinberger, Institute of Immunology, Vienna

Tabel 2: Fusionproteins

Cell Culture conditions

For cell culture RPMI (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 streptamycin (NBK, Novartis Research Institute, Vienna) was used. Cells

were cultured at 37°C with 5% CO^2.

Freezing of cells

- Freezing buffer: RPMI 1640 +20% FCS + 100U/ml penicillin + 100 µg/ml streptamycin and 2mM L-glutamine + 10% DMSO
- Nunc ^{TM}Cryo Tube Vials (Nunc, Roskilde, Denmark)
- NALGENE^{TM} Cryo 1°C Freezing container

1. Freshly isolated cells or cell lines were adjusted to a cell number of $1x10^7$/ml freezing buffer.
2. 1ml of cell-suspension were filled into a Cryo-tube. The tubes were stored in a 2-Propanol filled cryo container at –70°C for 24hours.
3. After 1 day the tubes were transferred to the N_2-tank.

Thawing of cells

1. The tubes were taken from the N_2-tank and thawed with lukewarm tap-water.
2. To avoid overheating we left a rest frozen and put the tube on ice for 10 minutes.
3. After 10 minutes the cells were transferred to a 15 ml tube and droplets of supplemented cell culture medium were added in an interval of 1min: Starting with 3 drops at 0 minutes, 6 drops at 1 minute, 12 drops at 2 minutes etc. up to 48 drops.
4. The cells were washed twice: The tube was filled up and centrifuged for 5 minutes at 900g, the supernatant was discarded and the cells were resuspend and so on.
5. We determined the cell number.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood of normal healthy donors by density gradient centrifugation using Ficoll-Paque. After centrifugation granulocytes and erythrocytes gathered at the bottom of the tube, mononuclear cells in the interphase. For the Isolation of monocytes and T-cells Magnetic Cell Sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) was applied.

Production of ct-CD45 fusion protein (ct-CD45)

To produce these proteins, bacteria (*E. coli*) were transformed with the vector containing our fusion proteins as an insert. Then the plasmids were isolated with a maxiprep. kit and were used to transfect Phoenix cells.
After transfection of Phoenix cells with the fusion protein coding genes, the supernatant was harvested after 3 and 6 days.
To increase the protein concentration of the fusion protein we used a protein isolation procedure to increase the concentration. The protein increase of the concentration of our fusion proteins was determined after the isolation with an ELISA (data not shown).

Transformation of E. coli *cells*

- Luria broth agar: 32 mg Lennox L Agar + 5 g NaCl per litre of distilled water; boil to dissolve and autoclave before use.
- Luria broth media: 25 mg Luria Broth Base per litre of distilled water; boil to dissolve and autoclave before use.
- Luria broth Amp agar: LB agar containing ampicillin in a final concentration of 100 µg/ml.
- The plasmids containing the fusion protein insert were provided by Mag. Schrauf.

For transformation 100 µl competent E.coli (stored at -80°C) were thawed on ice, mixed with 10 ng plasmid DNA or 3 µl of a ligation mix and then incubated on ice for 10 min.
The bacteria were heat shocked at 42°C in a water bath for 2 min, chilled on ice, diluted in 300 µl LB, incubated at 37°C for about 1 h under constant shaking and plated on LB-Amp dishes. Bacteria were allowed to grow over night at 37°C.

Transfection of Phoenix cells

$CaCl_2$ Transfection

HBS-Buffer (pH 7,05):

- 140 mM NaCl
- 1,5 mM Na_2HPO_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_2O and $CaCl_2$ (see table below). The transfection mix was sterile filtered using a 0,22 µm filter and then 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 after 3 and 6 days. For generation of immunoglobulin fusion proteins 6 to 10 ∅10 cm plates were transfected with the respective plasmid construct.

	Cell number/ml	DNA	ddH_2O	$CaCl_2$ 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 **) for spin-infection add 1,5 µg MLV + 1,5 µg Plasmid-DNA					

Table 3: Transfection-mix.

Plasmid purification

For large scale up preparation of plasmids, bacteria were grown under shaking over night at 37°C in 250 up to 300 ml LB media supplemented with ampicillin (70 µg/ml). Plasmid maxipreparation was performed with the Qiagen Plasmid Maxi Kit, according to the manufacturers protocol. Bacterial cells were harvested by centrifugation at 5 000 x g (4 000 rpm) for 15 minutes at 4°C. The pellet was resuspendend in 10 ml buffer P1 (Qiagen) for cell lysis.

Then 10 ml of buffer P2 (Qiagen) were added, the solution was gently mixed and incubated at room temperature for 5 minutes. In the next step 10ml of buffer P3 (Qiagen) were added to stop cell lyses.

Lysates were incubated on ice for 15 minutes and then the cell debris was removed by centrifugation at 5 000 x g (4 000 rpm) for 30 minutes at 4°C. The supernatant was 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 buffer QC (Qiagen), DNA was eluted with 15 ml buffer QF (by Qiagen) and precipitated by adding 10,5 ml isopropanol. The precipitated DNA was collected by centrifugation for 30 minutes at 4°C at 12 000 x g (10 000 rpm). DNA pellet was washed twice 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 260 nm and diluted to a final concentration of 1 µg/µl. Plasmid preparations were stored at -20°C.

Protein Purification

Buffers and solutions:
All buffers were sterile filtered (0,45 µm filter) before usage
Binding buffer (wash buffer): 20 mM sodium phosphate, 3M NaCl (pH: 7) on ice
Elution buffer 0,1 M sodium citrate pH 3
1M Tris-HCl pH 9
PBS + 20% EtOH
Dialyse buffer: 50mM HEPES, pH 7,0, 0,15M NaCl, 4mM DTT, 0,0035% Tween 20

For protein purification of fusion proteins from cell-culture supernatants the HiTrap rProtein A FF column 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 CD45 Ig fusion proteins were centrifuged at 2200 rpm for 5 minutes to remove cells. Before applying to the column the samples were filtered using a 0,45 µm filter.
Using a constant flow rate of 1 ml/minute the column was equilibrated with 10 ml binding buffer, 10 ml elution buffer and 10 ml binding buffer again.

Then the sample was loaded to the column. Before elution, the column was washed with 10 ml binding buffer, then 5 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 10 ml binding buffer. For further storage (4°C) the column was washed with 20% ethanol in PBS.
After purification the protein concentation was measured at OD280. Then all fractions which contain protein were pooled and dialysed over night against the dialyse buffer under stirring. The fusion proteins were aliquoted and stored at -80°C.

Magnetic cell sorting (MACS)

Cells can be purified from a heterogeneous cell suspension by magnetically labelled antibodies. The mAbs we used were indirectly labelled with biotin. These biotinylated antibodies recognizing cell-type specific surface molecules were mixed with the cells to be separated. In a second incubation step paramagnetic beads (50 nm in diameter) were coupled via a biotin-streptavidin interaction to the monoclonal antibodies which had been specifically bound to a distinguished cell population. 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.
For the separation of Monocytes we applied positive selection utilizing an antibody against the monocyte-marker CD14. The PBMC were incubated with a biotinylated anti-CD14 Antibody. Monocytes retained in the column and were eluted after removal from the magnet.

T-cells were purified by negative selection: Populations expressing CD11b, CD14, CD16, CD19, CD33 and MHCII were labelled and retained in the column while T-cells passed through.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

- Heparin-Medium: 500 ml RPMI 1640 (+10% FCS + 100 U/ml penicillin + 100 µg/ml streptamycin and 2 mM L-glutamine) + 10 U/ml Heparin (stock: 5000 U/ml, Baxter, Vienna)
- MACS-buffer (stored at 0°C): 1000 ml 1x PBS def. + 25 ml HSA (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0,5 M); filtrated sterile
- Ficoll-Paque (Pharmacia, Uppsala,Sweden)

Blood from normal healthy donors 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 900 g without brake.

The interphase contained all the PBMCs, whereas the erythrocytes and granulocytes gathered at the bottom of the tube: The white interphase ring was transferred into a new tube and spun down (5 minutes at 900g). The supernatant was discarded.

The cells were washed twice with MACS-buffer:

The pellet was resuspend in a few ml of buffer, filled up, spun down. The supernatant was discarded, and the cells again resuspend. The number of PBMCs was determined and the cells were used for monocyte and T-cell selection.

Purification of T-cells

For the isolation of monocytes up to 1 x 10^9 PBMNCs were incubated with 250 µl of biotinylated CD14 (VIM13) and positively enriched (positive selection), whereas T-cells

were isolated by collecting the flowthrough of PBMNCs depleted 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, negative selection).

Freshly isolated PBMNCs were resuspended in 750 µl MACS buffer and incubated with 250 µl 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 anti-Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 minutes at 4°C. In the mean time a CS column (Miltenyi biotec) was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. Afterwards the labelled PBMNCs were loaded onto the column and for monocyte enrichment washed with 40 ml MACS buffer. The flow through was collected as monocyte negative fraction. The column was further washed four 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 flowthrough was collected, washed with MACS buffer and counted. 20 µl of this solution were pipetted into a tube for cell counting with the coulter. Before measuring the cell number two drops of Zap-Oglobin® (Beckman Coulter, Miami, FL) are added to remove residual erythrocytes.

T-cell proliferation assays

For proliferation assays with Ig-fusion proteins, plates were coated with 3 µg/ml of both anti-mouse IgG (3 µg/ml; Invitogen) and anti-human IgG, Fc-specific (3 µg/ml; Jackson ImmunoResearch Laboratories, West Grove, Pa) overnight at 4°C, washed, and then incubated with 4 µg/ml of the respective fusion protein (ctCD45Fc and CTLA4Fc) plus anti-CD3 (1 µg/ml; OKT3; Ortho Pharmaceutical Corporation) or the combination of anti CD3/CD28 or anti CD3/CD63 mAb. After another washing step, T-cells (1 x 10^5 /well) were added. Proliferation was monitored by measuring tritiated thymidine ((methyl-^{3}H)TdR; Valeant Pharmaceuticals, Irvine, CA) incorporation on day 3 of culture. Cells were harvested 18 hours later and radioactivity was determined on a microplate scintillation counter (PerkinElmer Life and Analytical Science, Waltham, MA). Assays were performed in triplicate.

Flow cytometry

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 488nm, 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

Binding of mAbs to Fcg-receptors was blocked by incubation of cells with a mouse anti VIAP, a non-binding calf intestine alkaline phosphatase-specific antibody. As negative control Beriglobin, a human immunoglobin, was applied. For secondary labelling we used R-Phycoerythrin-conjugated goat anti-human antibody.

- PBS/BSA: 1x PBS def + 1% BSA
- Fusion protein: 20µg/ml
- Secondary antibody: R-Phycoerythrin-conjugated AffinityPure F(ab`)$_2$ Fragment Goat anti-Human IgG, Fc Fragment Specific antibody (Jackson ImmunoResearch) 20µg/ml in PBS/BSA (Molecular Probes; Eugene, Oregon)

1. The cell suspension (2x10^5 /assay) was spun down. 5´ at 300g
2. The pellet was resuspended with 50µl mouse anti VIAP antibody/assay and kept 25´on ice.
3. 20µl of Antibody were prepared in Micronic-tubes and 50µl of the cell suspension was added, mixed and incubated 30´ at 4°C.
4. Each assay was washed twice with PBS/BSA: resuspended in PBS/BSA, spun down (5´at 300g), the supernatant was discarded...

