DIPLOMARBEIT

Titel der Diplomarbeit

The human Ig-family member CD147 regulates T cell activation

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag[a]. rer.nat.)

Verfasser:	Zojer Verena
Matrikel-Nummer:	0302684
Studienrichtung (lt. Studienblatt):	A490
Betreuer:	Dr. Wolfgang Paster Dr. Herbert Schiller Univ. Prof. DI Dr. Hannes Stockinger Assoc. Prof. Dr. Pavel Kovarik

Wien, November 2008

DANKSAGUNG

Ein großer Dank gilt Univ. Prof. Dr. Hannes Stockinger dafür, dass ich die Möglichkeit hatte in seinem Labor an meiner Diplomarbeit zu arbeiten. Bedanken möchte ich mich außerdem für die professionelle Betreuung durch intensive Besprechungen und erfahrene Ratschläge, die mich und damit auch meine Diplomarbeit sehr bereichert haben.

Besonders bedanken möchte ich mich hiermit auch bei meinen Betreuern Dr. Wolfgang Paster und Dr. Herbert Schiller für die Einschulung in alle angewandten Labortätigkeiten und ihre mitreißende Motivation. Danke auch, für die Bereitschaft zu jeder Zeit meine Ideen und Vorschläge zu reflektieren.
Ganz herzlich möchte ich mich bei allen Laborkollegen/Innen bedanken, dass sie mir immer mit Rat und Tat zur Seite gestanden haben und deren Sinn für Humor ein wunderbares Arbeitsklima geschaffen hat!

Mein größter Dank gilt meinen lieben Eltern, Monika und Hans, die mich immer in allen Lebenslagen unterstützt und mir Rückhalt gegeben haben. Darüber hinaus haben sie mein Interesse an der Naturwissenschaft geweckt und kreative Ideen gefördert. Daher, soll diese Arbeit auch ihnen gewidmet sein.

Herzlich bedanken möchte ich mich hiermit auch bei meinem Freund Roland Supper für das Verständnis und die Geduld während der Diplomarbeit.

Zuletzt ein herzliches Dankeschön an meine Familie, meine Verwandten und meine Freunde, dass man, abseits der Diplomarbeit, mit ihnen soviel Spaß haben kann und sie immer für mich da sind!

TABLE OF CONTENTS

1 Abstract

CD147 belongs to the family of type I transmembrane immunoglobulin (Ig) like glycoproteins and is upregulated upon T cell activation[1]. Several findings point to role of CD147 as negative regulator of T cell activation[2-4]. In line with these findings, previous studies in Prof. Stockinger's laboratory revealed that CD147 knockdown T cells exhibit higher interleukin-2 (IL-2) production and a T helper 1 (Th1) cytokine bias.

Here I analyzed which structural determinants of CD147 are responsible for CD147 antagonistic function on T cell stimulation. By reconstituting CD147 silenced Jurkat T cells with small interfering RNA (siRNA) resistant CD147 deletion and swap mutants, we could show that the extracellular and the transmembrane domain of CD147 cooperated to decrease the IL-2 promoter activity upon T cell stimulation. This effect was at least partially due to negative regulation of the nuclear factor of activated T cells (NFAT). Further experiments revealed that also the activation of nuclear factor k B (NF-kB) and the activity of the focal adhesion kinase (FAK) was influenced by CD147.

The fact that the CD147 mutant lacking the cytoplasmic domain could inhibit the IL-2 promoter activity indicated that CD147 exerts its function via another transmembrane protein. Therefore, we analyzed the contribution of potential interaction partners of CD147 to the regulation of the IL-2 promoter activity. The surface expression level of the CD147 of CD43[5] and CD98[6,7], and surprisingly also of the co-stimulatory molecule CD28, was found to be upregulated upon CD147 silencing. Therefore, silenced T cells for CD28, CD43 and CD98 were generated and were tested for the IL-2 promoter activity. CD28 and CD98, but not CD43 silenced cells, displayed decreased transcriptional activity of the IL-2 promoter, making the deregulation of these molecules a likely cause for the enhanced IL-2 production in the CD147 knockdown cells. We speculate that CD147 might affect the function of these receptors during T cell activation, thereby dampening the immune response.

2 Zusammenfassung

CD147 gehört zur Familie der Typ I transmembranen Immunglobulin (Ig) ähnlichen Glykoproteine, und wird bei der T-Zell Aktivierung hochreguliert[1]. Einige Entdeckungen deuten darauf hin, dass CD147 die T-Zell Aktivierung negativ reguliert[2-4]. Passend dazu zeigten vorangegangene Experimente in Prof. Stockinger's Labor, dass CD147 Knockdown T-Zellen erhöht Interleukin-2 (IL-2) produzieren, und eine Tendenz zur T-Helfer 1 (Th1) Zytokin-Antwort aufweisen.

In dieser Studie untersuchte ich, welche strukturelle Determinanten von CD147 die antagonistische Funktion von CD147 in der T-Zell Aktivierung ausführen. Mittels Rekonstituierung von CD147 Knockdown Jurkat T-Zellen mit siRNA resistenten CD147 Deletions- und Swap-Mutanten, stellten wir fest, dass die extrazelluläre und die transmembrane Domäne von CD147 kooperieren, um die IL-2 Promotor Aktivität infolge der T-Zell Aktivierung zu verringern. Dieser Effekt war zumindest teilweise von der negativen Regulation des „nuklearen Faktor aktivierter T-Zellen" (NFAT) abhängig. Weitere Experimente zeigten, dass auch die Aktivität des „nuklearen Faktors k B" (NF-kB) und die Aktivität der „fokalen Adhäsionskinase" (FAK) durch CD147 beeinflusst werden.

Da die CD147 Mutante ohne cytoplasmatischer Domäne die IL-2 Promotor Aktivität verringern konnte, übt CD147 seine Funktion vermutlich über ein anderes Transmembranprotein aus. Deshalb wurde der Beitrag potenzieller CD147 Interaktionspartner zur Regulation der IL-2 Promotor Aktivität analysiert. Die Oberflächenexpression von CD43[5] und CD98[6,7], und überraschender Weise auch die des co-stimulatorischen Moleküls CD28, war in CD147 Knockdown Zellen erhöht. Deshalb wurden CD28, CD43 und CD98 Knockdown Zellen hergestellt, und deren IL-2 Promotor Aktivität getestet. CD28 und CD98, nicht aber CD43, Knockdown Zellen wiesen eine verringerte transkriptionelle Aktivität des IL-2 Promotors auf. Daher könnte die Deregulation dieser Moleküle für die verstärkte IL-2 Produktion in CD147 Knockdown verantwortlich sein. Wir

spekulieren, dass CD147 die Funktion dieser Transmembranproteine während der T-Zell Aktivierung beeinflusst und dabei die Immunantwort verringert.

3 Introduction

The adaptive immune system is a tightly regulated, swiftly alternating system to defend vertebrates against pathogens by highly evolved antigen specificity of its mediators. The major players executing an adaptive immune response are the B and T cells. The B cells secrete antibodies upon stimulation, and thereby mediate adaptive humoral responses against extracellular pathogens. The T cells can be further categorized into cytotoxic T cells and T helper cells (Th1, Th2, Th17 and regulatory T (Treg) cells). Complementary to the B cells, the cytotoxic T cells conduct cellular mediated immune responses against intracellular pathogens by directly interacting with infected cells, leading them into cell death. Depending on the T helper cell population evolving after antigen presentation, either humoral or cell mediated, or even down regulation of immune responses will be evoked by a distinct "cytokine cocktail"

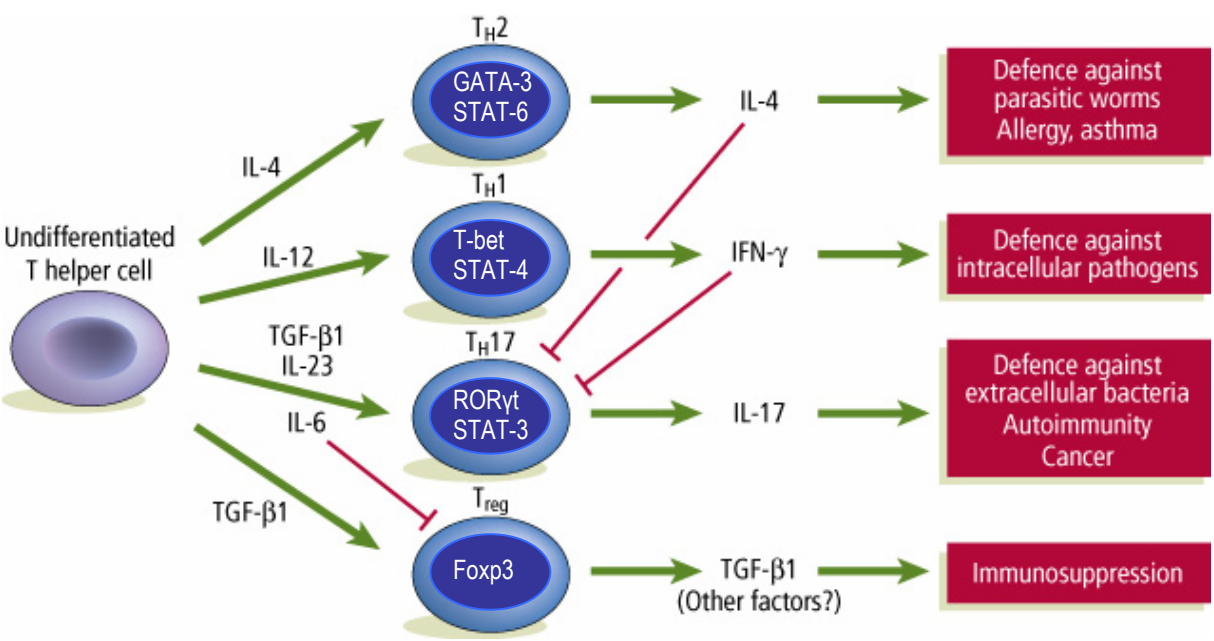


Figure 1: T helper cell subsets.

Figure was modified from Beth Israel Diaconess Medical Center homepage (http://www.bidmc.harvard.edu/display.asp?node_id=8815)

(Figure 1). This highly intelligent adaptive mechanism is necessary for the body to react in the right way with a specialized set of cells to pathogens. The T helper cells are responsible for stimulating either humoral (Th2 mediated) or cell mediated (Th1 mediated) adaptive immune responses. Fascinatingly, the different populations of T helper cells evolve through different cytokine exposure, secreted from antigen presenting cells during antigen presentation in secondary lymphoid organs[8]. Additionally, the affinity of the presented antigen to the T cell receptor takes influence in the development of T helper cells into the different T helper cell subsets[9].

3.1 *Th1 cells*

Upon development in the thymus, the naïve CD4+ T helper cells travel to various secondary lymphoid organs where they are awaiting antigen presentation. As mentioned before, depending on the properties of the pathogen, the antigen presenting cell is secreting special cytokines into the immunological synapse. In case of intracellular pathogens, the APC will secrete interleukin-12 (IL-12) during cross-presentation of the antigen via major histocompatibility complex II (MHCII) to the naïve CD4+ T cells. The secreted IL-12 binds to its receptor on T helper cells to modulate T cell receptor mediated activation and cytokine expression. Complex signaling events, that will be described later, lead to interferon-γ (IFN-γ) and IL-2 production, inducing positive feedback loops necessary for Th1 differentiation. Due to the IFN-γ and IL-12 dependent differentiation, Th1 cells display high levels of IFN and IL-12 receptors on their surface. Finally, differentiated and activated effector Th1 cells arise, which secrete IFN-γ, IL-2, tumor necrosis factor (TNF) and leukotrienes to enforce cytotoxic T cell function, enhance macrophage mediated cytotoxicity and phagocytosis and will further stimulate naïve Th precursor cells to become Th1 cells[8].

3.2 *Signaling pathways leading to Th1 cytokine production IFN-γ and IL-2 in T cells*

3.2.1 Interferon-γ

The IFN-γ production in T cells is triggered by concerted stimulation of the signal transducer and activator of transcription (STAT) pathway by IL-12, T cell receptor (TCR) mediated signaling cascades via MHCII presented antigen and the costimulatory molecule CD28[8]. The TCR/CD3 mediated signaling pathways will be discussed in detail later. Interleukin-12 crosslinks the two IL-12 receptor chains IL-12Rβ1 and IL-12Rβ2, which leads to tyrosine phosphorylation of the receptor associated kinases Janus kinase 2 (Jak2) and tyrosine kinase 2 (Tyk2). These two kinases phosphorylate the IL-12 receptor, creating src homology 2 (SH2) domain binding sites[10].