5. 20µl R-Phycoerythrin-conjugated secondary antibody was added to the cells, and again incubated for 30´at 4°C.
6. The tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA)

Quantification of cytokines via LUMINEX100

The Flourokine MAP system, using LUMINEX xMAP technology (R&D Systems Inc., Minneapolis, MN), provides a tool to simultaneously measure multiple cytokines in a single sample. Analyte-specific antibodies are pre-coated onto color-coded beads. Standards and samples are pipetted into the wells and analytes of interest are bound by the immobilized antibodies. After washing away any unbound substances, labelled antibodies specifid to the analytes of interest are added. Following a wash to remove any unbound labelled antibodies, the beads are resuspended in buffer and read using the LUMINEX100 analyzer. Advanced optics capture the color signals and digital signal processing sorts and translates the signal into real-time, quantitave data for each reaction. Supernatants remaining from various experiments were measured via the Luminex100 System (R&D Systems Inc., Minneapolis, MN) for their content of IL-2, IL-10, IL-13, IL-17 and IFN-gamma.

Polymerase Chain Reaction (PCR)

The principle of the PCR technique is the amplification of a specific DNA segment in vitro. In the first step a double stranded template DNA is heat denaturated and separated. Two primers specific for the flanking region of the desired DNA fragment were selected. The specific primer annealing temperature depends on the GC content of the primer sequences, and is usually around 50°C. Finally, by elongating the primers in 5’ → 3’ direction, the heat-resistant Taq-Polymerase copies the DNA template. Repetitive

cycles of template denaturation, primer annealing and primer extension lead to an exponential amplification of the desired DNA fragment.

Agarose Gelelectrophoresis

Buffers

- running buffer 1 x TAE:
  - 40 mM Tris Acetate
  - 20 mM EDTA
  - pH 8,5
- sample buffer:
  - 20 % Glycerol in 1 x TAE
  - Orange G

DNA molecules migrate in an electric field towards the positive pole due to the negative charge of their backbone. Depending on the length of the analyzed DNA-Fragments 0,8 % - 1,5 % agarose gels were used. For gel preparation agarose was dissolved in 1xTAE by boiling. After cooling 500 µg/l ethidiumbromid were added. GeneRuler™ 1 Kb plus DNA ladder was used as size marker.

Gelelectrophoresis was performed at a constant voltage (according to the gel size) of 80 V to 120 V using 1 x TAE as running buffer. DNA molecules can be detected by irradiation with UV-light as intercalated ethidiumbromid renders the DNA fluorescent.

Restimulation assay

For restimulation assays with Ig-fusion proteins, 24 well plates were coated with 3 µg/ml of both anti-mouse IgG (3 µg/ml; Invitogen) and anti-human IgG, Fc-specific (3 µg/ml; Jackson ImmunoResearch Laboratories, West Grove, Pa) overnight at 4°C, washed, and then incubated with 4 µg/ml of the respective fusion protein (ctCD45Fc and CTLA4Fc) plus anti-

CD3 (1 µg/ml; OKT3; Ortho Pharmaceutical Corporation) or the combination of anti CD3/CD28 or anti CD3/CD63 mAb. The cells were cultured for 5 days, washed twice counted and rested for 5 days in new media and 24 well plates without stimulation or fusion proteins.
After this resting periode the cells were counted again and restimulated in new CD3, CD3CD28 or CD3/CD63 mAb coated plates. Thymidine incorporation was measured 4 days after stimulation.

FACS cell sorting

All reagents have to be sterile before use!
To isolate ct-CD45 receptors the human T cell cDNA library was screened using the generated Ig fusion proteins as baits.
For this purpose approximately 30 x 10^6 cells of the human T cell cDNA library were stained using ctCD45Fc fusion protein (3 µg/ml) and goat anti human PE conjugated secondary antibody (20 µg/ml). Cells were washed between the antibody incubation steps with sterile FACS buffer. All incubation steps were made at 4°C. To exclude isolation of Fcγ receptor expressing cells, counterstaining with a mAb to CD64 (20 µg/ml) was done. Cells were resuspended in 100 up o 300 µl of sterile FACS buffer. Sorting was done using a FACS Aria (Becton Dickinson). Cells were sorted in eppendorf tubes supplemented with 300 µl cRPMI 1640. Sorted cells were spined down, resuspended in 100 µl c RPMI 1640 and finally replated in 96 well plates.

siRNA knockdown

For each transfection, a cell suspension containing 5x10^5 BW cells in 500 µl of growth medium with serum but without antibiotics was added to a well of a 24 well plate. 2 solutions were prepaired:
Solution A: 2 µl siRNA (20nM/ml) were diluted in 50 µl OptiMEM I Medium.

Solution B: 1µl Lipofectamine™ 2000 were diluted in 50 µl OptiMEM I Medium.

Both solutions were incubated for 5 min. Then they were combined and incubated for additional 20 min. After that the combined solutions were added to each well and mixed gently. Then those cells were incubated at 37°C in a CO_2 incubator. 48 and 72 hours later those cells were stained with ctCD45Fc as a primary reagent and PE conjugated goat anti human Fc-specific antibodies (Jackson ImmunoResearch Laboratories, West Groove, PA) as a second reagent.

Results

Analysis of the functional consequences of the inhibitory signal delivered by ct-CD45 to T cells

ct-CD45 inhibits the proliferation of T cells under certain conditions

In order to analyse the influence of ct-CD45 on T cell proliferation, we conducted proliferation assays in an antigen presenting cell free system and used ct-CD45-Fc fusion proteins, which consist of the cytoplasmatic part of CD45 an the Fc part of a human antibody. For these assays bulk T cells, bulk T cells without regulatory T cells ($CD25^-$ T cells), cord blood T cells and isolated $CD4^+$ and $CD8^+$ T cell were used. The bulk T cells, the $CD25^-$ T cells and the $CD4^+$ and $CD8^+$ T cells were isolated from human peripheral blood. The cord blood T cells were purified from cord blood. These different T cell populations then were stimulated with an anti CD3 monoclonal antibody (OKT3) and anti CD3 monoclonal antibody (mAb) in combinations with anti CD28 or anti CD63 monoclonal antibodies. OKT3 binds to the CD3 molecule, which initiates T cell receptor signalling. The binding of this anti CD3 mAb to CD3 activates the T cell receptor and provides Signal 1 which is essential for T cell proliferation. The binding of the anti CD28 and the anti CD63 antibody deliver the costimulatory signal which is also necessary for proper T cell activation.

The experiments with bulk T cells showed the exact same results as the experiments with $CD25^-$ T cells. CD3 and CD3/CD63 stimulated $CD25^-$ T cells showed a strong inhibition of T cell proliferation when ct-CD45 fusion proteins (ctCD45Fc) but not CTLA4 fusion proteins (CTLA4Fc) were added (Figure 2). CTLA4 fusion proteins, which consist of the human CTLA4 protein and the Fc part of human antibodies, were used as a con-

trol to ensure that the inhibitory effect is not due to the Fc-part of those fusion proteins. The $CD25^-$ T cells that were CD3/CD28 stimulated showed no inhibition of T cell proliferation after ctCD45Fc was added.

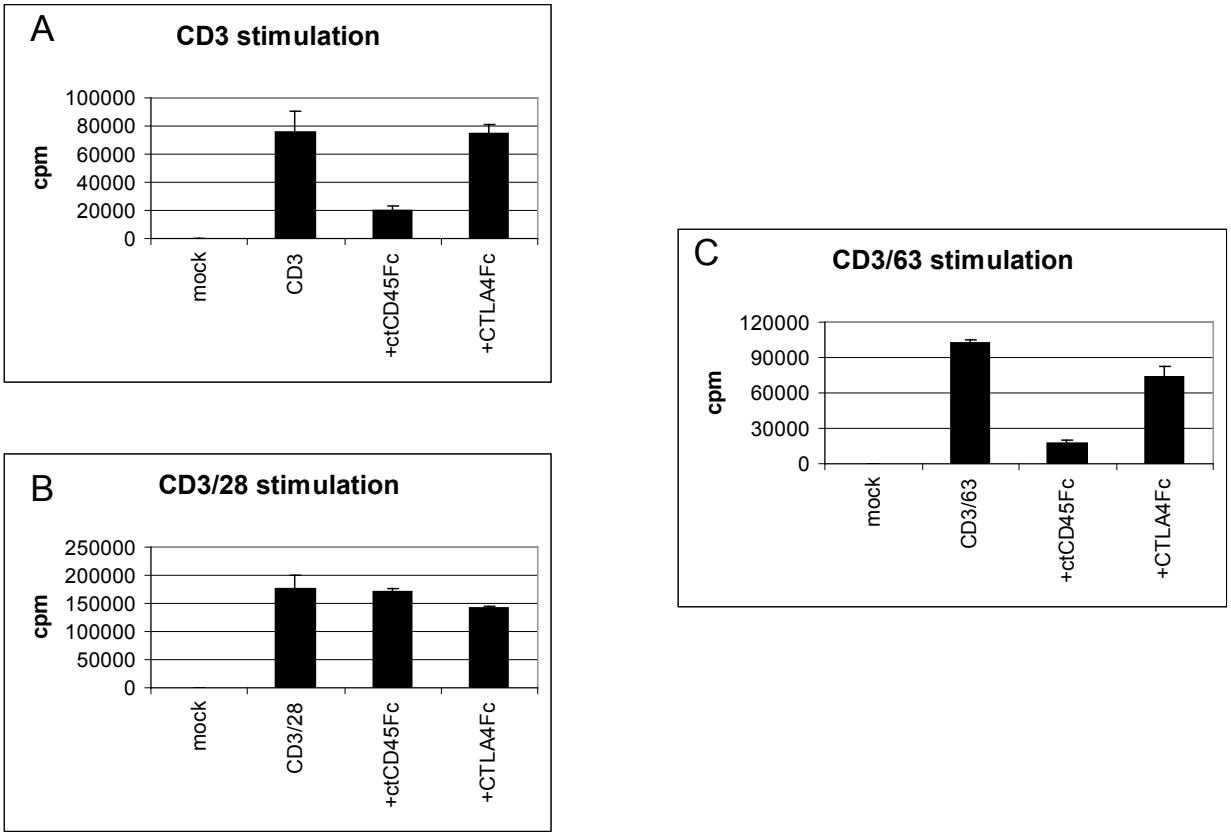
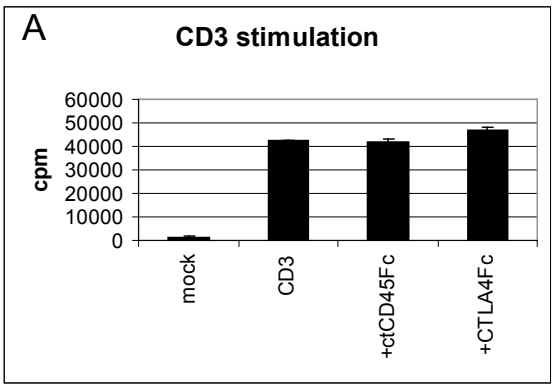
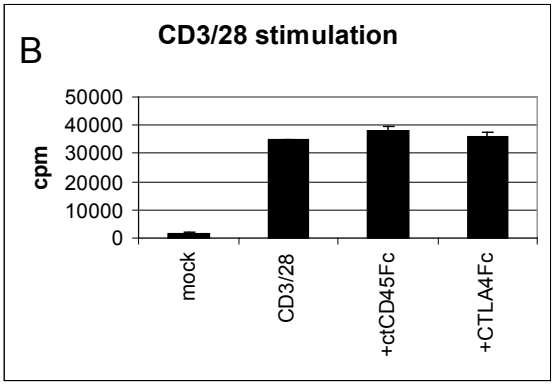


Figure 2: ct-CD45 inhibits the proliferation of $CD25^-$ T cells, if they are CD3 or CD3/CD63 activated but not if they are CD3CD28 activated

$CD25^-$ T cells were stimulated with platebound anti CD3 monoclonal antibody (OKT3) and anti CD3 mAb in combinations with anti CD28 or anti CD63 mAb or no stimulation at all (mock) for 90 hours and cultured with or without platebound ct-CD45 or CTLA4 fusion protein. The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. A) $CD25^-$ T cells with or without CD3 activation; B) $CD25^-$ T cells with or without a combination of anti CD3 and anti CD28 mAb; B) $CD25^-$ T cells with or without a combination of anti CD3 and anti CD63 mAb; The results of one representative of seven independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

Figure 2 shows representative examples of $CD25^-$ T cell proliferation after stimulation with plate bound antibodies. Figure 2 A shows CD3 stimulated $CD25^-$ T cells. A strong inhibition of proliferation in these T cells can be seen when the ct-CD45 fusion protein is added. Figure 2 B shows CD3/CD28 stimulated $CD25^-$ T cells. In this setup ct-CD45 shows no inhibitory effect on those cells. CD3/CD63 activated $CD25^-$ T cells showed an inhibition of T cell proliferation when ctCD45Fc was added (Figure 2 C). The control with the CTLA4 fusion protein shows no inhibition of T cell proliferation. The CTLA4 fusion protein showed no effect on T cell proliferation in any of these three experiments. The unstimulated T cell showed no sign of proliferation in all experiments.





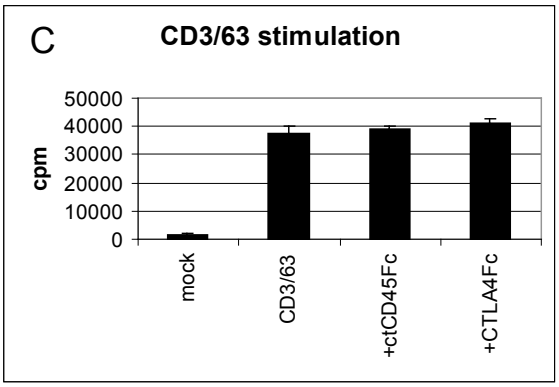


Figure 3: ct-CD45 does not inhibit the proliferation of CD3, CD3/CD63 and CD3/CD28 stimulated cord blood T cells

Cord blood T cells were stimulated with platebound anti CD3 monoclonal antibody (OKT3) and anti CD3 mAb in combinations with anti CD28 or anti CD63 mAb or no stimulation at all (mock) for 90 hours and cultured with or without platebound ct-CD45 or CTLA4 fusion protein. The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. A) cord blood T cells with or without anti CD3 activation; B) cord blood T cells with or without a combination of anti CD3 and anti CD28 mAb; B) cord blood T cells with or without a combination of anti CD3 and anti CD63 mAb; The results of one representative of two independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

Cord blood T cells were used to determine if ct-CD45 has a different effect on naive T cells. For this purpose the same three proliferation assays were conducted with those T cells (Figure 3). The CTLA fusion protein was used as a control again.

Representative examples of cord blood T cell proliferation under three different activation conditions are shown in Figure 3. Figure 3 A shows CD3 stimulated cord blood T cells. The CD45 fusion protein had no inhibitory effect on the proliferation of those T cells. The proliferation of the cord blood T cell, which were CD3/CD28 (Figure 3 B) and CD3/CD63 (Figure 3 C) activated could not be inhibited with the ct-CD45 fusion protein as well. Neither the CD45 fusion protein nor the CTLA4 fusion protein showed any effect on cell proliferation under all three different activation conditions. The cord blood T cells, which were cultivated without activation (mock) showed a very low proliferation.

In order to find out if ct-CD45 has a different effect on $CD4^+$ and $CD8^+$ T cells, $CD4^+$ (Figure 4) and $CD8^+$ T cell (Figure 5) were activated sepa-

rately with anti CD3 mAb and anti CD3 mAb in combinations with anti CD28 or anti CD63 monoclonal antibodies.

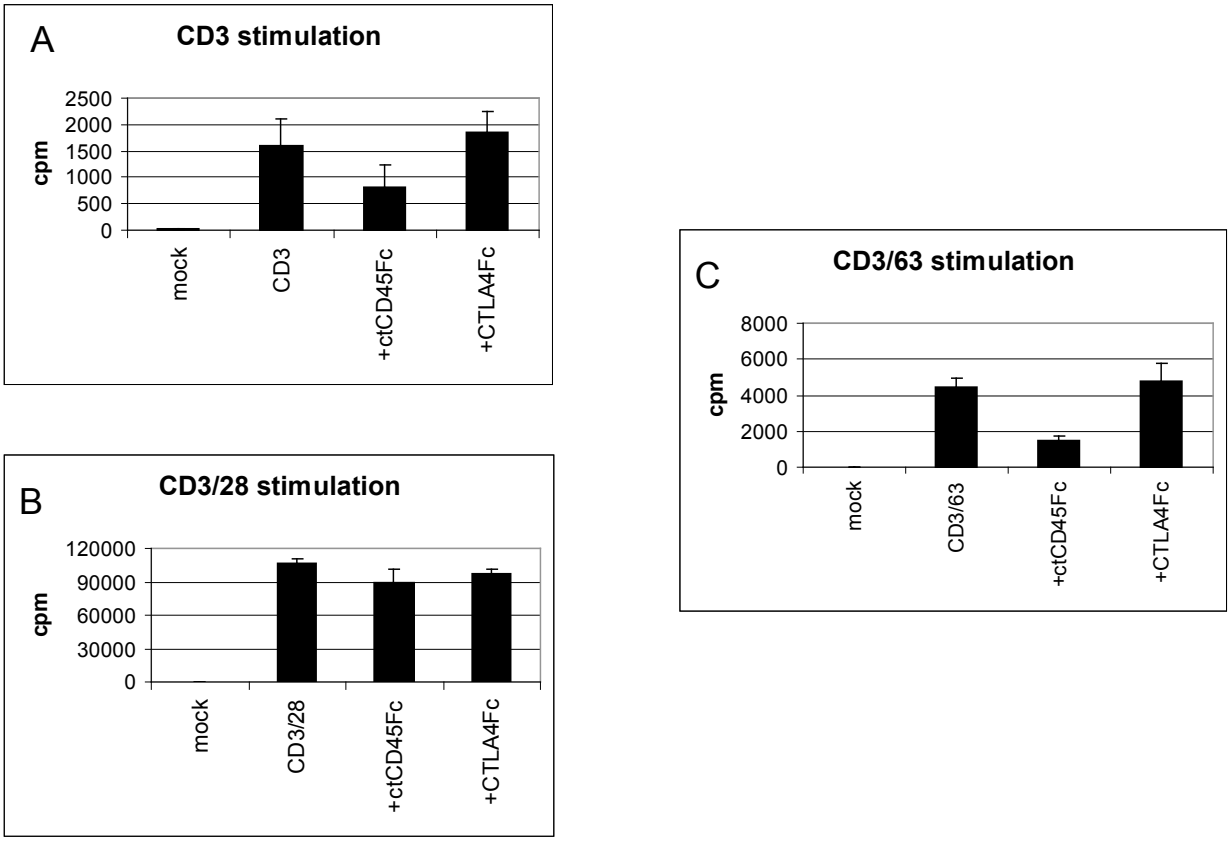


Figure 4: ct-CD45 inhibits the proliferation of CD3 and CD3/CD63 but not CD3/CD28 activated $CD4^+$ T cells

$CD4^+$ T cells were stimulated with platebound anti CD3 monoclonal antibody (OKT3) and anti CD3 mAb in combinations with anti CD28 or anti CD63 mAb or no stimulation at all (mock) for 90 hours and cultured with or without platebound ct-CD45 or CTLA4 fusion protein. The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. A) $CD4^+$ T cells with or without anti CD3 activation; B) $CD4^+$ T cells with or without a combination of anti CD3 and anti CD28 mAb; B) $CD4^+$ T cells with or without a combination of anti CD3 and anti CD63 mAb; The results of one representative of two independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

Figure 4 A shows CD3 activated $CD4^+$ T cells. The ct-CD45 fusion protein inhibits the proliferation of those T cells. This effect can not be seen when the CTLA4 fusion protein is added. When the $CD4^+$ T cells are CD3/CD28 activated (Figure 4 B) proliferation can not be inhibited by ctCD45Fc. The proliferation of CD3/CD63 activated $CD4^+$ T cells could be inhibited by ctCD45Fc. The CTLA4 fusion protein had little to no effect on the proliferation of $CD4^+$ T cells under these three conditions. Those cells that were cultivated without activation showed no proliferation.

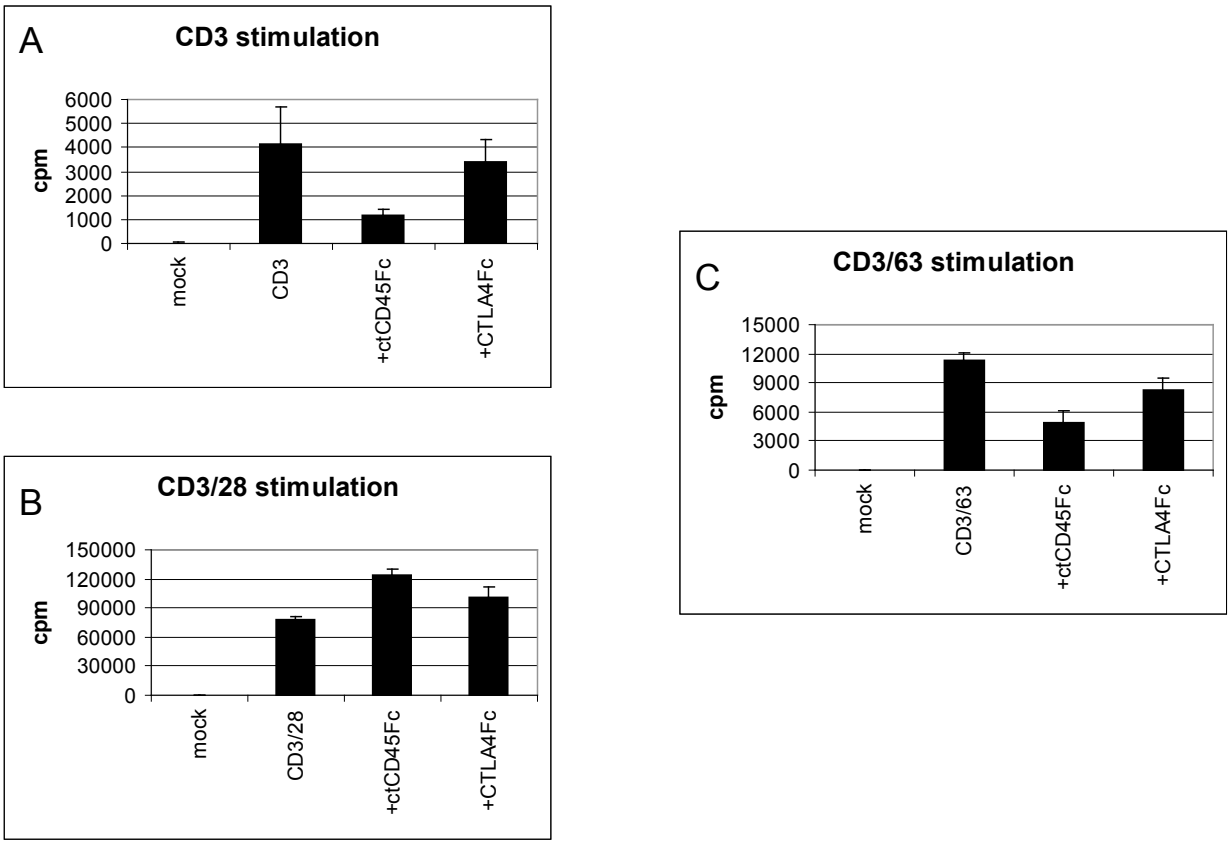


Figure 5: ct-CD45 inhibits the proliferation of CD3 and CD3/CD63 stimulated CD8 positive T cells but it increases the proliferation of CD3/CD28 stimulated $CD8^+$ T cells