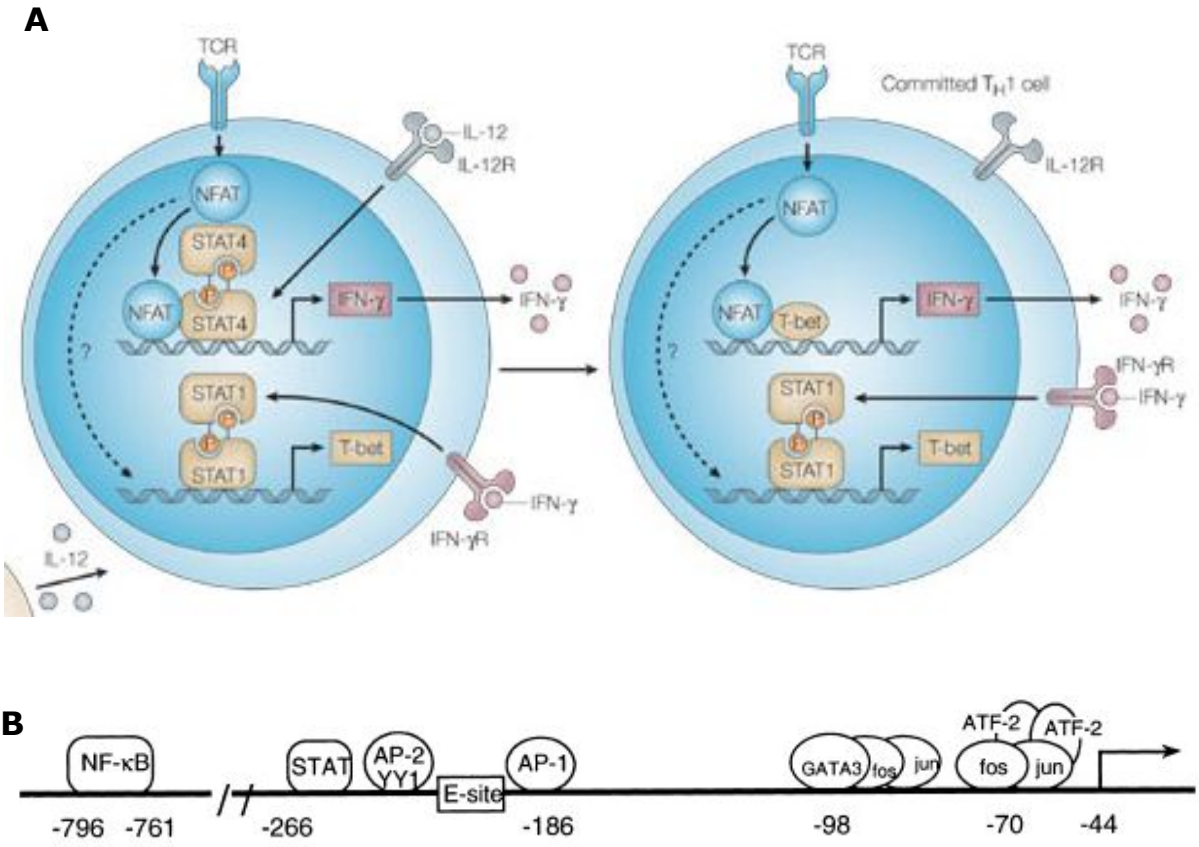


Figure 2: Activation of IFN-γ promoter in Th precursor cells. A) STAT mediated commitment to Th1 development[11]. B) Transcription factors binding to IFN-γ promoter[8].

Thereafter, STAT4, associated with the SH2 domains of IL-12R, will be tyrosine phosphorylated by Jak2 and Tyk2[12] (Figure 2a). An alternate mechanism leading to STAT4 tyrosine phosphorylation and activation can be induced by type 1 interferons mediated by STAT2[13,14]. For transcriptional activity, STAT4 needs to get phosphorylated on a serine residue via a mitogen activated kinase kinase 6 (MKK6)/p38 dependent pathway[15] leading to expression of IFN-γ and several other genes e.g. Ets related molecule (ERM) transcription factor[16,17]. Via a positive feedback loop, IFN-γ signaling leads to the activation of the Brahma/SW12-related gene (Brg) chromatin remodeling enzyme and thereby changes DNA accessibility for the transcription factors STAT1, interferon regulatory factor 1 (IRF1) and p300 by histone modifications[18-20]. STAT1, for instance will lead to T box expressed in T cells (T-bet) activation, that triggers – supported by NFAT or inhibited by GATA binding protein 3 (GATA-3) - the transcription of many other genes as IL-2Rβ, IL-12R, chemokine (C-C motif) ligand 3 (CCL3), chemokine (CXC motif) receptor 3 (CXCR3), IL-2 and even IFN-γ[11,21-24]. Thereby, the activation of the STAT pathway via IL-12 and TCR mediated signaling pathways leading to the activation of Fos, cJun, activating transcription factor 2 (ATF-2) and NF-κB. The coordinated binding of the transcription factors to the IFN-γ promoter, elicits IFN-γ expression, see Figure 2b.

3.2.2 Interleukin-2

Interleukin-2 (IL-2) is a cytokine that is predominantly secreted by CD4+ naïve T cells and by Th1 effector cells upon stimulation via the T cell receptor and the CD28 costimulatory molecule[8]. It induces proliferation of effector T cells leading to clonal expansion, and was recently reported to be involved in peripheral T_{reg} cell generation, maintenance and survival[25]. Depending on T cell activity and maturity, the IL-2 production and secretion in response to stimulation is differentially regulated. On the one hand, this is caused by developmentally regulated chromatin remodeling

affecting IL-2 promoter accessibility[26]. On the other hand, the activation pattern of IL-2 transcription factors can be modulated upon differential expression of e.g. stimulatory or inhibitory coreceptors, as CD28, inducible T cell costimulatory (ICOS), lymphocyte function-associated antigen 1 (LFA-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), CD7, programmed death 1 (PD-1) or very late antigen 4 (VLA-4)[27,28].

The most prominent transcription factors necessary for IL-2 promoter activation are the inducible transcription factors cNFAT, NF-κB and activator protein 1 (AP-1) consisting of Fos, cJun and the constitutive factor octamer binding transcription factor 1 (Oct-1)[8,26-28]. Recently also p300/ CREB binding protein (CBP) was found to bind to the IL-2 promoter. By association with Fos at Fos binding sites p300/CBP act as coactivator for Fos[29]. T cell receptor/CD3 mediated signaling pathways - modulated by signals from coreceptors - activate all three inducible transcription factors for IL-2 expression, as well as Oct-1 and p300/CBP, see
Figure 3.

Upon interaction with MHCII presented antigen, TCRζ chains, cluster with TCR α and β chains, CD3ε,γ and δ chains, CD4 and other coreceptors to form peripheral microclusters. Thereby the lymphocyte specific protein tyrosine kinase (Lck) gets localized to its site of action, to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs)[27,30]. From today's point of view, Lck, attached to CD4, is activated by dephosphorylation of an inhibitory phospho-tyrosine residue through developmentally regulated transmembrane-phosphatase CD45[27,31,32]. The Lck-phosphorylated ITAMs serve as docking sites for the SH2 binding protein zeta-chain-associated protein kinase 70 (ZAP70) that becomes phosphorylated and thereby activated. Active Zap70 induces several targets such as the scaffold proteins SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells (LAT). These adaptor proteins then recruit kinases, G-proteins and guanidine exchange factors[27]. At this point, the TCR signaling gets strongly amplified and

further integrated with enhancing or inhibitory signals from costimulatory receptors. The costimulatory receptors LFA-1 or CD28, for instance, mainly trigger phosphoinositol or diacylglycerol mediated signaling pathways by recruitment and activation of phospholipases [33-36].

There are three main signaling routes transducing the events from the membrane to the nucleus, leading to IL-2 promoter activation. The signals are forwarded either by increased cytosolic calcium leading to NFAT activation, by the mitogen activated (MAP) kinase pathways leading to AP-1 activation or by activation of NF-κB[27], see
Figure 3.

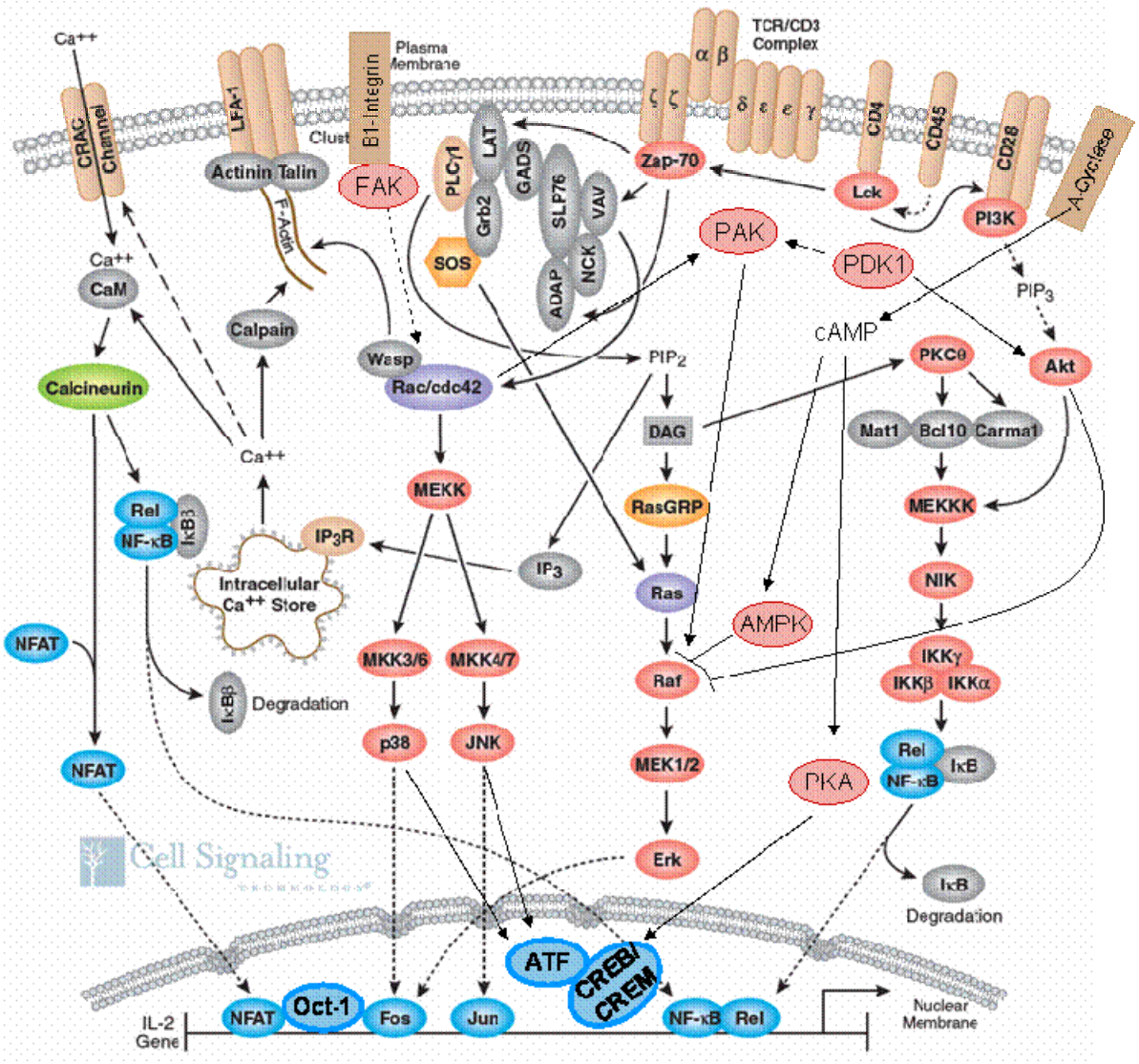


Figure 3: Transcription factors binding to IL-2 promoter

This picture was taken from Cell Signaling Technology and was slightly modified.

By activation of phospholipases C or D (PLC, PLD) via LFA-1 or the T cell receptor, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)[33] are generated. DAG serves as binding site for the protein kinase C (PKC) that will be discussed later.

Via IP3 operated calcium channels, calcium can be released from the endoplasmatic reticulum (ER) to cytosol, which is necessary to mediate calcium dependent mechanisms in the cytosol. Additionally, elevated cytosolic calcium levels lead to the opening of calcium release activated calcium (CRAC) channels at the plasma membrane, which will further increase and prolong intracellular Ca^{2+} levels during TCR stimulation. Upon calcium binding, calmodulin changes its conformation and thus can activate the phosphatase calcineurin. Thereafter NFAT is dephosphorylated by calcineurin and can enter the nucleus to bind to the IL-2 promoter[27].

The activation of the extracellular signal-regulated kinase (ERK) MAP kinase pathway is initiated directly at the TCR/CD3 supramolecular signaling cluster. ZAP70 phosphorylates LAT, thereby a SH2 binding site for the growth factor receptor-bound protein 2 (Grb-2) is created. RAS, recruited to the membrane and is activated via the guanidine exchange factor son of sevenless (SOS) that associates with Grb-2. Via a phosphorylation cascade of MAP kinases Ras triggers ERK phosphorylation, which can then translocate to the nucleus to further phosphorylate Elk. The phosphorylated transcription factor Elk will then activate Fos transcription, a component of the AP1 transcription factor.

Upon recruitment to the microclusters via phosphorylated SLP76, the guanidine exchange factor Vav activates rho GTPases. The rho GTPases induce cytoskeletal rearrangement and the stress activated protein kinases p38 and the cJun N-terminal kinase (JNK). After activating phosphorylation, p38 induces Fos expression and JNK increases the activity of cJun. Fos and cJun heterodimerize to form the AP-1 transcription factor and bind to the IL-2 promoter. Additionally, JNK[37] and p38[38] are capable to activate ATF-2 by phosphorylation. ATF-2

heterodimerizes with either cAMP-response-element binding protein (CREB) to bind to ATF/CRE motifs or with cJun to bind to AP-1 motifs and can thereby modulate the IL-2 production[29,39].

During T cell activation CD28 crosslinking generates an SH2 binding motif[40] for phosphoinositol-3-kinases (PI3K). The major function of PI3K is to create phosphatidylinositols at the plasma membrane, which is essential for the recruitment and activation of phosphoinosite-dependent protein kinase-1 (PDK-1). PDK-1 and DAG in turn recruits the protein kinase C (PKC) and a complex containing the caspase recruitment domain membrane-associated guanylate kinase 1 (Carma1), the B cell lymphoma 10 (Bcl10) and the mucosa associated lymphoid tissue lymphoma translocation protein 1 (MALT1) The Carma1/Bcl10/MALT1 complex induce the E3 ligase activity of TNF receptor-associated factor 6 (TRAF-6)[41]. TRAF-6 ubiquitinates and thereby activates the NF-κB essential modifier (NEMO), the regulatory subunit of the IκB kinase (IKK)[42]. IKK is then phosphorylating the inhibitor of NF-κB (IκB) and leads to IκB's proteolytic degradation. Upon IκB degradation, NF-κB is proteolytically processed to yield active NF-κB fragments that dimerize with Rel proteins, translocate to the nucleus and bind to the IL-2 promoter[27,43,44].