$CD8^+$ T cells were stimulated with platebound anti CD3 monoclonal antibody (OKT3) and anti CD3 mAb in combinations with anti CD28 or anti CD63 mAb or no stimulation at all (mock) for 90 hours and cultured with or without platebound ct-CD45 or CTLA4 fusion

protein. The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. A) $CD8^+$ T cells with or without anti CD3 activation; B) $CD8^+$ T cells with or without a combination of anti CD3 and anti CD28 mAb; B) $CD8^+$ T cells with or without a combination of anti CD3 and anti CD63 mAb; The results of one representative of two independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

The proliferation of CD8 positive T cells, which are activated with anti CD3 (Figure 5 A) or the combination of anti CD3 and anti CD63 antibodies (Figure 5 C), can be inhibited if the ct-CD45 fusion protein is added. In contrast to that, ctCD45Fc slightly increased the proliferation of the CD8 positive T cells upon activation with a combination of anti CD3 and anti CD28 antibodies. The CTLA4 fusion protein had only little effect on the proliferation of CD8 positive T cells under these three conditions. The CD4 positive cells that were cultivated without activation showed no proliferation.

ct-CD45 inhibits cytokine production of CD3/CD63 stimulated peripheral T cells

The finding that ctCD45Fc strongly inhibits the proliferation of peripheral T cells which were stimulated by anti CD3/CD63 mAb raised the question if ctCD45Fc effects cytokine production and T cell polarisation as well.
The cytokines Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 10 (IL-10), Interleukin 13 (IL-13), Interleukin 17 (IL-17) and Interferon gamma (IFN gamma) are important for the function, proliferation and polarisation of T cells. Therefore the influence of ct-CD45 on the expression of those cytokines in peripheral T cells after stimulation with different stimuli was evaluated. To do that, peripheral T cells were isolated from human peripheral blood and stimulated with an anti CD3 mAb in combination with anti

CD63 (Figure 6) mAb. Then these cells were incubated with or without ct-CD45 (ctCD45Fc) or CTLA4 fusion proteins (CTLA4Fc), which were used as a control. After five days the concentration of IL-2, IL-10, IL-13, IL-17 and IFN gamma in the supernatant was measured using an E.L.I.S.A.. The concentration of IL-4 was determined by intracellular staining after five days of cultivation.

Il-2 is produced by activated T cells and stimulates the proliferation and effector functions of NK cells, B cells and T cells. It is also involved in the development of regulatory T cells. The main sources for IL-4 are CD4 positive T cells of the TH2 subset. It induces the differentiation of TH2 cells from naive CD4 positive precursors, stimulates the IgE production and suppresses IFN gamma dependent macrophage function. IL-10 is produced by activated macrophages and some T cells. Its function is to control immune reactions by inhibiting activated macrophages. Il-13 mimics the effect of IL-4 on macrophages but has less effect on T and B cells. It is produced by $CD4^+$ Th2 cells. IL-17 is a proinflamatory cytokine and is produced by Th17 cells. IFN gamma is the principal macrophage activating cytokine, promotes the differentiation of naive $CD4^+$ T cells to the Th1 subset and inhibits the proliferation of Th2 cells. Those Th1 cells the produce IFN gamma in a positive feedback loop.

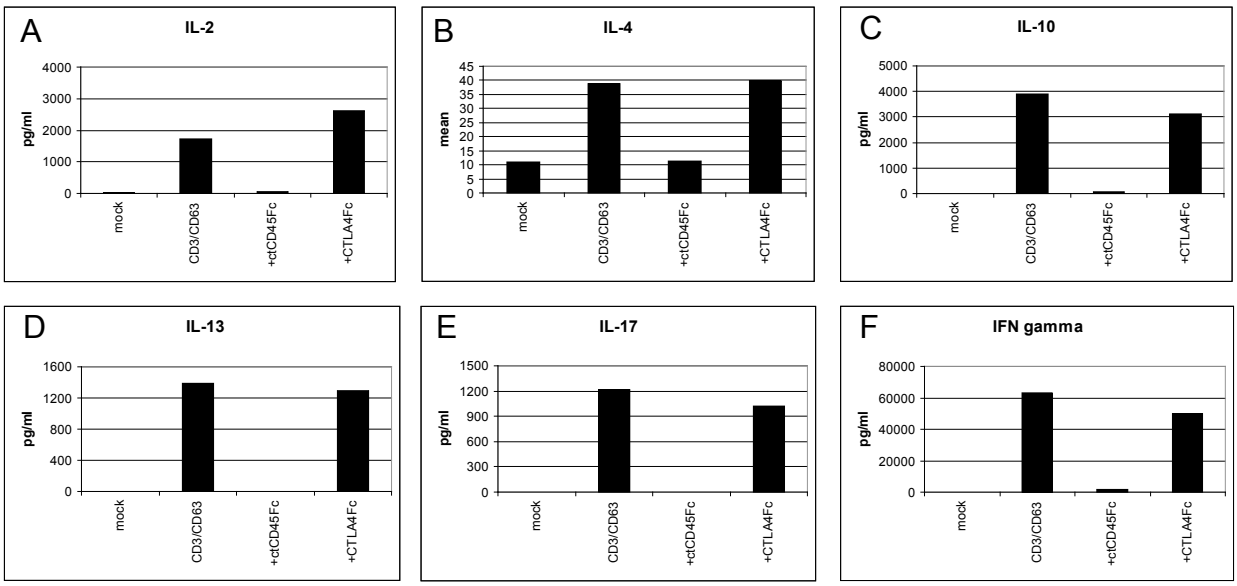


Figure 6: ct-CD45 inhibits the cytokine production of CD3/CD63 activated peripheral T cells.

Peripheral T cells were stimulated with platebound anti CD3 mAb (OKT3) in combination with an anti CD63 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion proteinor not stimulated at all (mock). After five days the concentration of IL-2 (A), IL-10 (C), IL-13 (D), IL-17 (E) and IFN gamma (F) in the supernatant was measured via Luminex. The expression of IL-4 (B) was determined by intracellular staining: Peripheral T cells were stimulated in the presence of platebound anti CD3 mAb (OKT3) and anti CD63 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein. After four days monensin was added to block the release of IL-4. 18 hours later the concentration of IL-4 was determined by intracellular staining. The results of one representative of five independent experiments are shown.

The concentration of IL-2 is almost as low in the supernatant of the CD3/CD63 activated T cells (Figure 6 A), which were cultivated in the presence of the ct-CD45 fusion protein (ctCD45Fc), as in the supernatant of the T cells, which were not stimulated at all (mock). There is a slight increase in the IL-2 concentration in the supernatant of the peripheral T cells, which were cultivated with CTLA4 fusion proteins (CTLA4Fc). ctCD45Fc inhibits the expression of IL-4 while CTLA4Fc seems to have no

influence (Figure 6 B). The concentration of IL-10 (Figure 6 C), IL-13 (Figure 6 D), IL-17 (Figure 6 E) and IFN gamma (Figure 6 F) is almost as low in the supernatant of the cells which were cultivated in the presence of ctCD45Fc as in the supernatant of the negative control (mock). The presence of the CTLA4 fusion protein has only little to no effect on the expression of those cytokines.

ct-CD45 inhibits cytokine production of CD3/CD28 stimulated peripheral T cells

Since ctCD45Fc inhibited cytokine production or release of CD3/CD63 stimulated peripheral T cells, the question was if the cytokine production of anti CD3/CD28 mAb activated T cells is also influenced by ctCD45Fc. Figure 7 shows that ctCD45 has a quite similar effect on the cytokine production or release of CD3/CD28 activated peripheral T cells than it has on the cytokine production of CD3/CD63 activated peripheral T cells.

The concentration of IL-2 is much lower in the supernatant of those T cells (Figure 7 A), which were cultivated in the presence of the ct-CD45 fusion protein (ctCD45Fc), than without. There is an increase in the IL-2 concentration in the supernatant of the peripheral T cells, which were cultivated with CTLA4 fusion proteins (CTLA4Fc). ctCD45Fc inhibits the expression of IL-4 while CTLA4Fc increases it (Figure 6 B). The concentration of IL-10 (Figure 6 C), IL-13 (Figure 6 D), IL-17 (Figure 6 E) and IFN gamma (Figure 6 F) is much lower in the supernatant of the cells which were cultivated in the presence of ctCD45Fc. The presence of the CTLA4 fusion protein has only little to no effect on the expression of those cytokines.

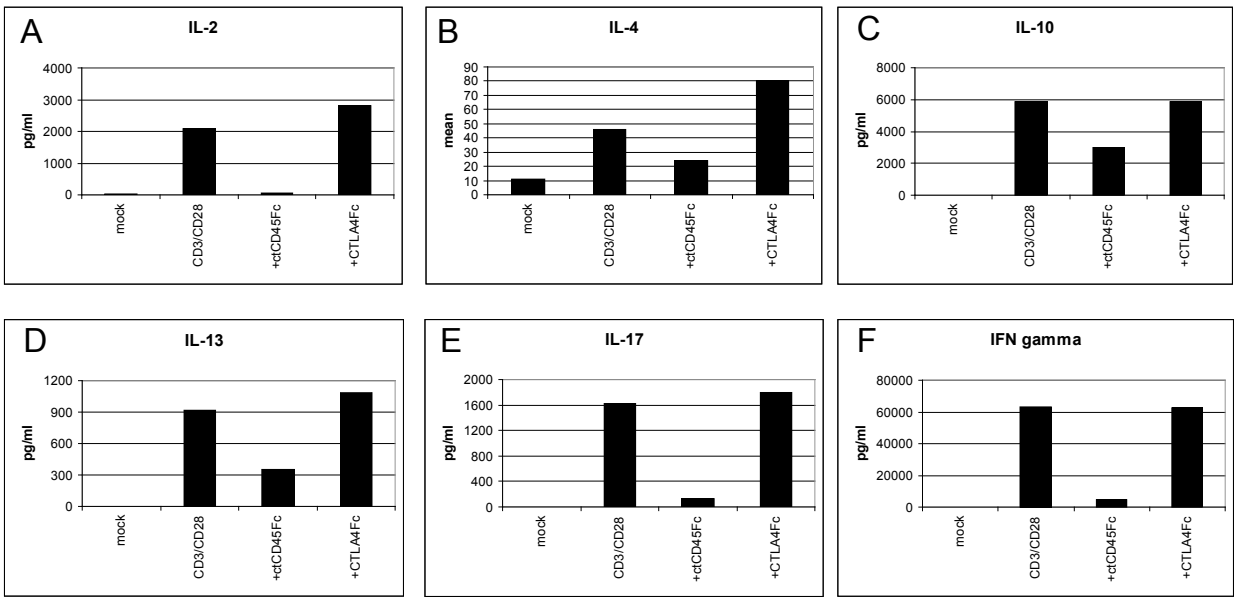


Figure 7: ct-CD45 inhibits the cytokine production of CD3/CD28 activated peripheral T cells.

Peripheral T cells were stimulated with platebound anti CD3 mAb (OKT3) in combination with an anti CD28 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein or not stimulated at all (mock). After five days the concentration of IL-2 (A), IL-10 (C), IL-13 (D), IL-17 (E) and IFN gamma (F) in the supernatant was measured via Luminex. The expression of IL-4 (B) was determined by intracellular staining: Peripheral T cells were stimulated in the presence of platebound anti CD3 mAb (OKT3) and anti CD28 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein. After four days monensin was added to block the release of IL-4. 18 hours later the concentration of IL-4 was determined by intracellular staining. The results of one representative of five independent experiments

ct-CD45 inhibits cytokine production of naive T cells

Since ctCD45Fc inhibits even the cytokine production of the CD3/CD28 stimulated peripheral T cells, whose proliferation was not inhibited by ctCD45Fc, we wanted to find out if it inhibits the cytokine production of naive T cells as well. To do that cord blood T cells were isolated from cord blood and stimulated with the combination of either anti CD3 and anti

CD63 (Figure 8) or the combination of anti CD3 and anti CD28 antibodies (Figure 9). Then those cells were cultivated in the presence of either ctCD45Fc, CTLA4Fc, which was used as a control or no fusion protein at all.

Figure 8 shows the CD3/CD63 stimulated naive T cells. The concentration of all the measured cytokines in the supernatant of those cells, which were cultivated in the presence of ctCD45Fc, was almost as low in the supernatant of those T cells which were not stimulated with the exception of IL-4, which was down regulated by about 30% (Figure 8 B). The presence of CTLA4Fc has almost no influence on the cytokine production of those cells.

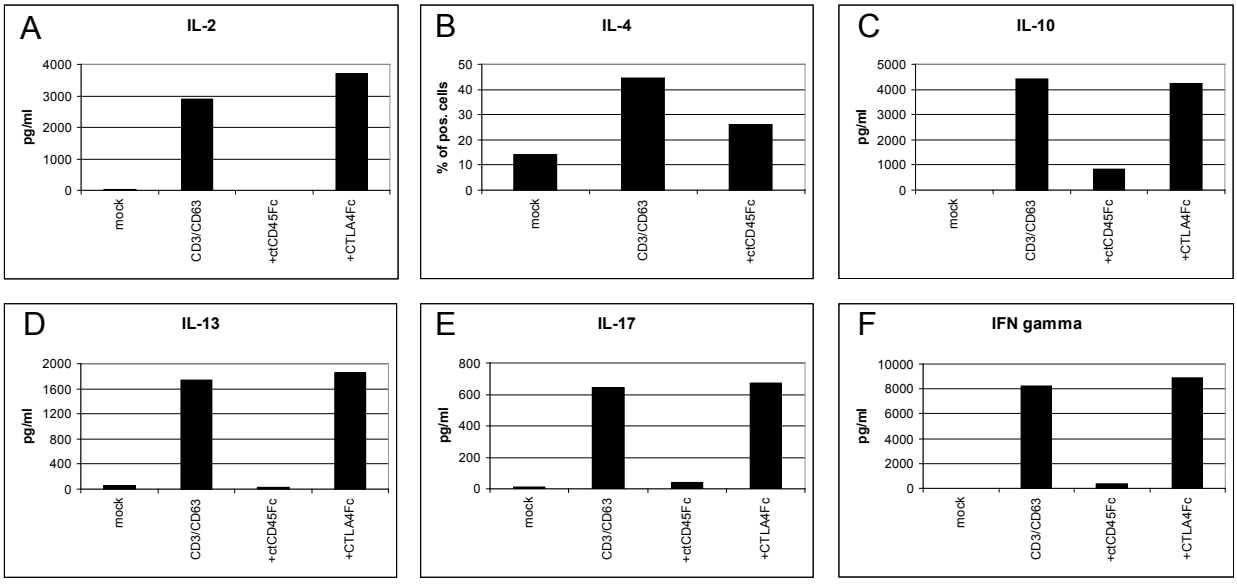


Figure 8: ct-CD45 inhibits the cytokine production of CD3/CD63 activated cord blood T cells.

Cord blood T cells were stimulated with platebound anti CD3 mAb (OKT3) in combination with an anti CD63 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein or not stimulated at all (mock). After five days the concentration of IL-2 (A), IL-10 (C), IL-13 (D), IL-17 (E) and IFN gamma (F) in the supernatant was measured via Luminex. The expression of IL-4 (B) was determined by intracellular stain-

ing: Cord blood T cells were stimulated in the presence of platebound anti CD3 mAb (OKT3) and anti CD63 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein. After four days monensin was added to block the release of IL-4. 18 hours later the concentration of IL-4 was determined by intracellular staining. The results of one representative of two independent experiments are shown.

Figure 9 shows cord blood T cells, which were stimulated with the combination of anti CD3 and anti CD28 mAb. The concentration of IL-2 in the supernatant of the CD3/CD28 activated cord blood T cells was almost as low as in the supernatant of those cells without any activation (Figure 9 A) and the concentration was reduced to zero in the presence of ctCD45Fc. The expression of IL-4 was downregulated by about 30% in the presence of ctCD45Fc (Figure 9 B). The concentration of IL-10 (Figure 9 C), IL-13 (Figure 9 D) and IL-17 (Figure 9 E) was greatly reduced in the supernatant of those cord blood T cells, which were cultivated with ctCD45Fc compared to the supernatant of the T cells cultivated with CTLA4Fc or no fusion protein. ctCD45Fc inhibits the production of IFN gamma while CTLA4Fc increases it (Figure 9 F).

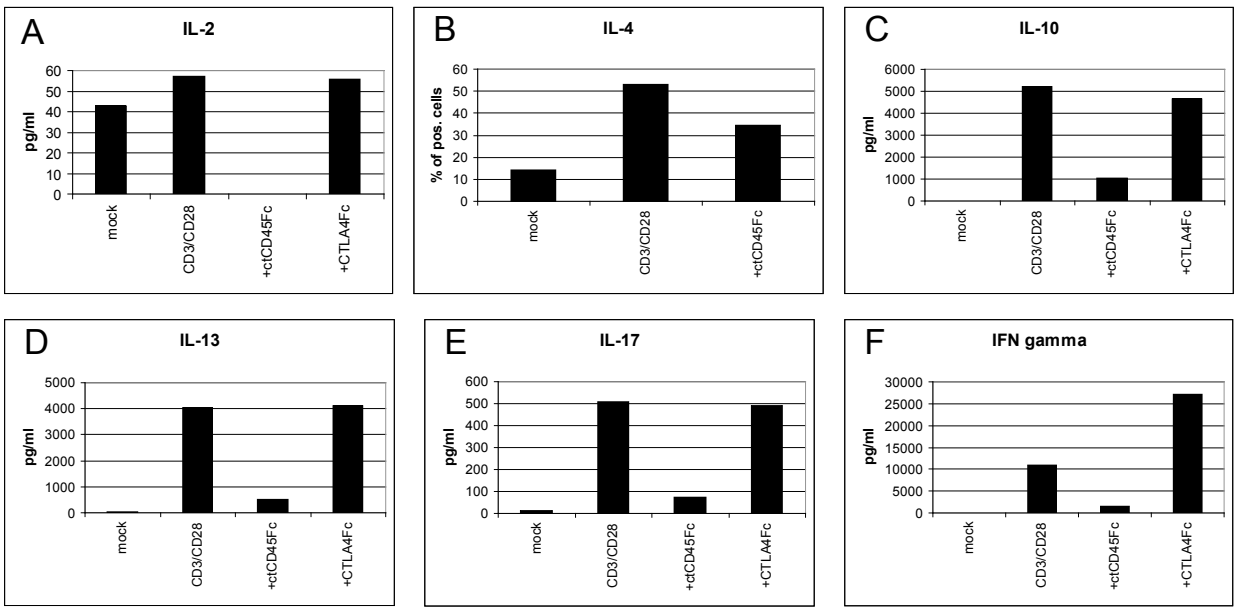


Figure 9: ct-CD45 inhibits the cytokine production of CD3/CD28 activated cord blood T cells.

Cord blood T cells were stimulated with platebound anti CD3 mAb (OKT3) in combination with an anti CD28 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein or not stimulated at all (mock). After five days the concentration of IL-2 (A), IL-10 (C), IL-13 (D), IL-17 (E) and IFN gamma (F) in the supernatant was measured via Luminex. The expression of IL-4 (B) was determined by intracellular staining: Cord blood T cells were stimulated in the presence of platebound anti CD3 mAb (OKT3) and anti CD28 mAb with or without platebound ct-CD45 (ctCD45Fc) or CTLA4 (CTLA4Fc) fusion protein. After four days monensin was added to block the release of IL-4. 18 hours later the concentration of IL-4 was determined by intracellular staining. The results of one representative of two independent experiments are shown.

Expression of activation marker molecules on T cells in the presence of ctCD45Fc

To examine if the ctCD45Fc induced inhibitory effects are caused by an altered regulation of cell surface molecules involved in T cell activation, we had a look on the expression of cell-surface molecules on activated peri-

pheral and naive T cells cultivated in the presence of ctCD45Fc or CTLA4Fc.

The main function of MHC II is to present processed antigens and it is expressed on activated human T cells. CD25 is a part of the IL-2 receptor and its expression is up regulated during T cell activation like the expression of CD97. CD69 is one of the earliest cell surface molecules expressed by T cells after activation.

In peripheral T cells the up regulation of the activation markers CD25 and CD97 was strongly reduced in the presence of ctCD45Fc (Figure 10). The co cultivation of the activated T cells with ctCD45Fc had only a minor effect on the expression of CD69. The CTLA4 fusion protein CTLA4Fc had only very little influence on the expression of any of these surface molecules on activated peripheral T cells.

There is a strong reduction of CD25 and MHC II expression if the activated cord blood T cells are co cultivated with the ctCD45 fusion protein (Figure 10). ctCD45Fc only had only little effect on the expression of CD97. CTLA4Fc reduced the expression of CD25 of activated cord blood T cells while it had only minimal effect on the other surface molecules.

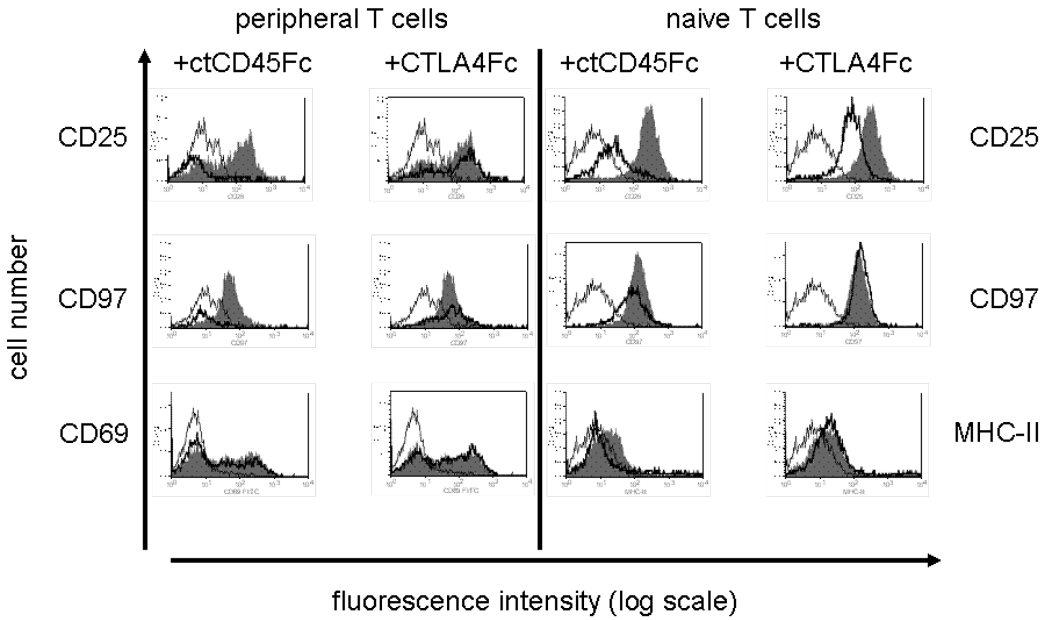


Figure 10: expression of surface molecules in the presence of different fusion proteins.