By coordinated binding of the major IL-2 transcription factors cNFAT, AP-1 (Fos/cJun) and NF-κB the IL-2 expression can be stimulated.

3.3 *Immunoglobulin family member CD147*

The type I Ig-like protein CD147 is a 45-65kDa transmembrane glycoprotein – also known as emmprin, hbasigin, HAb18G or M6 - with strongly conserved orthologues in other species called neurothelin, OX-47, gp42, basigin, CE9, 5A11, or HT7[45]. CD147, is known for its important role in activation of the matrix metalloproteinase (MMP) system[46-51] and its interaction with cyclophilins[7,52-56], integrins[7,57-59], monocarboxylate transporters (MCTs)[60-63] and amino acid transporters[6,7,58]. CD147 is

expressed in various tissues and can be upregulated upon T cell activation[1].

3.3.1 Evidence for CD147 influencing T cell activation

There are several indications, that CD147 has an impact on T cell activation. First, Kasinrerk et al. (1992)[1] showed that CD147 is upregulated upon stimulation of naïve lymphocytes, a finding that was later supported by microarray data[64]. Interestingly T cells from CD147 knockout (KO) mice were found to be hyperproliferative. By investigating the effect of CD147 monoclonal antibodies (mAb) on T cell proliferation, Koch et al (1999)[3] identified one CD147 antibody with antiproliferative capacity on human T cells. This CD147 antibody, called MEM-M6/6 is directed to an epitope within the membrane proximal Ig domain (D2), in contrast to the non-inhibitory CD147 antibodies which are directed to the N-terminal Ig domain (D1)[3].
The same antibody was capable of changing the lipid raft composition of human T cells[4]. Furthermore, LFA-1 mediated adhesion of leukocytes was shown by Khunkaewla et al. (2008) to be regulated by CD43 and CD147[5]. Interestingly LFA-1 and the ras related G-Protein Rap1 were suggested to reciprocally modulate adhesion and T cell receptor signaling[34].

To elucidate the mechanism underlying CD147's regulatory function, previous studies in Prof. Stockinger's laboratory already focused on the impact of CD147 on cytokine production in human T cells. A stable knockdown of CD147 was generated in Jurkat T cell line and was analyzed for its cytokine production. Thereby it was found, that CD147 inhibits IL-2 expression and exerts a negative regulatory effect on Th1 cytokine production (IFN-γ), as shown in Figure 4.
These previous findings lead to the idea that CD147 might serve as a mediator of negative feedback regulation during the T cell activation.

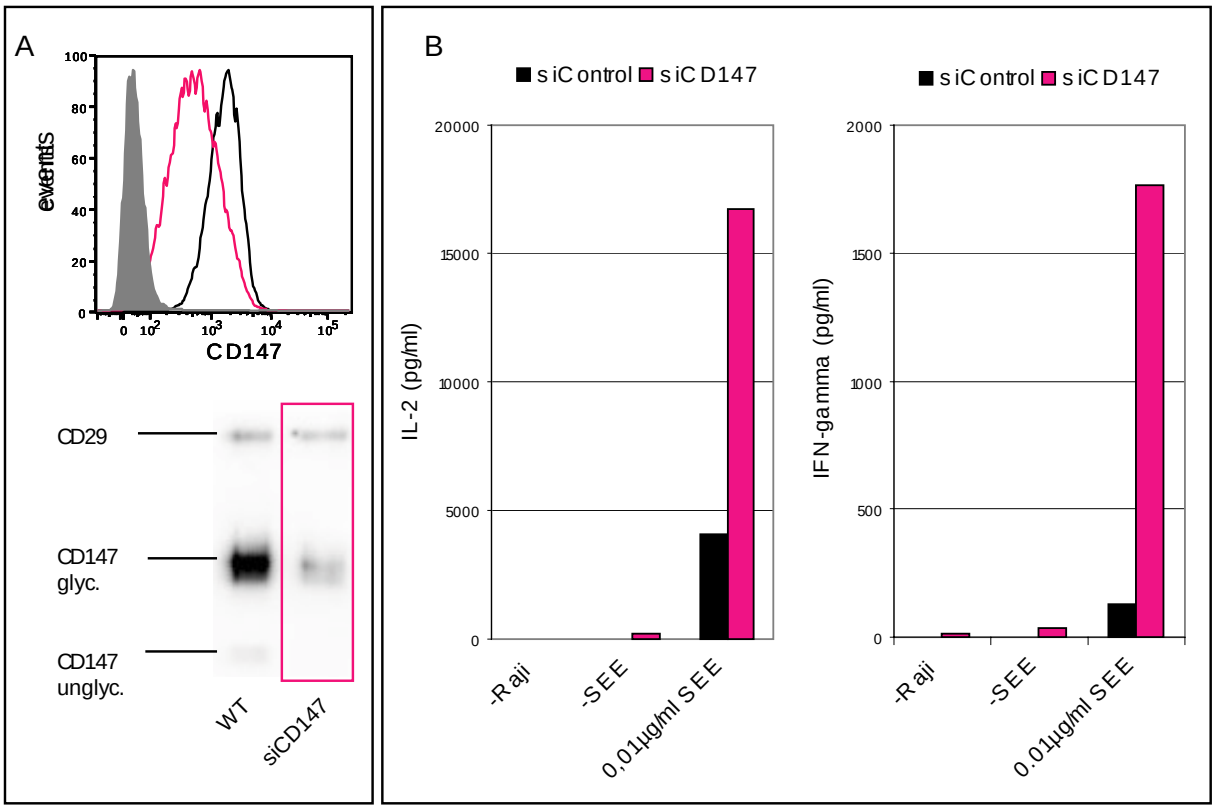


Figure 4: Phenotype of CD147 knockdown Jurkat T cell line. A) Analysis of CD147 silencing efficiency. The upper panel shows flow cytometric analysis of CD147 surface expression (pink: CD147 knockdown, black: sh-control cells, gray (filled): isotype control). Total CD147 expression is shown in the lower panel by Western blot analysis of CD147. CD29 was stained as loading control. B) The IL-2 and IFN-γ production upon CD147 silencing was assessed by ELISA.

3.3.2 CD147 expression, structure, modification and localization

The CD147 gene consists of 7 exons stretching over a length of 7.5kb[65]. CD147 is transcriptionally regulated by the selective promoter factor 1 (Sp1), inducing its expression, and by pinin (pnn), inhibiting its expression[66,67]. After T cell activation, CD147 was reported to be upregulated[1].

By alternative splicing, four different transcript isoforms of 618 to 1158bp in size are generated in man. Three transcripts give rise to CD147 protein isoforms 1, 2 and 4, while the transcript isoform 3 is degraded by nonsense mediated decay mechanism. The longest protein isoform is the isoform 1, which is exclusively expressed in the retina[68] and consists of

385 amino acids. The protein isoform 4 is the shortest CD147 isoform, consisting of 205 amino acids and having a different N-terminus than isoform 1. The CD147 isoform 2 comprises 269 amino acids and lacks the sequence of one exon, without differing in the N- and C-termini from isoform 1. Isoform 2 is the isoform investigated in this study. The N-terminal 200(-300) amino acids of CD147 are exposed extracellulary. Depending on the isoform, the extracellular domain contains two (isoform 2, 4) to three (isoform 1) Ig like domains[68]. Recently, the crystal structure of CD147's extracellular domain (isoform 2) was described. The N-terminal Ig C2-set domain was shown to be flexibly linked (116.4° to 163.0°) to the membrane proximal Ig I-set domain[69]. The I-set domain is similar, but shorter than V-set domains, which were also found in CTLA-4[69,70]. Homophilic interactions were found to take place in a C2-C2 or C2-I mode[69].

The transmembrane domain was calculated to be composed of a 18-23 amino acids containing helix, somewhere between the residue 206-229[55]. The presence of a highly conserved glutamic acid residue and a leucine zipper motive in the CD147's hydrophobic transmembrane region, points to a possible interaction site with various transmembrane proteins[71,72]. The cytoplasmic domain is made up of 40 C-terminal amino acids.

In the Golgi apparatus CD147 becomes glycosylated by N-coupling to the asparagine residues 44 in the membrane proximal Ig domain. and 152 and 186 in the distal Ig domain. CD147 has two glycosylation states: the low glycosylated (LG) form with high mannose type carbohydrate catalyzed by α-mannosidase-II, and the high glycosylated (HG) form with complex type carbohydrate performed by β1,6-*N*-acetylglucosaminyltransferase V. In fibroblasts, the regulation of CD147's glycosylation was shown to be regulated by caveolin[73]. As lymphocytes lack caveolin, the glycosylation of CD147 has to be regulated in another way.

The mechanism, how CD147 is transported to the cell surface, was suggested to be caveolin, cyclophilin and/or MCT dependent.

Caveolin was shown to affect the ratio between LG- and HG-CD147 at the surface. It was shown to bind LG-CD147's membrane proximal Ig domain (D2). Thereby it inhibits the further glycosylation of LG-CD147 to HG-CD147. Furthermore, it was hypothesized, that caveolin shuttles LG-CD147 but not HG-CD147 to the surface[73].

Because CD147 surface expression is highly sensitive to cyclosporine A and to a mutation in proline 211 of CD147, it was suggested, that CD147 interacts with cyclophilins via residue 211 for its surface expression. It was shown that Cylcophilin60 co-precipitates with CD147 and thus might be responsible for its surface expression[54,55]. It was further hypothesized, but not proven yet, that the translocation of CD147 to the plasma membrane needs the peptidyl-prolyl isomerase activity of cyclophilin60[74].

Additionally, the transport of CD147 and MCT's to the plasma membrane was shown to depend on CD147's conserved glutamic acid residue 218 in the transmembrane domain[63].

CD147 was shown to be released from the cell surface either by proteolytic cleavage or by microvesicle shedding and subsequent release during microvesicular degradation[75,76]

3.3.3 Interaction partners and functions of CD147

3.3.3.1 CD147 influencing proteolytic processes

First, homophilic interactions both in cis and in trans were shown for CD147. The CD147 isoform 1, the rare 3 Ig-like domain isoform of CD147 expressed exclusively in the retina, showed high similarity to neuroplastin p65 and could interact homophilic via its extracellular part[68]. Isolated, immobilized CD147 D1 extracellular domain was found to induce adhesion of CD147 transfected COS cells, supporting a homo- or heterophilic interaction in trans[48]. To exert its inhibitory effect on T cell activation, CD147 has to dimerize or to cluster[3,77].

Clustering of CD147 on cancer cells was shown to stimulate matrix metallo proteinase MMP-1 and MMP-2 expression on stromal fibroblasts[46] via a p38 MAP kinase[47], phospholipase A2 and 5' lipoxygenase mediated pathway[50]. It was found, that CD147 N-terminal Ig domain and its glycosylation are necessary for MMP induction[48,49]. These findings make CD147 a prominent MMP activator and thus CD147 is also called EMMPRIN, the extracellular matrix metallo proteinase inducer. Monocyte associated MMPs, induced upon adhesion to fibronectin, were shown to be necessary for transmigration through activated endothelium[78]. Therefore, CD147 upregulation upon T cell stimulation might enable T cells to transmigrate the endothelium, to reach infected areas.

Interestingly, CD147 was reported to be an integral component of the γ-secretase complex, negatively modulating presilin-1 proteolytic activity[79]. The γ-secretase was recently shown to process CD43 sialoglycoprotein[80], an interaction partner of CD147[5]. CD43 was reported to enforce T cell receptor stimulation by interaction with guanidine exchange factor Vav and tyrosine kinase Fyn via its cytoplasmic domain. This leads to the activation of PKC and subsequent Casitas B-lineage lymphoma b (Cbl-b) activation and increased cytosolic calcium concentration[81-84]. Upon removal of CD43's extracellular domain by metallo proteinases, the CD43 fragment is further processed to a transmembrane and a cytoplasmic fragment. The cytoplasmic fragment, harboring a nuclear localization signal (NLS), translocates to the nucleus and binds to β-catenin to increase β-catenin mediated gene expression e.g. c-Myc and CyclinD1[80,85].

3.3.3.2 CD147's function as receptor

As already mentioned before, CD147 was shown to interact with cyclophilins for different purposes.

First, the transport of CD147 from the Golgi apparatus to the plasma membrane is dependent on the interaction with cylcophillin60. Cyclophilin60, localized in the lumen of endoplasmatic reticulum, has

access to CD147's proline 211[54,55], binds to CD147 and serves as its chaperone.

Second, CD147 was suggested to act as a signaling receptor for extracellular cyclophilinA and B, thereby mediating chemotaxis[86]. When CD147 is glycosylated, cyclophilinA can bind to proline 180 and glycine 181 residues in CD147's extracellular domain. Thereby cyclophilin's peptidylproline *cis–trans*-isomerase activity was shown to be necessary for triggering the ERK MAP kinase pathway through CD147[56]. CyclophilinB was shown to promote CD147-Syndecan-1 association followed by induction of the ERK MAP kinase pathway[53]. Recently, cyclophilinB was reported to trigger integrin mediated adhesion via a CD147, CD98 and β1 integrin complex, as will be discussed later[7,87].

Third, an infection with the HIV virus, displaying cyclophilinA on its surface, can be facilitated by a cyclophilinA/CD147 dependent mechanism[52]. Interestingly, this mechanism was shown to be independent from the ERK MAP kinase signaling pathway[88].

3.3.3.3 CD147's role in metabolism

The translocation of the monocarboxylate transporters (MCT-1 and MCT-4), from the perinuclear regions to the plasma membrane was shown to be highly dependent on their interaction with CD147. This interaction takes place via CD147's transmembranous glutamic acid residue 218 and MCT's transmembranous arginine residue[61,63]. Thereby CD147 might play a crucial role in the proper localization of MCT-1 and MCT-4. Additionally, CD147 was also found in the mitochondrial reticulum to interact with MCT-1 and cytochrome oxidase (COX) in a rat skeletal muscle cell line[62]. Cells that are metabolically active, highly producing lactate, have to upregulate monocarboxylate transporter to export lactate from the cell[89,90]. Interestingly, increased lactose concentration in T cells was shown to deregulate the cytotoxic T cell function and cytokine expression[91].

It was suggested, that CD147 and MCTs are components of a greater complex composed of CD98, the large amino acid transporter type 1 light chain (Lat-1), the ASC-system amino acid transporter-2 (ASCT2) and the epithelial cell adhesion molecule (EpCam). CD147 was found to interact via its N-terminal Ig domain (D1) with the extracellular domain of CD98, the heavy chain of the amino acid transporter. Further it was shown, that the CD98 expression positively correlates with CD147 expression and with cell proliferation in fibroblasts[6]. Therefore, the CD147-CD98 complex plays an important role in energy metabolism.

3.3.3.4 Impact of CD147 on cytoskeleton and membrane dynamics

Apart from the energy metabolism, the CD147–CD98 complex plays a role in cell adhesion, when associated with β1-integrins. This complex was shown to mediate cyclophilinB mediated adhesion to fibronectin a in T cell line[7,58,92]. On the one hand, crosslinking of CD98 stimulates the PI3K/protein kinase B (PKB) pathway via the transmembrane domain of integrin β1[7,93,94]. Besides, crosslinking of CD98 promoted anchorage independent growth. On the other hand, cyclophilinB, as mentioned above, triggers CD147-Syndecan-1 association, which leads to the induction of the ERK MAP kinase pathway[53]. This interplay of cyclophilin B and CD147, CD98 and β1-integrins in a complex gives rise to integrin mediated signaling by activation of the PI3K and PKC-δ pathways[7].

Generally, β1-integrin signaling depends on phosphorylation of the β1-integrin cytoplasmic tail and subsequent FAK activation by autophosphorylation[95]. β1 integrins were shown to trigger TCR costimulation by activation of Crk-associated substrate like protein (CasL) via FAK[96,97]. It was shown that CD147 also interacts directly with β1-integrins associated with α3 and α6 light chains[49,57]. So far its thought, that CD147 does not directly interact with β2-integrins, although CD147 has an impact on LFA-1 (CD11α/CD18) activity[5,98]. Recently, Khunkaewla et al. (2008)[5] showed that CD147 stimulates adhesion through LFA-1 by

association with CD43, thereby blocking the CD43 inhibitory effect on LFA-1 mediated adhesion[5].

Lately CD147 was shown to inhibit the NFAT activity by influencing Vav-1/Rac-1 dependent signaling pathway. It was suggested that the cytoplasmic domain of CD147 leads to changes in JNK and the p21 activated kinase (PAK) activity downstream of the Vav-1/Rac-1 signaling pathway[99].

Further interaction partners of CD147, such as caveolin-1, annexin II or enigma, point to an important role of CD147 in membrane dynamics and cytoskeletal rearrangements.

Caveolin is a component of membrane invaginations called caveolae, which can give rise to vesicles to perform endocytosis. Caveolin-1 was shown to interact with low but not with high glycosylated forms of CD147. This interaction was shown to be glycerol dependent and was found in an intermediate sucrose density fraction. Caveolin-1 is thought to shuttle LG-CD147 from the Golgi apparatus to the plasma membrane. By inhibiting glycosylation of LG-CD147 to HG-CD147, caveolin-1 diminishes surface expression and clustering of HG-CD147 accompanied by decreased induction of MMPs[49,73]. Surprisingly, yet no impact of CD147 on caveolae formation was observed.

Moreover, it was reported, that CD147's activity can be regulated by annexin II[100]. Annexins are phospholipid and calcium binding proteins that are known to regulate membrane dynamics and rearrangement of the actin cytoskeleton [101-103].

Enigma, a PDZ and LIM containing scaffold protein, was found by yeast to hybrid screen to interact with CD147. The enigma protein family binds protein kinases via its LIM domains and targets them to actin binding proteins by association with its PDZ domain[104-106]. Interestingly, the enigma family member Z-band alternatively spliced PDZ motif (ZASP) protein was reported to be necessary for the proper assembly of functional integrin adhesion sites[107].

3.3.3.5 Role of CD147 in cell death and proliferation

As already mentioned above, Lymphocytes from CD147 KO mice were hyperproliferative[2], pointing to an antiproliferative effect of CD147. The CD147 extracellular domain presented on phage surfaces induced apoptosis selectively in a monocytic cell line and thus supports a pro-apoptotic function in trans[108].

In contrast, endogenous CD147 was shown to inhibit anoikis in cancer cells by a hyaluronan dependent mechanism. CD147 was shown to stimulate hyaluronan production, leading to increased phosphorylation of FAK, PKB and ERK and the pro-apoptotic protein Bcl associated death promoter (BAD)[109]. Further hyaluronan was shown to trigger ErbB2 mediated anti-apoptotic signaling via interaction with CD44[110]. In a separate study CD147 inhibited anoikis by reducing expression of pro-apoptotic Bcl-2 interacting mediator of cell death (Bim)[111]. Thus, CD147 plays an important role in anchorage independent growth in cancer cells.

3.3.4 CD147's role in pathology

3.3.4.6 Cancer

Cancer is a collective of cells exhibiting deregulated properties in growth and tissue remodeling, leading to a dysfunction of the affected organs. As the cancer grows, it will get short of oxygen leading to hypoxic conditions. Under these conditions, cells produce hypoxia-inducible factor (HIF-1), which mediates the expression of MCT and vascular endothelial growth factor (VEGF)[112]. MCTs export the product of anaerobic glycolysis, lactate, out of the cells and VEGF induces angiogenesis.

Cancerous cells display CD147 upregulation, which correlates with tumor aggressiveness[113,114] and is probably linked to MCT expression. CD147 was also shown to positively influence tumor angiogenesis and the metastatic potential of cancer cells. Thereby, CD147 was found to induce tumor angiogenesis by stimulating VEGF production in an MMP dependent

manner[100,115]. Furthermore, the induction of MMPs by CD147 on cancer associated fibroblasts[47,48] was shown to support the invasive properties of cancer cells[48,116]. Additionally cancer cell invasive potential is further increased by CD147 mediated anchorage independent growth. Thereby CD147, as mentioned before, triggers integrin-like signaling and inhibits components of apoptotic pathway[109-111]. These functions make CD147 a key-mediator in tumor formation and progression.

3.3.4.7 Autoimmune diseases

Systemic lupus erythematosus (SLE) is an autoimmune disease based on hyper reactive lymphocytes and increased autoantibody production against nuclear components. It's typical characteristics are inflammatory lesions in the joints, skin, kidneys and the nervous system[117]. Recently, SLE patients were found to express CD147 to a higher level in CD3+ cells in contrast to healthy donors. Peripheral blood mononuclear cells (PBMCs) of SLE patients displayed increased activity upon treatment with the CD147 antibody MEM-M6/1 in combination with CD28 and CD3 antibodies. Additionally, MMP-9 production was upregulated in SLE patients. Thus CD147 was suggested to play a role in systemic lupus erythematosus by mediating TCR signaling and MMP-9 production[118].

3.3.4.8 Alzheimer's disease

Alzheimer's disease is characterized as a cognitive decline that can lead to dementia. This characteristics result from neuronal cell death caused by non degradable β-amyloid plagues and neurofibrillary tangles from Tau protein. Interestingly, CD147 knockout mice showed similar deficits in spatial learning and memory as found in Alzheimer mouse model[119,120]. First, it was suggested, that the amount of extracellular β-amyloid is regulated by clearance mechanisms through proteinases and peptidases[121]. Later, data came up, that CD147 directly interacts with and

thereby inhibits γ-secretase complex activity, in HELA cells[79,122,123]. Recently, CD147's regulatory function in β-amyloid plaque formation was clearly formulated new. The γ-secretase activity, measured by the formation of the intracellular cleavage fragment, was shown to be independent from CD147 expression level. Instead, β-amyloid stability was found to be decreased in supernatants of CD147 overexpressing cells. Thus, this results support the earlier hypothesis that CD147's MMP inducing function is mediating β-amyloid degradation[124,125].
Furthermore, it was found, that the CD147/α6β1-integrin complex stimulates microglia cell activation upon β-amyloid peptide binding[126].

3.3.4.9 Heart disease

As described earlier, CD147's most prominent function is the induction of MMPs. MMPs were shown to be important for the breakdown of collagen in the extracellular matrix surrounding the vessels. Thereby, CD147 is thought to mediate the turnover of collagen, thus inhibiting accumulation of collagen in deposits, leading to hypertension.
However, exactly this feature of CD147 can lead to congestive heart failure. Deregulated degradation of collagen in the left ventricle leads to loss of organization of the cardiomyocytes along the collagen matrix, leading to dysfunctional pumping of the heart[127].
Second, MMPs produced by cardiovascular cells were also shown to cause acute myocardial infarction, also known as heart attack[128]. Increased CD147 expression on monocytes was found to trigger MMP production by cardiovascular smooth muscle cells. Thereby MMPs are thought to destroy atherosclerotic plaques leading to leakage in the hearts blood supply.

3.3.4.10 Stroke

As mentioned before, MMPs have an important role in the clearance of collagen deposits, decreasing the possibility of vessel occlusion. Still occlusions occur within the vessels leading to loss of oxygen supply of affected tissues. Under hypoxic conditions, cells upregulate MCTs to export the anaerobic glycolysis product lactate. The surface expression of CD147 correlates with that of MCTs, as CD147 is necessary for the transport of MCTs from the Golgi apparatus to the plasma membrane. The upregulation of CD147 induces MMP activity that was shown to degrade extracellular matrix. Thereby CD147 mediated MMP induction further increases the damage of the brain tissue upon vessel occlusion[129,130].

3.4 *Aim of the study*

In this study, I aimed to elucidate the molecular mechanism(s) involved in the negative regulatory function of CD147 during T cell activation:

First, to determine the immunomodulatory subdomain of CD147, several siRNA resistant swap and deletion mutants of CD147 were tested for their impact on cytokine expression upon T cell stimulation.
Second, I wanted to studied potential lateral or intracellular interaction partners. Thus, I silenced these potential interaction partners and analyzed the cytokine response of the silenced T cells. Further, I wanted to figure out, which signaling pathway linked to cytokine expression could be affected by CD147. Therefore, I biochemically analyzed signaling components influencing the IL-2 promoter activity down-stream of the TCR and CD147's interaction partners in CD147 silenced Jurkat T cells.
With the help of co-immunoprecipitation, I wanted to prove the interaction of CD147 and its interaction partner(s).

The final goal of the study was to characterize the mechanism underlying CD147 function to find new drug targets for therapeutic modulation of the adaptive immune response.

4 Material and Methods

4.1 *Antibodies*

The following monoclonal mouse antibodies were kindly provided by Dr. Vaclav Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic): AFP-01 to alpha-fetoprotein, MEM-83 to CD11α, MEM-154 to CD16, MEM-48 to CD18, MEM-101A to CD29, MEM-59 to CD43, MEM-156 to CD98 and MEM-M6/1, MEM-M6/3 and MEM-M6/4 to CD147. The mouse antibodies clone Leu28 to CD28 and clone 5 to annexin II were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). The polyclonal rabbit antibodies specific for phospho-Lck (Tyr416), phospho-Lck (Tyr505), phospho-Akt (Thr308), phospho-Akt (Ser473), phospho-GSK3β (Ser9), p44/42 (=ERK1/2), phospho-p44/42 (Thr202/Tyr204), phospho-Raf (Ser296), phospho-Raf (Ser259), phospho-Raf (Ser338), cRaf, phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), phospho-GSK-beta (Ser9), phospho-PAK1 (Thr423) PAK2 (Thr402), phospho-PDK1 (Ser241), FAK, α/β-Tubulin, as well as the monoclonal phospho-IκB (Ser32) (clone 14D4), phospho-NF-κB (Ser536) (clone93 H1) and Zap70 (clone 99F2) were purchased from Cell Signaling (Danvers, MA, USA). The monoclonal mouse antibody specific for NFATc2 (clone 4G6-G5) was from Santa Cruz Biotechnology (Santa Cruz, California, USA). The polyclonal rabbit antibody against phospho-FAK (Tyr397) was obtained from Invitrogen (Inchinnan Business Park, Paisley, UK). The polyclonal rabbit antibody against phospho-paxillin (Tyr118), the clone2A7 to FAK and the clone HB1.1 to CD29 were purchased from Millipore (Billerica, MA, USA). The monoclonal mouse antibody α-Flag (Clone M2), the polyclonal rabbit antibody α-actin and the goat α-mouse IgG antibody were purchased from Sigma (St. Luis, MO, USA). The Lck antibody Lck-01 was from Abcam (Cambridge, MA, USA). For blocking of Fc-Receptors, Beriglobin from Aventis Behring (King of Prussia, PA, USA) was used.

The following antibody fluorophor conjugates were used for analysis by flow cytometry: goat α-mouse IgG+IgM (H+L) FITC labeled conjugate from "An der Grub" (Kaumberg, Lower Austria, Austria) and APC-conjugated AffiniPure F(ab')2 fragment goat α-mouse IgG + IgM (H+L) conjugate from Jackson Immuno Research (West Grove, PA, USA) and directly labeled conjugates α-IL-2-APC (MQ1-17H12) and IgG2a-APC from eBioscience (San Diego, CA, USA).

Secondary antibodies used for chemiluminescence were horseradish peroxidase (HRPO)-labeled IgG antibody conjugates goat α-rabbit-HRPO (Biorad, Hercules, CA, USA) and goat α-mouse-HRPO (Sigma, St. Luis, MI, USA). For analysis of immunoblotting by "Odyssey - infrared imaging system" (Li-cor Bioscience, Lincoln, Nebraska, USA) the following secondary fluorophore-labeled IgG antibody conjugates were used: Alexa Fluor 680 labeled goat α-mouse antibody (Invitrogen, Paisley PA4 9RF, UK), IRDye 800CW conjugated goat α-rabbit antibody (LI-COR Bioscience, Lincoln, Nebraska, USA).