Peripheral and naive (cord blood) T cells were stimulated with plate bound CD3/CD28 mAb for 5 days in the presence of ctCD45Fc, CTLA4Fc or without any fusion protein; black thin line: negative control (CD3/28 mAb activated T cells stained with an anti VIAP mAb); grey histogram: CD3/28 mAb stimulation without any fusion protein; thick black line: CD3/28 mAb stimulation with ctCD45Fc or CTLA4Fc. CD69 was stained 48 hours after activation with CD3/28 mAb. Facs histogram plots of 1 representative out of 2 experiments are shown.

ctCD45Fc treated T cells can not be restimulated with CD3/CD28 or CD3/CD63

Since T cells which are cultivated with ctCD45Fc are still alive [139], but do not proliferate and show reduced cytokine levels, the question was if these cells could be restimulated with platebound anti CD3/CD28 or CD3/CD63 mAb.

T cells were stimulated with the combination of an anti CD3 with either an anti CD28 (Figure 11 A) or anti CD63 mAb (Figure 11 B) in the presence of ctCD45Fc, CTLA4Fc or no fusion protein. Then after resting for five

days they were restimulated the same way as before but without fusion protein. Under both conditions the T cells which were preincubated with ctCD45Fc did not proliferate anew upon stimulation while the CTLA4Fc treated T cells showed the same proliferation as the T cells which were not preincubated with fusion protein.

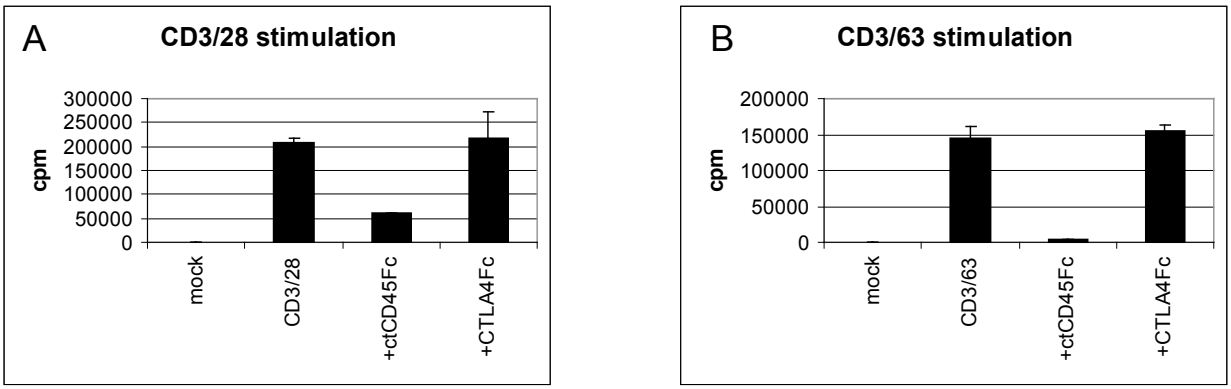


Figure 11: Restimulation of T cells

T cells were stimulated with plate bound CD3/CD28 or CD3/CD63 mAb for five days and incubated with ctCD45Fc, CTLA4Fc or without any fusion protein. After five days these cells were washed and incubated for another five days without any stimulation in fresh media. The T cells were then restimulated with either CD3/CD28 (Figure 11 A) or CD3/CD63 (Figure 11 B) mAb like they were before or not stimulated at all (mock). The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. The results of one representative of 5 independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

The addition of exogenous IL-2 can only partly reverse the ctCD45Fc induced hyporesponsive state

Since ctCD45Fc strongly reduced the concentration of IL-2 in the supernatant of stimulated T cells and given the fact that IL-2 is one of the most important factors for T cell proliferation, we wanted to find out if the addition of exogenous IL-2 could reverse the ctCD45Fc induced hyporespon-

sive state and trigger proliferation in ctCD45Fc treated T cells. Figure 12 A and B show that the addition of IL-2 partly reverses the inhibitory effect of ctCD45Fc and increases the T cell proliferation. However Figure 11 C and D show that even though IL-2 increases the proliferation of the T cell which were incubated with ctCD45Fc the T cells which were incubated with CTLA4Fc or without any fusion protein, in the presence of 10 Units/ml IL-2, proliferated much stronger than the ones which were incubated with ctCD45Fc. There seems to be no difference between the CD3/28 and the CD3/63 stimulations.

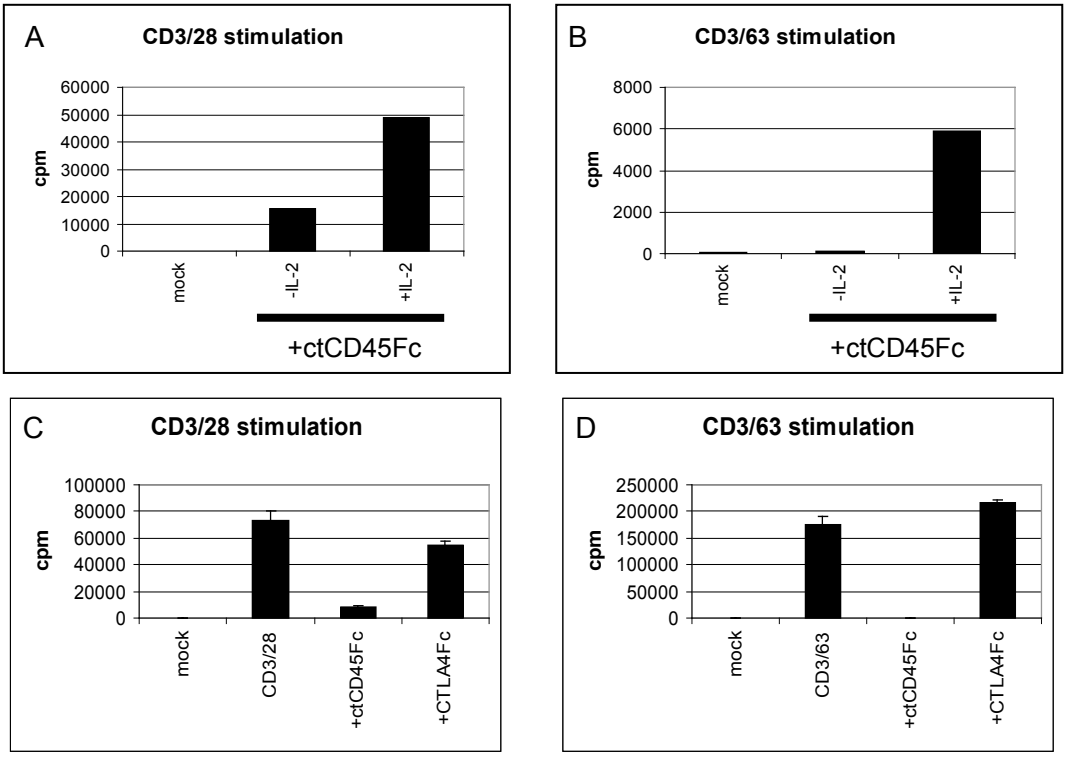


Figure 12: The addition of exogenous IL-2 can only partly reverse the ctCD45Fc induced hyporesponsive state

(A, B) T cells were stimulated with plate bound CD3/CD28 or CD3/CD63 mAb for five days in the presence of ctCD45Fc. After five days these cells were washed and incubated for another five days without any stimulation in fresh media. Then the T cells were

restimulated with or without 10 Units/ml IL-2 with either CD3/CD28 (Figure 11 C) or CD3/CD63 (Figure 11 D) mAb or not stimulated at all (mock). The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. The results of one representative of 3 independent experiments are shown.

(C, D) T cells were stimulated the same way as in Figure 12 A and B with the only difference that all the cells were restimulated in the presence of 10 Units/ml IL-2.The proliferation of those T cells then was monitored by adding [methyl-3H]TdR after 72 hours of cultivation and measuring [methyl-3H]TdR incorporation 18 hours later. The results of one representative of 5 independent experiments are shown. Mean values of triplicate determination ± standard deviation are shown.

Identification of the receptor for ct-CD45 on human T cells

Identification of ct-CD45 receptors by screening a cDNA library

ctCD45 has been described to inhibit the proliferation of CD3 activated T cells. To analyze the interaction between ct-CD45 and human T cells, binding studies were performed with fluorescence labelled ct-CD45 fusion proteins. In these experiments, ct-CD45 was found to bind to activated t cells. This binding was saturable and blockable with blocking antibodies or unlabeled ct-CD45 fusion protein. [139]

To identify activated T cell surface molecules involved in the interaction with ct-CD45, we used a cDNA library, derived from activated T cells. The cDNA library was expressed in the mouse B cell line BW. The target cell pool was then stained with ctCD45Fc as a primary reagent. Then the BW cells binding ctCD45Fc were labelled with a goat anti human Fc, PE conjugated mAb and FACS cell sorting was used to separate them from the rest of the cells (Figure 13). These ctCD45Fc binding BW cells were expanded for two additional sorting steps. After three rounds of FACS cell sorting 10,7% of the cells bound to ctCD45Fc from originally 0,3%. As a control we used two different cell sorting experiments. For the first control we used a combination of anti CD27 and anti CD54 mAb, for the second control we used the combination of two fusion proteins consisting of either ICOS-L or CD80 and the Fc part of human antibodies.

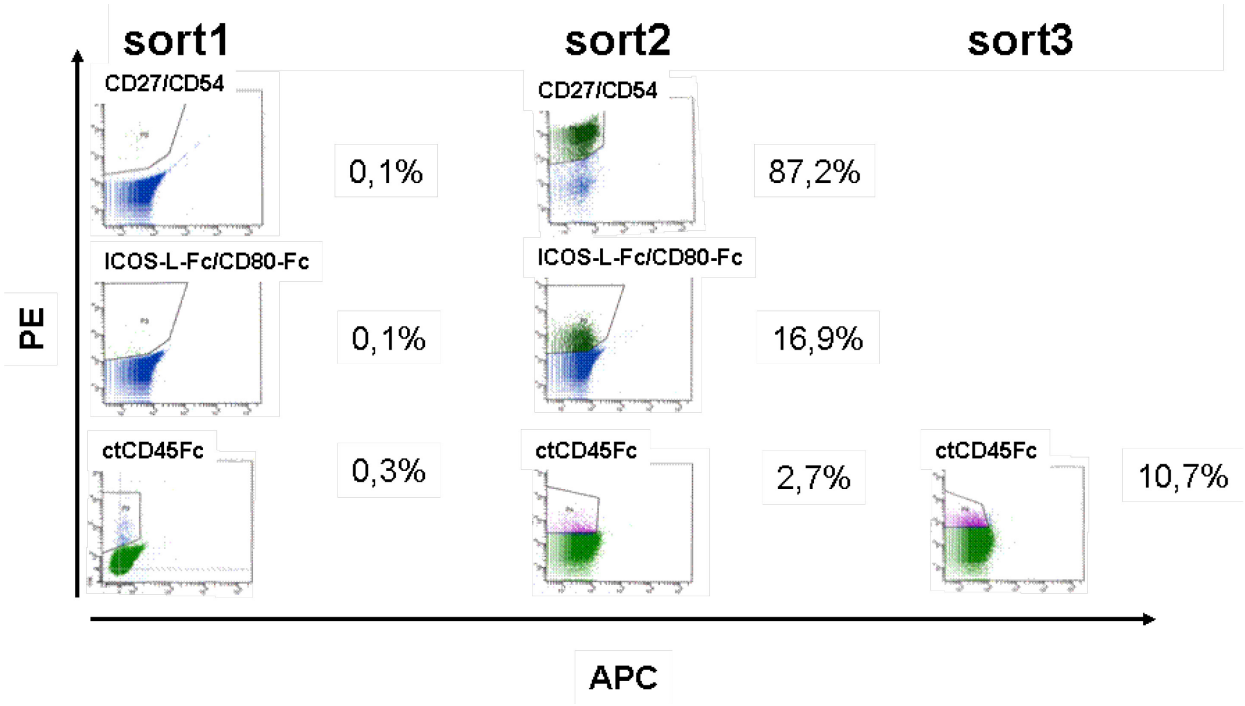


Figure 13: Identification of ct-CD45 receptor by cDNA library screen
ctCD45Fc-reactive cells were isolated from a cDNA library by performing three FACS sorting steps. The cDNA library was derived from activated human T cells expressed in BW cell line. Gates used for selection of ctCD45Fc- binding cells are shown. The cells were probed with ctCD45Fc following a staining step with a PE labelled anti human Fc mAb. Two controls were used to ensure that the sorting procedure worked. The first sorting control was with a combination of anti CD27/CD54 antibodies the second with the combination of ICOS-L-Fc/CD80-Fc fusion proteins.