4.2 *Reagents*

Complete protease inhibitor cocktail tablets were purchased from Roche (Basel, Germany). Imidoester crosslinker dimethyl 3,3'-dithiobipropionimidate 2 HCl (DTBP) was from Pierce (Rockford, IL, USA).

Benzonase nuclease (≥250 units/µL), polybrene (hexadimethrine bromide), chloroquine diphosphate salt, puromycin dihydrochloride from *S. alboniger*, phorbol 12-myristate 13-acetate (PMA), ionomycin calcium salt (Iono) from *S. conglobatus*, brefeldin-A and monensin sodium was purchased from Sigma (USA). Staphylococcal Enterotoxin E (SEE) was from Toxin Technology (Sarasota, FL, USA) and albumin fraction V was purchased from Roth (Karlsruhe, Baden-Württemberg, Germany).

4.3 *Cells*

The leukemia human Jurkat T cell lines, Jurkat E6.1, Jurkat IL-2-luciferase and Jurkat J14 SLP-76-Flag (kind gift of Oreste Acuto, Sir William Dunn School of Pathology, Oxford, UK) and the lymphoma human Raji B cell line were maintained in RPMI-1640 supplemented with 10% FCS, 5% glutamine, 5% penicillin & streptomycin (stock: 10.000IE/ml, 10mg/ml, respectively). Phoenix cells, a derivative of the human embryonic kidney cell line 293, were maintained in DMEM with 10% FCS, 5% glutamine, 5% penicillin & streptomycin.

4.4 *Cryopreservation of cells*

Stocks were prepared in medium with 30% FCS and 10% DMSO. They were then slowly frozen at -20°C for 4-10h, afterwards placed at - 80°C for 1 days and then stored in liquid nitrogen. If needed, cells were thawed by slowly adding prewarmed 10ml medium. After centrifugation and aspiration of the DMSO-containing freezing-buffer, cells were then incubated in 10ml medium in a standing 25cm² tissue culture flask. After 1 day, the medium was changed.

4.5 *Plasmids*

4.5.1 Lentiviral vectors

pLKO-CD147_333 (‘siCD147’), pLKO-CD98hc (‘siCD98’), pLKO-CD28p (‘siCD28’), pLKO-CD43 (‘siCD43’) containing short hairpin RNA (shRNA) construct specific for human CD147, CD98, CD28 and CD43 with puromycin resistance, were generated in Prof. Stockinger’s laboratory. pLKO-puro_ntCtr (‘siControl’), the non-target shRNA construct, 5bp mismatches to any known human and mouse gene, with puromycin resistance was from RZPD (Mountain View, CA, USA).
psPAX, the lentiviral packaging plasmid and pMD2.G, the envelope (VSV) was purchased from Addgene (Cambridge, MA, USA)

4.5.2 Retroviral vectors

Several CD147 deletion mutants or tagged CD147 forms were cloned into the retroviral pBMN-I-GFP vector backbone:

- full length CD147 with mutated wobble bases in siRNA postion 333 making it siRNA resistant (pBMN-I-CD147m)
- cytoplasmic tail deleted CD147, siRNA resistant (pBMN-I-CD147m-dcyt),
- CD16 extracellular domain, CD147 transmembrane and cytoplasmic domain chimera (pBMN-I-16:147TC),
- CD16 extracellular domain, CD7 transmembrane domain, CD147 cytoplasmic domain chimera (pBMN-I-16:7T:147c),
- CD147 extracellular domain, CD7 transmembrane domain chimera (pBMN-I-147:7t),
- full length CD147, siRNA resistant, N-terminal Flag-tagged (pBMN-I-Flag-CD147),
- full length CD147, siRNA resistant, C-terminal Flag-tagged (pBMN-I-CD147-Flag),

pgag-pol, the retroviral packaging plasmid without env and pMD2.G the envelope plasmid were from Addgene (Cambridge, MA, USA).

4.6 *Plasmid preparation*

To make the E.coli strain DH5alpha competent for transformation the KCM method was performed. Therefore, DH5alpha were cultured overnight in 500ml LB to an OD600 of 0.5. Then the bacteria were chilled for 2’ in an ice bath. After centrifugation for 10 at 4000g and 4°C, cells were resuspended in 50ml ice cold TSS (10% v/v PEG, 5% v/v DMSO, 20mM $MgSO_4$ in LB Medium, pH 6.5), aliquoted into prechilled eppendorf tubes and snap frozen in liquid nitrogen. The aliquots were stored at -80°C.

Competent DH5alpha (KCM method) were transformed with plasmids needed for virus production. Thus 100µl competent bacteria were incubated with the plasmids (10-100ng) and 100µl 1x KCM Buffer (0.1M KCl, 0.03M $CaCl_2$, 0.05M $MgCl_2$) for 10’ on ice and for 10’ on room temperature. For recovery, the bacteria were shaken for 1h at 37°C in

500µl LB-media. Afterwards 100µl bacteria were plated on a LB/Amp plate and incubated at 37°C. Single colonies were used to inoculate 5ml LB/Amp and prepare an overnight culture. Then 400 µl of the pre-culture were used to inoculate 200ml LB/Amp in a 1 l Erlenmeyer flask. After culturing over night, the bacteria were harvested and the plasmids were isolated by 'Genopure Plasmid Maxi Kit' (Roche, Basel, Germany) according to the manufacturers 'procedure for high copy number plasmids'.

4.7 *Virus production*

The day before transfection, Phoenix cells were split in 10cm tissue culture plates at $3.5x10^6$ cells per plate. The next day Phoenix cells were transfected with three different plasmid vectors:

a) plasmid containing the expression cassette (siRNA, CD147 deletion mutants,…) for transduction,

b) plasmid coding for envelope proteins,

c) plasmid coding for replication and packaging proteins.

For transfection of one 10cm dish of cells with lentiviral plasmids, 10µg pLKO-puro1, 5µg pMD2.G and 7.5µg psPAX2 were used. For the transfection with retroviral plasmids 10µg pBMN-I-GFP, 9µg pgag-pol and 3µg pMD2.G were applied. The plasmids were added to 1ml of 250mM $CaCl_2$. To generate precipitates 1ml 2xHBS was added drop wise to the DNA mixture while bubbling with a Pasteur pipette using a pipette boy. Bubbling was continued for additional 15''. To neutralize lysosomal DNases to avoid DNA degradation, 25µM chloroquine was added to the cells 5' before transfection. Then the transfection mixture was added to the Phoenix cells trying to disseminate the drops all over the 10cm tissue culture plate. The plates were shaken carefully before placing back to 37°C. The precipitates were checked under the microscope 30' after addition of transfection mix. The medium was exchanged 4h later by 5ml fresh medium.

The virus containing supernatant was harvested after 36-48h. The supernatant was centrifuged for 5’ at 300g at 4°C and afterwards filtered (pore size 0.45µm). The virus containing supernatants either were used immediately or were aliquoted and stored at -80°C.

4.8 *Infection*

The cells (3-6 x 10^6/well in 0.5ml in 6-well plate) were incubated with the same volume of virus suspension and with polybrene (10µg/ml) over night at 37°C. The next day the cells were washed and transferred to a 25cm² tissue culture flask. Upon infection with lentivirus, the cells were selected for siRNA expressing cells for 2 days by addition of 1µg/ml puromycin. Afterwards, cells were transferred to 75cm^2 tissue culture flask and used for experiments from day 7 to day 10 post infection, after confirmation of silencing or expression of CD147 deletion and swap mutants by flow cytometry.

4.9 *Immunofluorescence analysis and flow cytometry*

4.9.1 Cell surface staining

During the cell surface staining procedure every step was carried out on ice. Cells were first washed with ice cold staining buffer (1xPBS, 1%BSA, 0.02% sodium azide) and then, 1-3x10^5 cells were incubated for 30’ with 25µl staining buffer containing 2% Beriglobin for blocking Fc-receptors. For the staining, 25µl of this cell suspension (1-3x10^5) were transferred to a well of a 96-well plate (V-bottom). Then primary antibodies in staining buffer were added to a final concentration of 10µg/ml. After 30’ incubation, cells were washed once with staining buffer and incubated with FITC-labeled secondary α-mouse antibodies (concentration determined by titration) for 30’. Afterwards cells were washed 3 times with staining buffer and resuspended in 40µl staining buffer for flow cytometry analysis with LSRII from BD Bioscience Pharmingen (Franklin Lakes, NJ, USA).

4.9.2 Intracellular staining

Jurkat NFAT-luc cells were stimulated for 5h with PMA/Iono (10ng/ml, 1µM). After 2.5h of stimulation, 1.5µM monensin was added to the cells to stop cytokine secretion. After 5h stimulation, the cells were washed twice with PBS on ice and fixed with 4% PFA for 15' at room temperature. Afterwards, cells were washed again and permeabilized with PBS/0.1% saponin for 15' at room temperature. Then the cells were blocked with staining buffer (PBS/0.1%Saponin/5%FCS) for 5'. Upon blocking, the cells were transferred to a 96-well v-bottom plate (25µl/well) and stained with α-IL-2-APC (5µg/ml antibody conjugate in staining buffer). Afterwards, the cells were washed with and resuspended in staining buffer for flow cytometry analysis with LSRII from BD Bioscience Pharmingen (Franklin Lakes, NJ, USA).

4.10 *Preparation of cell lysates*

The cells were washed with washing buffer (20mM Tris-HCl, 150mM NaCl, 5mM EDTA) and centrifuged for 5' at 300g. The cell pellet was lysed in Lämmli-Buffer and shock frozen in liquid nitrogen. The lysates were then either stored at -20°C or used directly for further analysis.

4.11 *SDS-PAGE*

If reducing conditions were needed 1.5% β-mercaptoethanol was added to lysates. Samples were boiled for 5' at 95°C and loaded on 7.5 - 10% polyacrylamide gels. Electrophoresis was performed at 120V in PerfectBlue^{TM}Doppelgelsystem Twin ExW S (PEQLAB Biotechnologie GmbH, Germany). The polyacrylamide gels were either silver stained or used for Western blot analysis.

4.12 *Silver staining*

'SilverSNAP Stain for Spectrometry Kit' from Pierce (Rockford, IL, USA) was used for silver staining of the gels.

4.13 *Western blot analysis*

Polyacrylamide gel was semi-dry blotted in Western blot buffer (25mM Tris base, 200mM glycine, 20% methanol) to Immobilon-P or Immobilon-Fl transfer membranes (pore size 0.45µm) from Millipore (Billerica, MA, USA). Blotting was performed for 1h with constant current (2mA/cm^2 gel) in PerfectBlue^{TM}Semi-Dry-Electroblotter 'SEDEC M' from PEQLAB Biotechnologie (Erlangen, Germany). Afterwards, the membranes were blocked for 1h with 5% w/v non-fat dry milk in TBST (20mM Tris base, 150mM NaCl, pH7.6, 0.5‰ Tween 20) containing sodium-orthovanadate to avoid dephosphorylation of the proteins. Then they were incubated overnight with primary antibodies with 2.5% milk in TBST at 4°C. All primary antibodies were diluted 1:1000 in 2.5% milk in TBST, with exception of the α-cNFAT antibody, that was diluted 1:500 and α-actin antibody was used in a 1:2000 dilution. The next day membranes were washed 4 times for 5' with TBST and were incubated with HRPO or fluorescence-labeled secondary antibodies in 2.5% milk in TBST for 1h at room temperature. Afterwards, the membranes were washed 6 times for 5' and were either treated with luminol and were exposed to films or were analyzed by "Odyssey - infrared imaging system" from Li-cor Bioscience (Lincoln, Nebraska, USA). Upon stripping the membrane, it was blocked and reprobed for other proteins.

4.14 *Stripping at low pH*

The membrane was stripped for 30' with pH-Strip-Buffer (5,84g NaCl, 7,59 glycine, up to 0.5L, pH2.5) and was afterwards washed 4x10' with TBST.

4.15 *Luciferase reporter gene assay*

For this assay the '*Luciferase Reporter Gene Assay, high sensitivity - chemiluminescent assay for the quantitative determination of firefly luciferase activity in transfected cells'* from Roche (Basel, Germany) was used.

Jurkat IL-2-luciferase cells ($2x10^5$ cells/well) were stimulated with PMA/Iono (10ng/ml, 1µM) for 7h or with SEE pulsed Raji B cells ($2x10^5$ cells/well) for 7h and 24h in a 96-well plate. Raji B cells were pulsed with 100ng/ml SEE for 2h, washed twice and were then added to the T cells.

After stimulation, the cells were lysed with lysis buffer on ice for at least 30'. Debris was spun down under 16000g at 4°C and 100µl lysate was transferred to a special 96-well plate (for luminescence reader) on ice. Luciferase assay reagent (10µl/well) was added and mixed properly by rocking the plate. Luminescence was measured by liquid scintillation and luminescence reader Microbeta 1450 from Wallac (Turku, Finnland).

To normalize the samples, the protein concentration was determined by using a Bradford assay from Biorad (Hercules, CA, USA). Extinction was measured at 595nm.

4.16 *Immunoprecipitation*

First, a 96-well ELISA plate was coated with 10µg/ml goat α-mouse antibody for 2h at 37°C in PBS (pH 8.7). Then the plate was washed twice with PBS. Then eight wells each were incubated with one of the following antibodies (5µg/ml of α-AFP, α-Lck, α-FAK, or CD29 antibodies or with 10µg/ml of CD147 antibodies) for 2h at 37°C in PBS (pH8.7). Afterwards, the plate was washed twice, was blocked with PBS/1%BSA for 1h at 37°C. Jurkat IL-2-luciferase cells were washed once with PBS and $9x10^7$ were incubated with or without 500µg/ml of the crosslinker DTBP in PBS (pH8) for 30' at 4°C. Afterwards, the cells were washed once with PBS and once with 0.1M SPB pH 7.4. The cells were lysed in lysis buffer ($4.5x10^7$/ml) with detergent and inhibitors (0.02M SPB, 1% NP-40, 1x Complete, 1mM

orthovanadate, 50mM NaF, 2µl/ml benzonase) for 30’ at 4°C. Upon lysis the cell debris was spun down, 100µl aliquots of lysates were stored at -20°C and 50µl/well were transferred to the coated plates. The plate was shaken over night at 4°C. The next day the plate was washed 2-3 times and precipitate was eluted with 10µl Lämmli-buffer/well at 95°C.

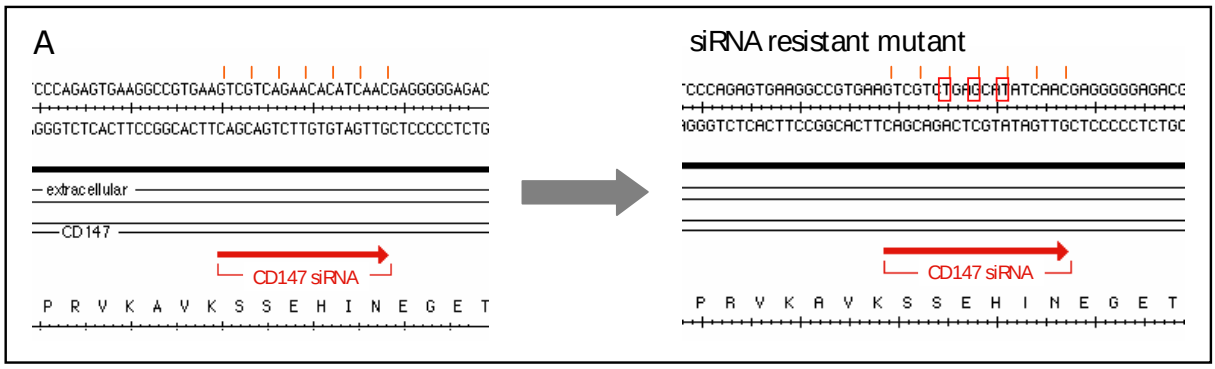
4.17 *Flag Immunoprecipitation*

Twenty million cells were stimulated in 3ml RPMI/10%FCS with PMA/Iono (10ng/ml, 1µM) for 0’ and 5’ and then washed once with washing buffer (20mM Tris, 150mM NaCl, 5mM EDTA). The cells were lysed for 30’ in lysis buffer ($2x10^7$/ml) with detergent & inhibitors (1%NP40, 1x Complete, 1mM orthovanadate, 50mM NaF, 20mM Tris, 150mM NaCl, 2mM EDTA) at 4°C. In the meanwhile, 50µl Flag-beads/$2x10^7$ cells (α-Flag m2 affinity gel, monoclonal IgG1) from Sigma (St. Luis, MO, USA) were washed 4 times with lysis buffer and once with lysis buffer with detergent & inhibitors. From the lysates, 100µl aliquots were stored at -20°C and the rest was kept in rotation with the beads for 1h at 4°C. Afterwards, 100µl aliquots from supernatant were stored at -20°C and the beads were washed twice with lysis buffer with detergent and inhibitors. Precipitate was eluted in two steps with 100µl elution buffer (50mM glycine in PBS pH2.7, 0.65% Tween-20, 1x Complete) for 5’ at room temperature.

5 Results

5.1 *Searching for the domain(s) of CD147 involved in negative regulation of T cell activation*

We aimed to define the functional structure of CD147 antagonizing T cell stimulation with the help of 5 differently composed deletion and swap mutants of CD147 (Figure 5) generated by Dr. Wolfgang Paster.



B

CD147 constructs	Description	Name
CD147e CD147t CD147c	siRNA resistant form of CD147	CD147etc
CD147e CD147t	siRNA resistant, cytoplasmic deletion mutant of CD147	CD147et
CD16e CD147t CD147c	CD16 extracellular domain / CD147 transmembrane and cytoplasmic domain swap mutant	CD16e/CD147tc
CD16e CD7t CD147c	CD16 extracellular domain / CD147 transmembrane and CD7 cytoplasmic domain swap mutant	CD16e/CD7t/CD147t
CD147e CD7t	siRNA resistant CD147 extracellular domain / CD7 transmembrane domain swap mutant	CD147e/CD7t

Figure 5: CD147 constructs resistant to short hairpin RNA. A) CD147 constructs were resistant to siRNA due to mutations of three wobble bases in the siRNA targeted sequence without changing the amino acid sequence. (Whither RNAi? (*Nature Cell Biology* 5, 489 - 490 (2003)). B) Composition of CD147 constructs used for overexpression and reconstitution experiments.

The constructs, were composed of CD147 domains combined with CD16 extracellular and CD7 transmembrane domains (swap mutants). The CD7 transmembrane domain was used to localize CD147 intra- or extracellular domains to the cell membrane. CD7 belongs to the superfamily of the immunoglobulins and is expressed upon T cell stimulation. Similar to CD147, it is excluded from lipid rafts, and is therefore an ideal transmembrane domain substitute, to achieve a physiologically relevant localization of the CD147 constructs in the cell membrane[131]. The extracellular domain of CD16, a Fc-receptor not expressed in Jurkat T cells, was used to make the CD147 transmembrane and cytoplasmic domain constructs detectable by flow cytometry upon cell surface staining. The expression level of all CD147 constructs, was also visualized by internal ribosome entry site (IRES) mediated expression of green fluorescent protein (GFP), which could be analyzed by flow cytometry in addition.

5.1.1 Overexpression of CD147 deletion and swap mutants

In a first approach, the expression level of the CD147 constructs was determined. Therefore, the full length CD147, a CD147 cytoplasmic deletion mutant and a CD147 extracellular domain/CD7 transmembrane swap mutant were overexpressed in Jurkat IL-2-luciferase cells. For this purpose, retroviruses were generated to transduce the CD147 constructs stably into the Jurkat cells. The Jurkat IL-2-luciferase cells were once, twice or thrice infected, to define optimal condition - where the cell viability is still unaffected and the construct expression level is the highest. Cell viability after infection was estimated by light microscopy and analyzed by flow cytometry using 7-AAD staining. To examine the construct expression levels, the cells were analyzed by flow cytometry or by Western blot (Figure 6). The analysis by flow cytometry revealed that the CD147 construct expression level could be increased by repeated infection from 30% up to 75% GFP positive cells.

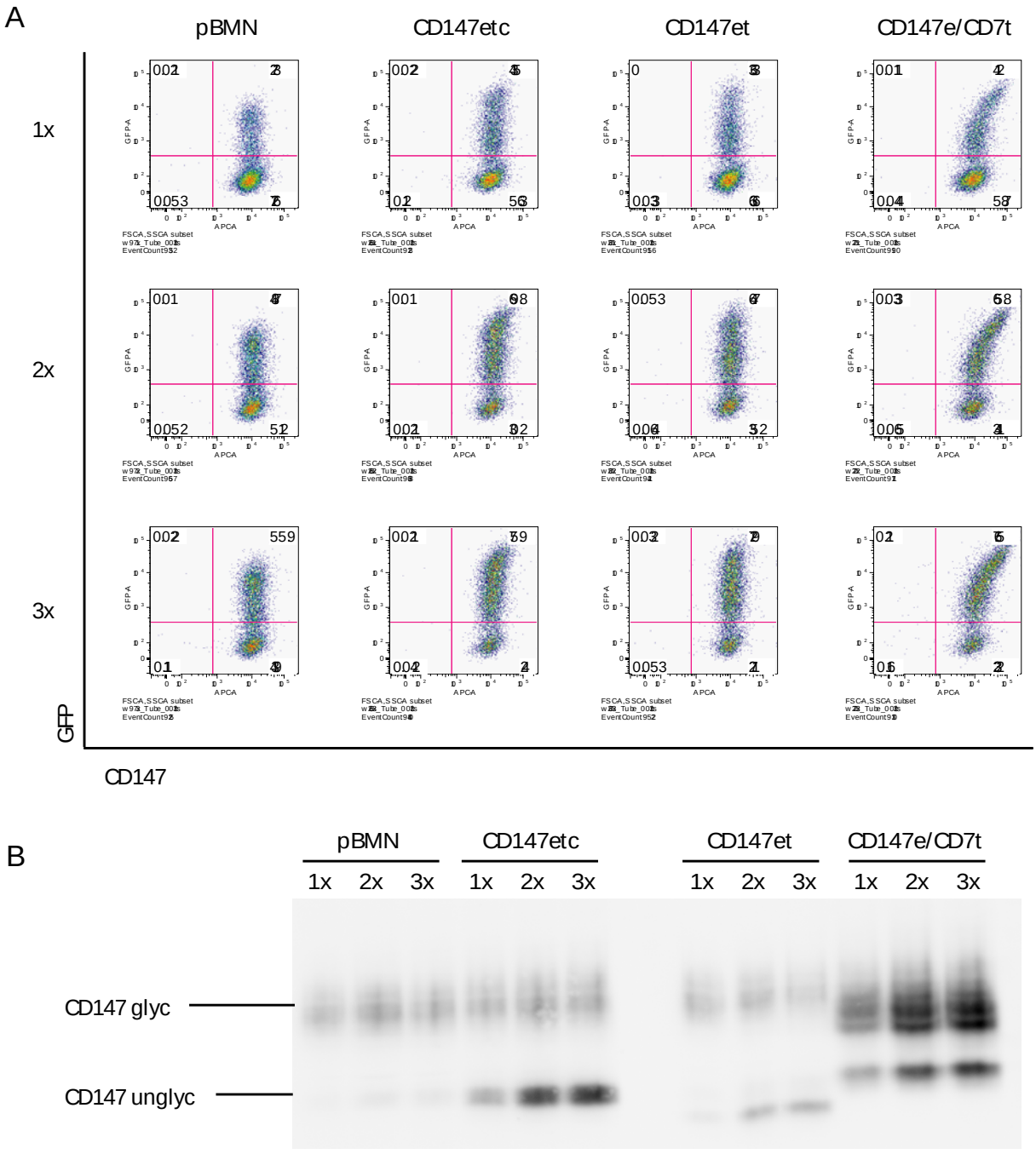


Figure 6: Overexpression of CD147 constructs A) Jurkat IL-2-luciferase cells, once to thrice transduced with CD147 constructs, were analyzed for their GFP expression and CD147 expression upon cell surface staining with MEM-M6/1. B) Cell lysates were investigated for CD147 expression level by non-reducing immunoblotting using CD147 mAb MEM-M6/1 and detection by chemiluminescence.

Interestingly, CD147 constructs containing the CD147 transmembrane domain could not be overexpressed on the cell surface in contrast to the swap mutant containing the substitute transmembrane domain from CD7 (Figure 6a). Moreover, we could show by Western blot analysis that the overexpressed CD147 constructs accumulated in the unglycosylated or low glycosylated form corresponding to a molecular weight of about 30kDa in size. Only the CD147e/CD7t construct, which appeared overexpressed at the cell surface accumulated also in the fully glycosylated form (Figure 6b). The substitution of the CD147 transmembrane domain by the CD7 transmembrane domain resulted in further glycosylation of the CD147e/CD7t, as the intensity of the 40-60kDa immunoreactive band for HG-CD147 increased with the level of overexpression. These results are in part consistent with the data published by Pushkarsky et al. (2005)[54] and Yurchenko et al. (2005)[55] in so far, that the transmembrane domain of CD147 might regulate the surface expression. Interestingly, according to our data the transmembrane domain might be crucial for regulation of the grade of glycosylation and not the extracellular domain, as was presented by Tang et al. (2004)[73]. Probably, CD147's transmembrane domain determines the glycosylation of CD147's extracellular domain and thereby its surface expression.

5.1.2 Clones overexpressing CD147 deletion mutants

To increase the probability for our CD147 deletion and swap mutants to become expressed at the plasma membrane, the expression level had to be further increased. Therefore, Jurkat IL-2-luciferase cells were freshly transduced with empty vector, CD147etc, CD147et, CD16e/CD147tc and CD16e/CD7t/CD147c. The cells were sorted for high GFP expression level and then clones were generated by single cell culturing. Upon flow cytometry analysis, two clones per CD147 construct displaying the highest GFP expression were selected for further analysis.