Single cell clones were established from the reactive cell pool. Two of these single cell clones bound to ctCD45Fc (Figure 14). These two cell clones (clone A and clone B) were stained with CTLA4 (CTLA4Fc), ICOS-L (ICOS-L-Fc) and ct-CD45 fusion proteins (ctCD45Fc). Neither CTLA4Fc nor ICOS-L-Fc show a binding reaction to those cells, only ctCD45Fc shows such a binding.

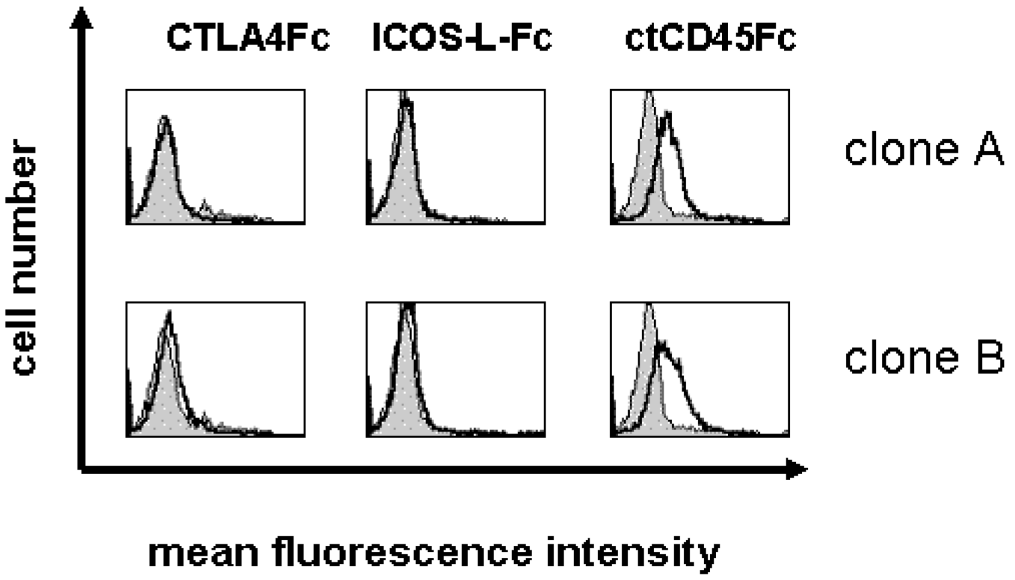


Figure 14: Two BW single cell clones bind ctCD45Fc

The BW single cell clones A and B were then stained with ctCD45Fc, CTLA4Fc or ICOS-L-Fc as a primary reagent. Then the BW cells binding to those fusion proteins were labelled with a goat anti human Fc, PE conjugated mAb. The gray histograms are the negative control with VIAP antibody and the black lines are the stainings with the different fusion proteins. Six histograms of one representative out of 5 different experiments are shown.

The cDNA inserts of those two single cell clones were retrieved from ctCD45Fc-binding BW cell clones by PCR. It turned out that both clones had the same six inserts (Figure 15 A). This DNA inserts were sequenced and they were found to be identical to five different mRNAs and one random chromosome 16 sequence (Figure 15 B). The only one that was described of having any function in the immune system was canopy 3 homolog protein PRAT4A (Figure 15 A: band F). This protein is described as an endoplasmatic reticulum resident protein, which is involved in the trafficking of toll like receptors (TLR) 1,2 and 9. Nevertheless staining of these BW cell clones with anti PRAT4A antibodies showed that PRAT4A is expressed on the cell surface of these BW cell clones (Figure 15 C). Anti VIAP mAb were used as control to verify that the binding of the anti

PRAT4A antibodies is not due to unspecific binding. We also stained these cells with ctCD45Fc and control fusion proteins (CTLA4Fc and ICOS-L-Fc) to be sure that these cells bind ctCD45Fc as well. By intracellular staining of T cells, it was shown that PRAT4A is expressed in resting as well as in activated T cells (Figure 15 D). However PRAT4A is stronger expressed on the cell surface of activated T cells then on resting ones.

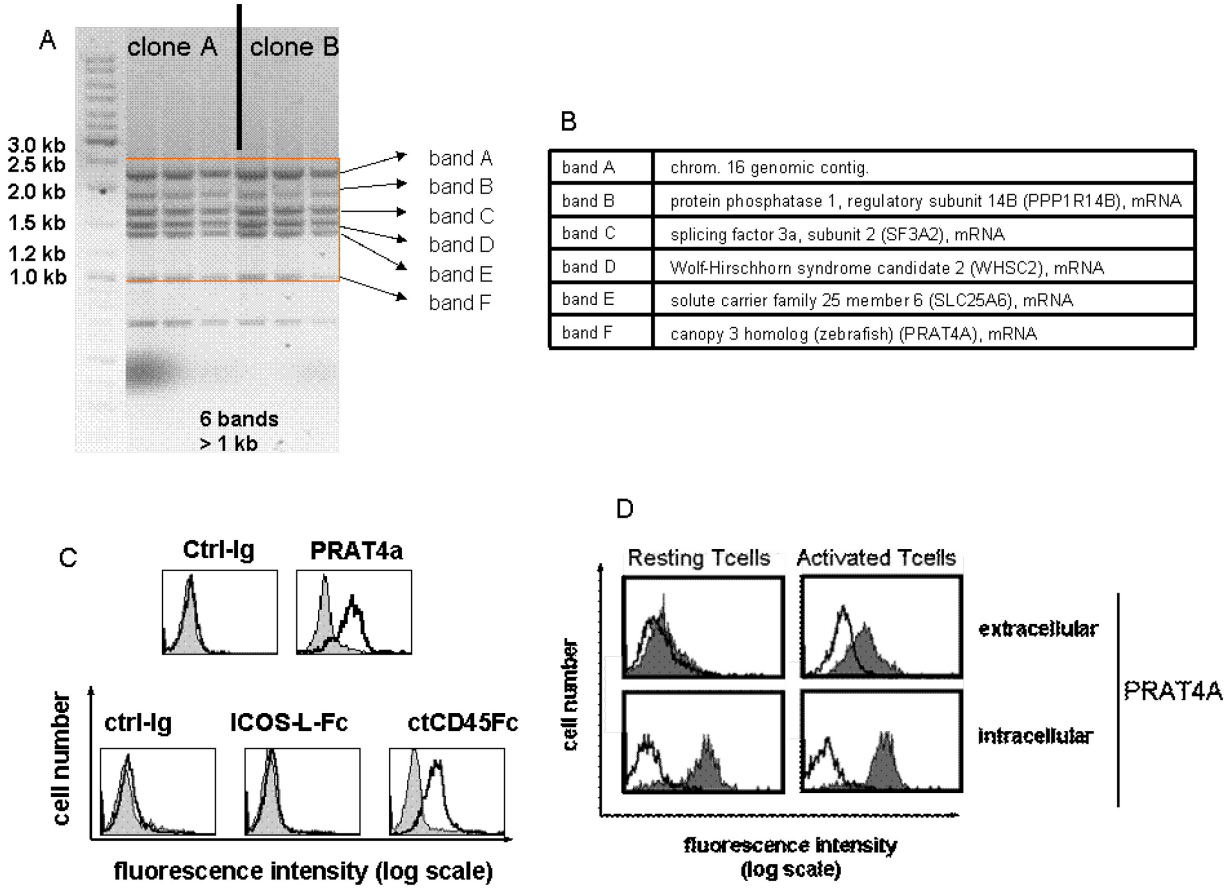


Figure 15: Identification of ct-CD45 receptors by screening a cDNA library

(A) PCR-amplified inserts from two different ctCD45Fc-binding single cell clones established from the selected cell pool. The 6 bands (in the red box), which were obtained in both products, was subjected to DNA sequence analysis. (B) List of the result of the sequence analysis of the 6 bands (Figure 15 A). (C) PRAT4A staining of ctCD45Fc binding BW single cell clone A was stained with anti PRAT4A antibody. Anti VIAP mAb and

ICOS-L-FC fusion protein were used to ensure that the binding of ctCD45Fc and PRAT4A antibodie is not due to unspecific binding. (D) CD3/CD28 activated and resting T cells were extra and intracellular stained with anti PRAT4A antibodies.

siRNA knockdown of PRAT4A inhibits binding of ctCD45Fc

To verify if the binding of ctCD45Fc to clone A and B is related to the expression of PRAT4A a siRNA knockdown was conducted. The BW cells were cultivated with Lipofectamin and with or without PRAT4A siRNA. Then a binding assay was used to determine if a knockdown of PRAT4A has any influence on the binding of ctCD45 to those BW cells.

Figure 16 shows that the binding of ctCD45 to the BW cells, in which PRAT4A was knocked down, is reduced compared to the ones, which were cultivated with just Lipofectamine. CTLA4Fc was used as a negative control to ensure that the binding of ctCD45 is not due to unspecific binding.

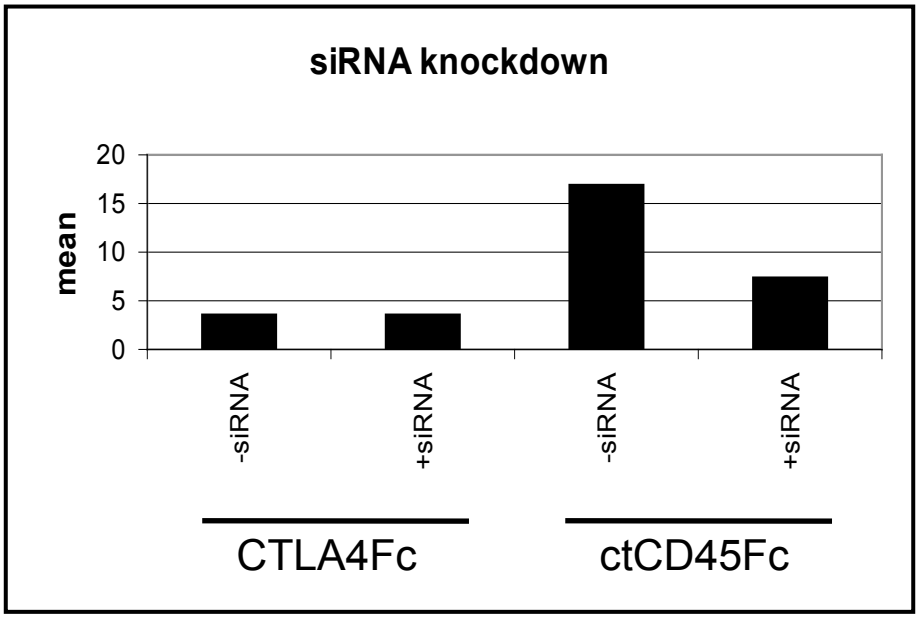


Figure 16: siRNA knockdown of PRAT4A inhibits binding of ctCD45Fc

ctCD45Fc-binding BW single cell clone A was incubated with µg Lipofectamine and with or without µg PRAT4A siRNA for 48 hours. Then these cells were stained with ctCD45Fc or CTLA4Fc as a primary reagent. The ctCD45Fc or CTLA4Fc binding cells then were labelled with PE conjugated goat anti human Fc mAb.