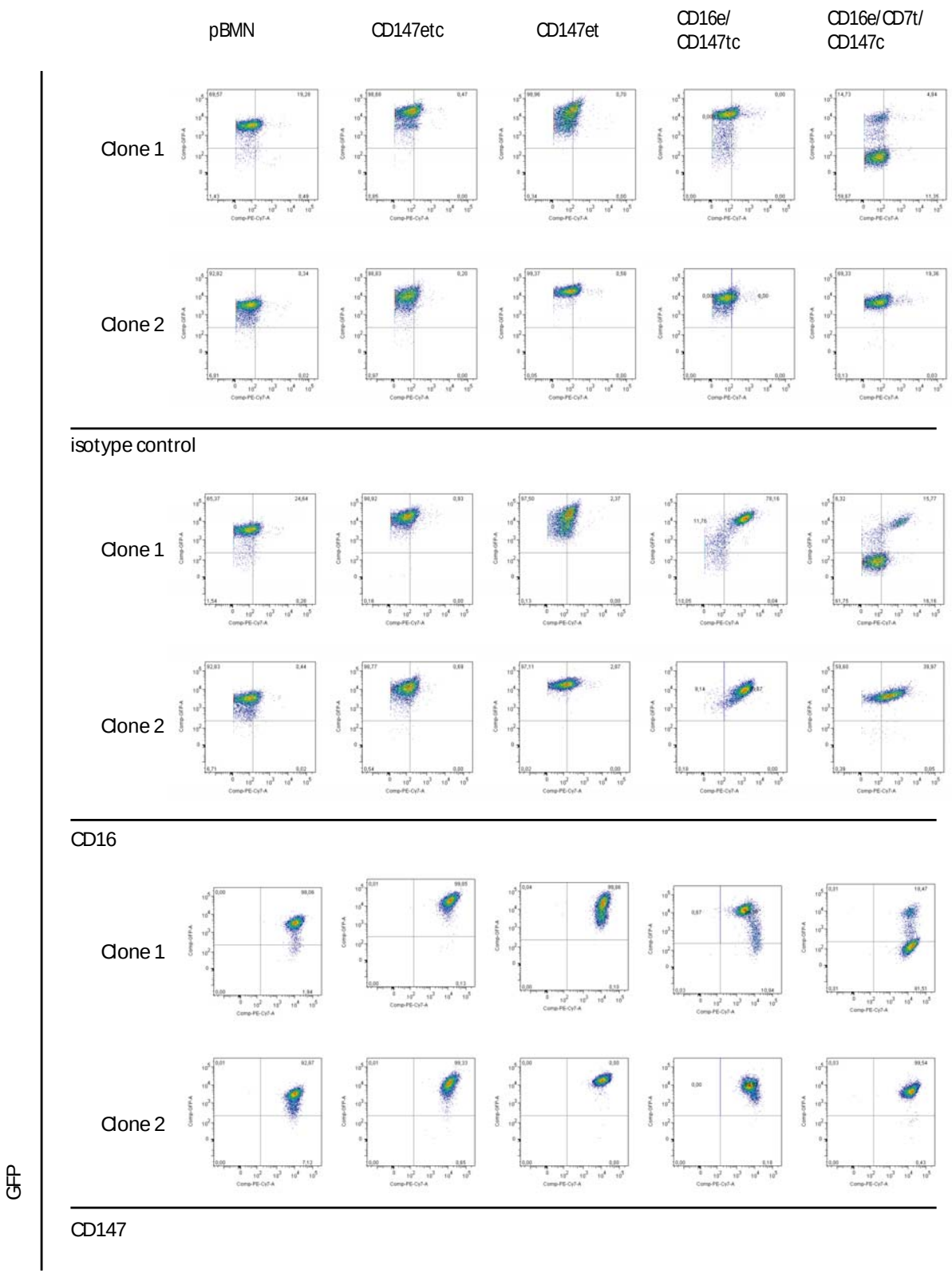


Figure 7: CD147 constructs clones. Analysis by flow cytometry of CD147 construct clones by GFP expression (y-axis) and surface expression of the deletion and swap mutants of CD147 construct clones by cell surface staining using mAbs MEM-154 to CD16 and MEM-M6/1 to CD147 (x-axis).

The clones were investigated by flow cytometry for CD147 and CD16 surface expression and again for GFP expression level (Figure 7). Surprisingly, one clone, CD16e/CD7t/CD147c_1, lost its GFP expression completely. All other clones still displayed high GFP and thereby high CD147 construct expression. By cell surface staining for CD16 extracellular domain, we could proof the expression of CD16 / CD147 swap mutants at the plasma membrane. However, the CD16 staining for construct CD16e/CD147tc was much higher than for the construct CD16e/CD7t/CD147c. Interestingly, the expression of the construct CD16e/CD147tc containing the CD147 transmembrane domain led to a decrease of endogenous CD147. The expression of the construct CD16e/CD7t/CD147c, lacking CD147 transmembrane domain, had no effect on endogenous CD147 surface expression level. This implies that the CD147 expression level at the plasma membrane is tightly regulated by the CD147 transmembrane domain.

To test the effect of the constructs on the IL-2 promoter activity, the clones were stimulated for 20 hours with PMA and ionomycin and analyzed by reporter gene assay (Figure 8). Since silencing of CD147 caused an increased IL-2 promoter activity (Figure 4), the overexpression of the full length CD147 was expected to have the contrary effect decreasing the IL-2 promoter activity. Indeed, the overexpression of the full-length CD147 construct inhibited the IL-2 promoter activity by 60% in the clone CD147etc_1. Surprisingly, the clone 2 expressing full length CD147 showed a 100% increase in the IL-2 promoter. Possibly the clone 2 contained more luciferase reporter gene copies than the other clones, which resulted in a stronger luciferase expression upon T cell stimulation. The CD147 construct lacking the cytoplasmic domain was still potent to reduce the IL-2 promoter activity by 60% to 80% in the clones CD147et_1 and CD147et_2.

The constructs containing the CD147 cytoplasmic domain alone or in combination with the CD147 transmembrane domain showed similar or even higher IL-2 promoter activity as clones containing the empty vector.

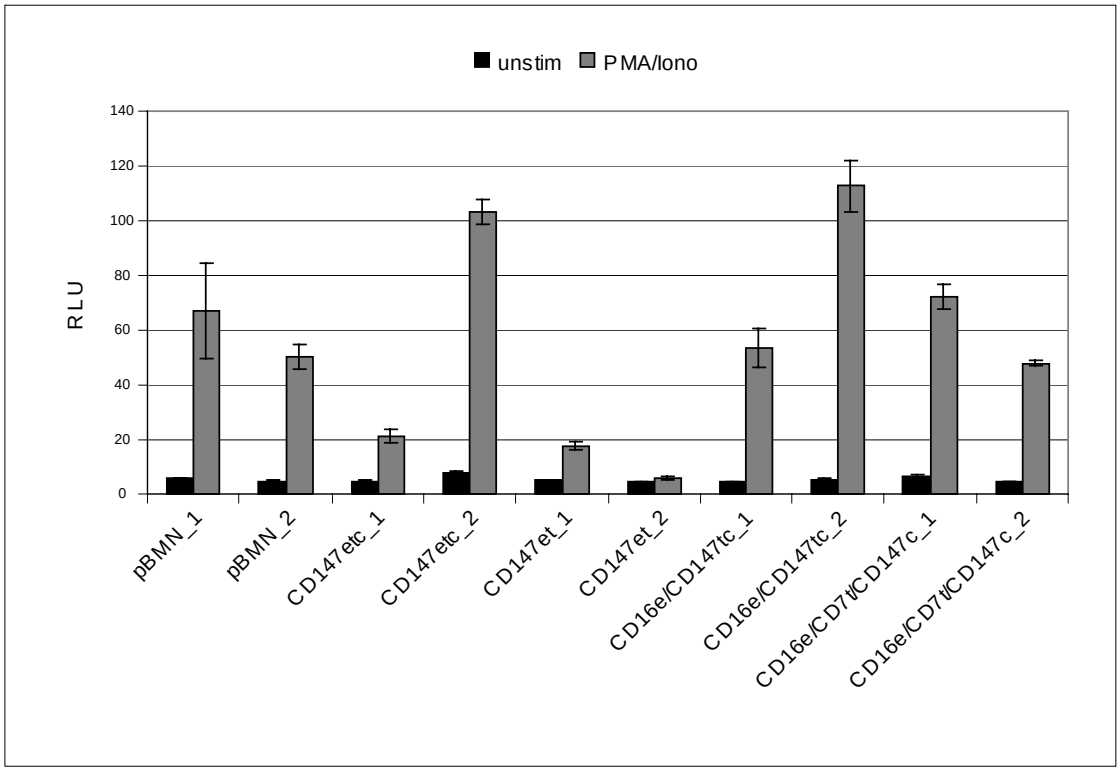


Figure 8: IL-2 promoter activity in Jurkat IL-2-luciferase clones transduced with the different CD147 constructs. The cells were stimulated for 20h with PMA/ionomycin (10ng/ml, 1µM). The numbers 1, 2 stand for clone 1 and 2. The mean values ± SD of three independent experiments are shown. Lysates were analyzed for luciferase activity.

5.1.3 Reconstitution with CD147 deletion and swap mutants upon CD147 silencing

After analyzing the effect of the CD147 constructs upon overexpression, we tested the reconstitution capacity of these constructs in CD147 silenced cells. Therefore, CD147 knock down Jurkat IL-2-luciferase cells were infected three times with retroviruses containing the different CD147 constructs. From day 4 after transduction of CD147 constructs, the cells were analyzed by flow cytometry, as shown in Figure 9. According to the GFP expression level, the CD147 silenced cells could express the CD147

constructs to a higher degree than cells containing siControl (non-target siRNA). The CD147 constructs containing CD147 extracellular and transmembrane domain could reconstitute the normal CD147 plasma membrane expression level in the silenced cells.

The construct lacking the CD147 transmembrane domain (CD147e/CD7 or CD16e/CD7t/CD147c) could even be highly overexpressed in silenced and control cells. Consistent with the data shown before, the expression of CD16e/CD147tc swap mutant obviously decreased the endogenous CD147 surface expression.

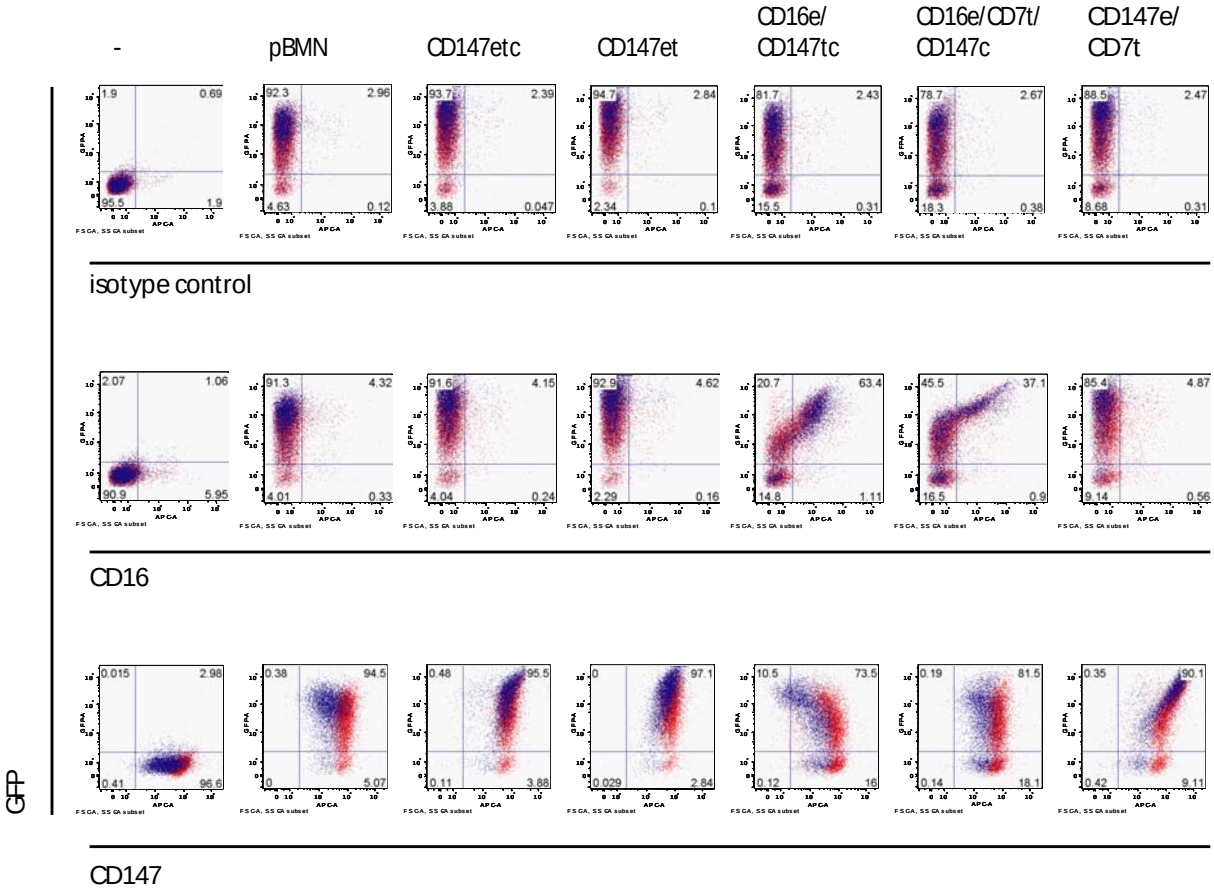


Figure 9: Expression level of CD147 constructs in CD147 silenced and control cells. SiControl (red) and siCD147 (blue) Jurkat IL-2-luciferase cells containing CD147 constructs were flow cytometrically analyzed. The cells were tested for GFP expression (y-axis) and for CD147 and CD16 surface expression (x-axis) upon cell surface staining with mAb MEM-154 to CD16 and mAb MEM-M6/1 to CD147.

Next, the IL-2 promoter activity was assayed by measurement of luciferase activity. Therefore, Jurkat IL-2-luciferase cells silenced for CD147 and reconstituted with CD147 constructs and non-target siRNA control cells overexpressing these mutant proteins were stimulated in separate assays for 7 to 20h. Phorbol 12-myristate 13-acetate (10ng/ml) combined with ionomycin (1µM) was used for stimulation over 7 hours. For 20h stimulation, Raji B cells were pulsed with 100ng/ml superantigen staphylococcal enterotoxin E (SEE) in advance. After stimulation, cells were lysed and the luciferase activity was measured (Figure 10a). Generally, CD147 silenced cells showed an increased IL-2 promoter activity in comparison to the control cells. However, the CD147 constructs containing CD147 transmembrane and extracellular domain could rescue the phenotype by decreasing the IL-2 promoter activity nearly to siControl levels. However, the constructs containing isolated extracellular, transmembrane or cytoplasmic domain did not affect IL-2 promoter activity.