Discussion

CD45 was one of the first signaling molecules identified on leukocytes and is used as a leukocyte marker molecule.[104-114] It is the prototypic member of transmembrane receptors like protein tyrosine phosphatases (PRTPs) and various forms of it are expressed on all nucleated hematopoetic cells.[111-114] CD45 plays an essential role in immune functions by dephosphorylizing different substrates.[119-122]

The starting point of the first part of my project was that recently an alternative function for the intracellular domain of CD45 (ct-CD45) was discovered. It was shown that CD45 is cleaved and ct-CD45 is released during activation of human monocytes and granulocytes by fungal stimuli. Furthermore was observed that ct-CD45 acts as a cytokine like factor, which inhibits T cell proliferation induced by dendritic cells or CD3 antibodies. The cytoplasmatic tail of CD45 can thereby act as an intercellular regulator between the innate and the adaptive immune system.[139] In order to analyze the functual consequences of the inhibitory signal delivered by ct-CD45 to T cells proliferation assays were conducted. They show that ct-CD45 inhibits the proliferation of CD3 as well as CD3/CD63 activated peripheral T cells. The proliferation of CD3/CD28 activated peripheral T cells however, was not inhibited together with the proliferation of CD3, CD3/CD63 and CD3/CD28 activated cord blood T cells. If the cells were CD4 or CD8 positive had no effect on the inhibitory effect of ctCD45.

The second question was if the cytokine production of T cells is affected by ct-CD45. The experiments show that the production or release of the cytokines IL-2, IL-4, IL-10, IL-13, IL-17 and IFN gamma which are important for activation and proliferation of all the different T cell subtypes, are strongly down regulated. Surprisingly even the CD3/CD28 activated peripheral T cells as well as the cord blood T cells which showed normal pro-

liferation in the presence of ct-CD45 had a reduced cytokine production with the only exception of IL-4, which showed an increased expression in cord blood T cells. Since IL-4 stimulates the development of Th2 cells from naïve CD4 pos T cells it seems that ct-CD45 induces the differentiation of cord blood T cells into Th2 cells. It inhibits the proliferation and the IL-4 production of peripheral T cells, though.

The down regulation of the activation markers on the cell surface of activated peripheral T cells in the presence of ct-CD45 together with the almost complete absence of effector cytokines shows that, even though their proliferation is not inhibited when they are stimulated with CD3/CD28, their function is severely impaired. Since CD3/CD28 provides a stronger activation signal then CD3 alone or CD3/CD63 the reason why the proliferation CD3/CD28 activated T cells is not inhibited when ct-CD45 is present might be because due to the strong activation signal the activation of these cells is powerful enough for the proliferation even though their cytokine production is inhibited. The same goes for the cord blood T cells which show a severe reduction of cytokine production except IL-4 but no inhibition of proliferation. Cord blood T cells showed a very strong proliferation in all the conducted experiments regardless of the means of activation in fact much stronger than peripheral T cells. So it could be that the activation signal is powerful enough for a normal proliferation even though ct-CD45 is present the only reason why those cells which were stimulated in the absence of this protein did not proliferate stronger was because their proliferation was already at its maximum.

It seems that ct-CD45 induces a hyporesponsive state in the T cells because even though T cells proliferated in the presence of ct-CD45 upon certain activating conditions none of those cells which were incubated with this protein showed proliferation upon restimulation. This hyporesponsive state appears to be different from normal T cell anergy because the addition of exogenous IL-2 during restimulation only has a minor effect on pro-

liferation. Clonal T cell anergy on the other side should be reversible by adding exogenous IL-2.

The second part of my diploma thesis was about finding a possible receptor candidate for ct-CD45 on T cells. The reason for searching for a receptor was the finding that ct-CD45 binds to T cells. Binding assays showed that this protein only binds to activated T cells and that this binding can be blocked by mAb. Furthermore it was shown that the binding of ct-CD45 to activated T cells is saturable.[139] These findings suggest that there is a specific receptor for this protein on the cell surface of those cells.
Binding assays with a cDNA library for activated T cells gave evidence for interaction of ct-CD45 and a protein associated with Toll-like receptor 4 (PRAT4A). PRAT4A was recently discovered as an ER-resident chaperon. It was initially described as a TLR4 binding chaperon (Figure 17), but PRAT4A also associates with TLR1. It was shown to be necessary for cell surface expression of these TLRs.[140] PRAT4A regulates not only the response of cell surface TLR but also that of intracellular TLR. In the absence of PRAT4A, the response to TLR9 ligand is completely abolished. It is required for the ligand induced trafficking of TLR9 from the ER to lysosomal components. TLR9 and TLR7 mediated responses are compleately dependent on PRAT4A, TLR3 on the contrary has been shown to respond to stimulation in the absence of PRAT4A.[140]

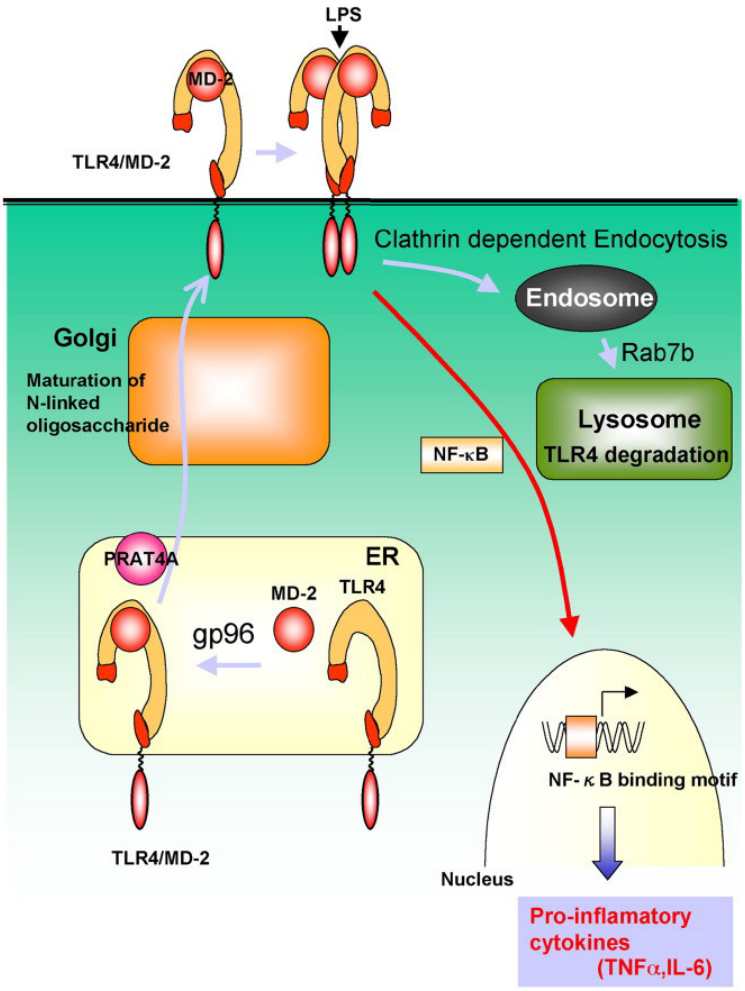


Figure 17:

PRAT4A interacts with the hypoglycosilated TLR4 bound to MD-2 to regulate the translocation of TLR4. After the interaction of PRAT4A with TLR4, TLR4 is glycosylated in ER and Golgi to the mature form of TLR4. TLR4/MD-2 is expressed on the cell surface and recognizes LPS to dimerize. LPS activation also induces TLR4/MD-2 endocytosis and translocates to endosomes/lysosomes to shut off the signaling by TLR4 degradation.[141]

Another unique characteristic of this protein is that it is necessary for inducting the endotoxin shock. Experiments have shown that PRAT4A$^{-/-}$ BM chimeric mice are capable to withstand toxic shock. In comparison to chimeric WT controls, it was revealed that serum cytokines level also follow a PRAT4A dependency.[140]

Although described as an endoplasmatic reticulum resident protein extra cellular staining showed that PRAT4A is also presented on the cell surface of activated T cells. Furthermore a siRNA knockdown of PRAT4A showed that the expression of PRAT4A and the binding of ct-CD45 are linked. Even though PRAT4A might not bind directly to ct-CD45, the experiments show that PRAT4A is involved in the signalling pathway of ct-CD45.

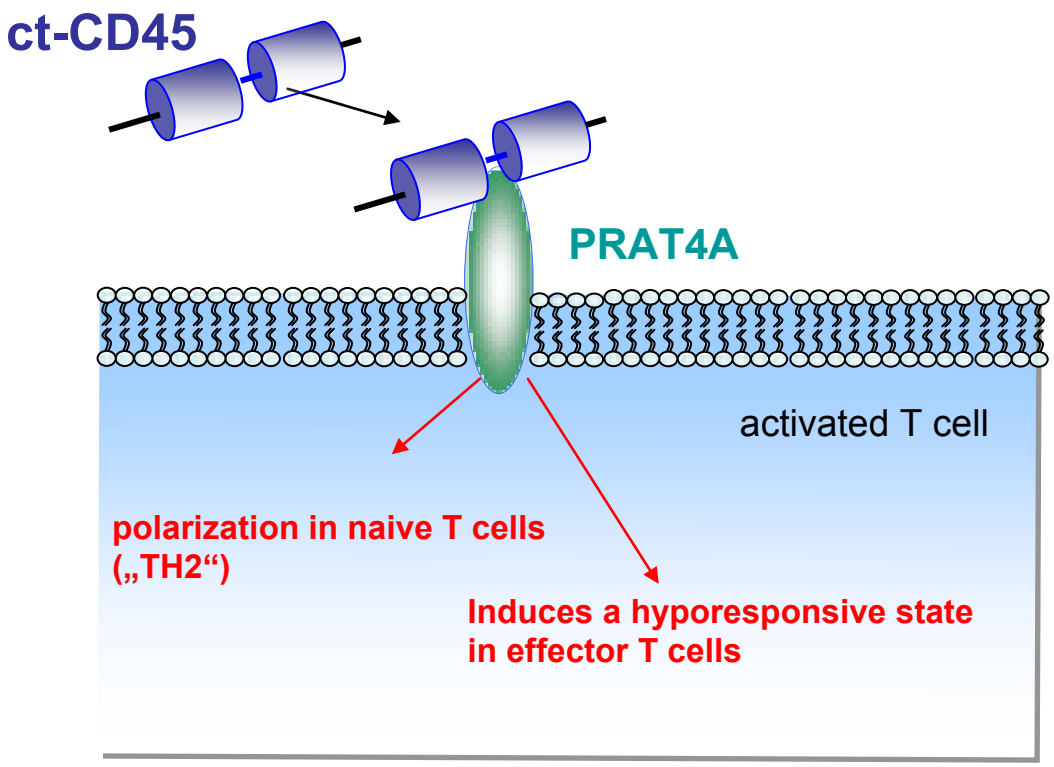


Figure 18: Summary of ct-CD45 binding to activated T cells

In summary ct-CD45 binds to naïve and activated T cells. PRAT4A is involved in this binding which leads to the Th2 linage commitment of naïve T cells and to the induction of a hyporesponsive, anergy like state in peripheral T cells. Since ct-CD45 is released upon celldeath of monocytes and granulocytes it is possible that this induced hyporesponsitivity serves as a regulatory effect to prevent a too strong T cell activation.

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