To proof, if this rescue in promoter activity has an impact on protein expression and secretion level, too, and to analyze IFN-γ expression, the cytokine profile in cell supernatants was analyzed by an enzyme linked immunosorbent assay (ELISA). Thus, the reconstituted and overexpressing cells were stimulated as described before. Supernatants were harvested and were tested for IL-2 and IFN-γ by Prof. Zlabinger's laboratory (Figure 10b). The CD147 silenced cells secreted about 10 times more IL-2 (10ng/ml) and about 20 times more IFN-γ (200pg/ml) in contrast to siControl cells. Interestingly, CD147 construct lacking the cytoplasmic domain showed the greatest inhibitory effect by a reduction of IL-2 and IFN-γ protein expression to 1.25ng/ml and 73pg/ml. The full-length CD147 construct was also capable of suppressing IL-2 and IFN-γ to a level of 5.7ng/ml and 150pg/ml. Surprisingly, the CD16/CD147tc construct had an inhibitory effect on the IFN-γ production (170pg/ml).

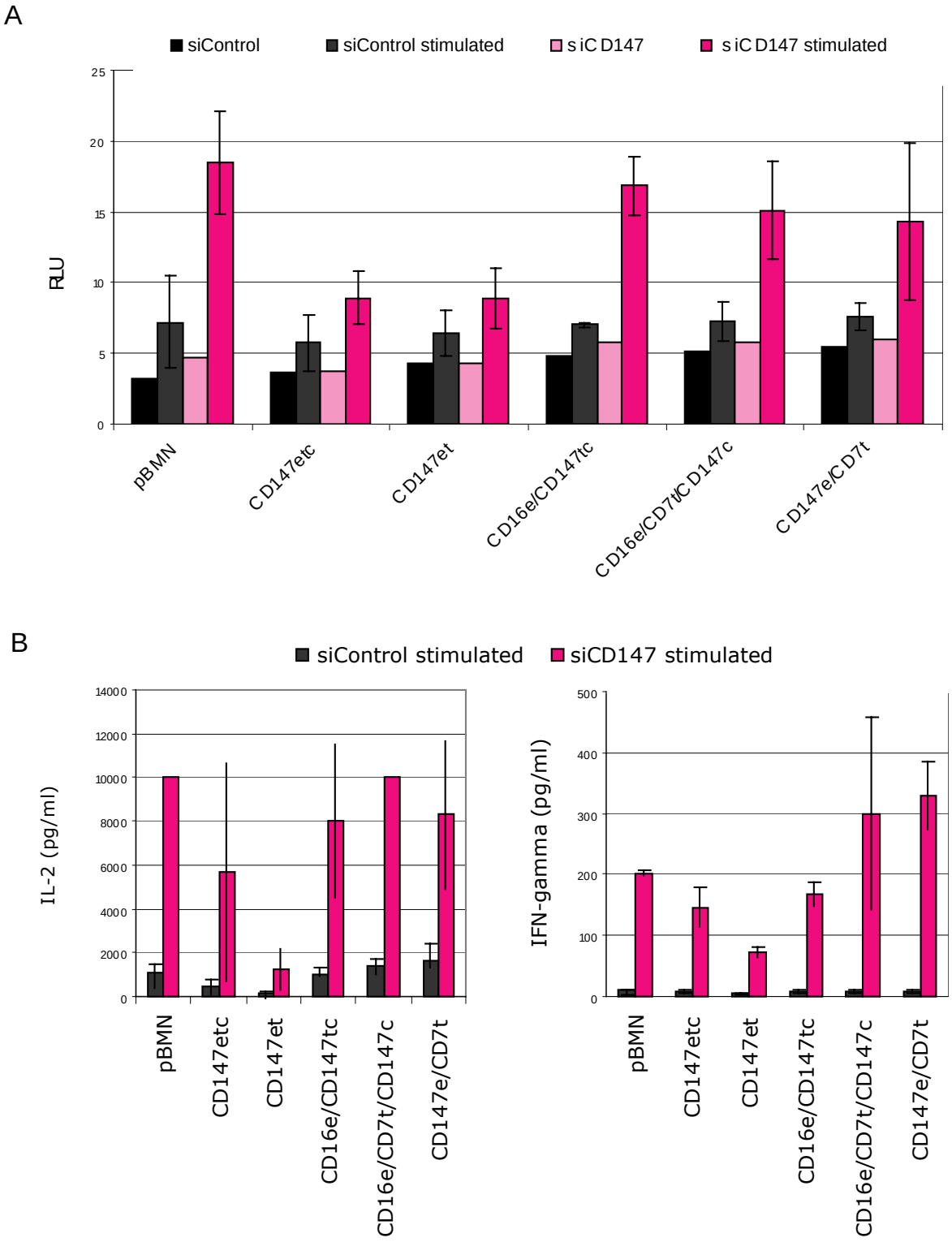


Figure 10: IL-2 and IFN-γ expression level upon reconstitution with CD147 constructs. A) Jurkat IL-2-luciferase cells, siCD147 (pink) or siControl (black), expressing CD147 constructs, were stimulated for 20h with Raji B cells pulsed with 100ng/ml SEE. Cells were lysed and the luciferase activity was measured. B) The same cells were stimulated with PMA/ionomycin (10ng/ml, 1µM) for 7h and the supernatants were analyzed by ELISA for IL-2 and IFN- γ.

Because CD147 was recently shown by Ruiz et al. (2008)[99] to regulate NFAT activity via its cytoplasmic domain upon overexpression, we investigated the effect of our constructs in a NFAT-luciferase reporter cell line. Therefore, CD147 silenced Jurkat NFAT-luciferase cells were transduced thrice with the different CD147 constructs. At day 5 after transduction or beyond, the cells were analyzed for the NFAT activity by a luciferase reporter gene assay and for the IL-2 expression level by flow cytometry. For the IL-2 expression analysis, the cells were stimulated for 5h with PMA/ionomycin (10ng/ml/1µM) and were treated with monensin for the last 2.5h of stimulation to prevent exocytosis of IL-2. The cells were fixed, permeabilized and stained intracellular with APC labeled IL-2 antibody conjugate. The stained cells were then analyzed by flow cytometry. In parallel, the cells were also analyzed for NFAT activity by a luciferase reporter gene assay upon 5h stimulation with PMA/ionomycin (Figure 11).

With the help of flow cytometric analysis of the intracellular IL-2 expression level, we could show that also in the Jurkat NFAT-luciferase reporter cell line CD147's transmembrane and the extracellular domain exert a negative regulatory function on the IL-2 expression level. This effect is also visible in the dot blot depiction in Figure 11a. Especially in the highly GFP positive cells of the CD147 silenced cells (pink box), the constructs CD147etc and CD147tc decreased the IL-2 expression upon stimulation with PMA/ionomycin. The results from flow cytometric analysis are additionally depicted in a bar chart in Figure 11b, left diagram. By means of the luciferase reporter gene assay, we could confirm a negative impact of CD147 on the NFAT activity, as was published by Ruiz et. al (2008)[99]. However, introducing the transmembrane and the extracellular domain – and not the cytoplasmic domain, CD147 could downregulate the NFAT activity to the control level (black) in CD147 silenced cells (pink) (Figure 11b, right diagram).

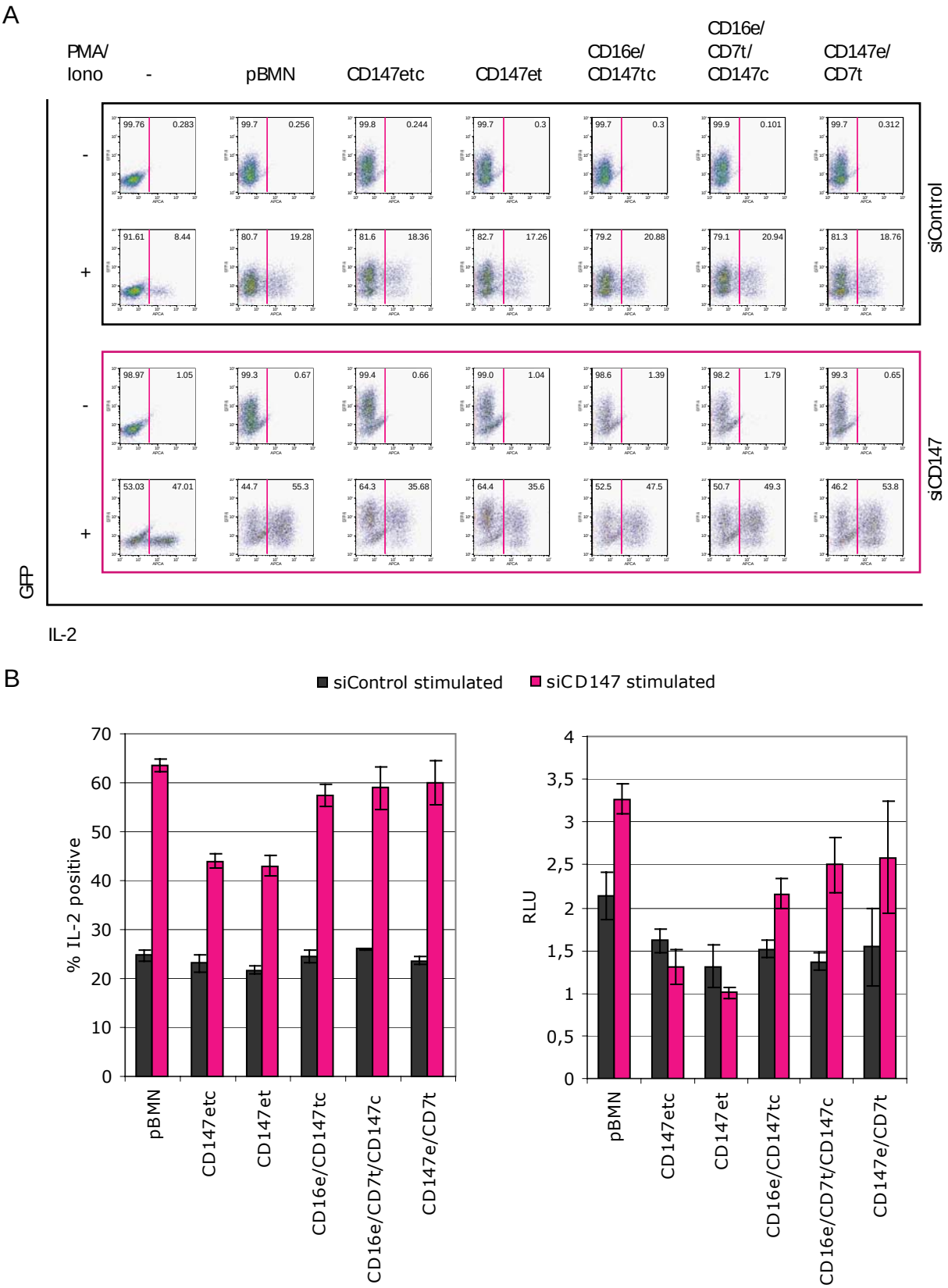


Figure 11: Effect of CD147 deletion mutants on NFAT activity. A) Flow cytometric analysis of Jurkat NFAT-luciferase cells reconstituted with (pink box) and overexpressing (black box) CD147 deletion mutants. Cells were stimulated for 5h with PMA/ionomycin (10ng/ml/1µM), treated for 2.5h with 1.5µM monensin and were analyzed for GFP and

IL-2 upon intracellular staining with α-IL-2-APC antibody conjugate. B) The left diagram shows the percentage of IL-2 positive cells from flow cytometric analysis. The right diagram displays NFAT activity in Jurkat NFAT-luciferase cells reconstituted with (pink) and overexpressing (black) CD147 swap and deletion mutants upon 5h stimulation with PMA/ionomycin (10ng/ml/1µM) and luciferase reporter gene assay.

By overexpression and by reconstitution with the CD147 constructs, we could show, that the combination of transmembrane and extracellular domain is the functional structure of CD147 regulating NFAT activity and thereby the IL-2 and IFN-γ expression. Further, we suggest, that the cytoplasmic domain attenuates CD147's regulatory effect, as the cytoplasmic deletion mutant displayed greater inhibitory effects as the full length CD147. Moreover, we got indications that CD147 surface regulation and glycosylation is regulated by its transmembrane domain.

5.2 *Deciphering CD147 dependent signaling pathway*

5.2.1 Analysis of potential lateral interaction partners of CD147

Due to the finding, that the regulatory capacity of CD147 on IL-2 expression is mediated via its extracellular and transmembrane domain, we focused now on molecules localized in the plasma membrane.

First, we analyzed the cell surface expression of known interaction partners of CD147 and molecules modulating T cell activation, such as CD28. Jurkat IL-2-luciferase cells were silenced for CD147 and at day 7 post infection they were flow cytometrically analyzed for CD18, CD28, CD29, CD43, CD98 and CD147 cell surface expression (Figure 12).

Interestingly, the known interaction partners of CD147 CD43 and CD98, and the T cell receptor signaling costimulatory receptor, CD28, were found to be upregulated upon CD147 silencing. In contrast, neither β1-Integrin CD29, another CD147 interaction partner[57], nor β2-Integrin CD18, that was shown to be indirectly regulated by CD147 via CD43[5], were deregulated in their surface expression upon CD147 silencing.

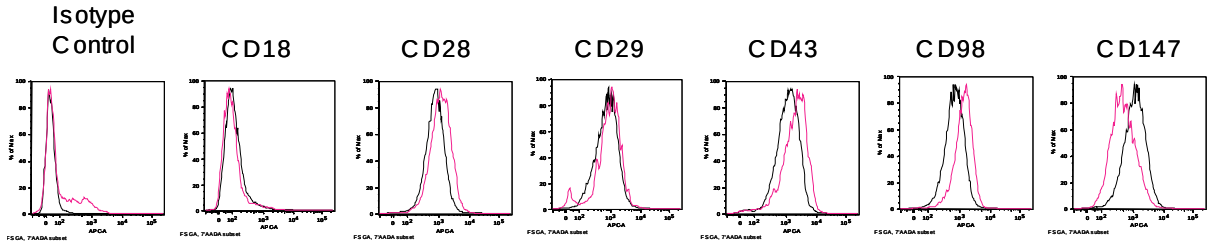


Figure 12: Effect of CD147 silencing on the surface expression of potential CD147 interaction partners. Flow cytometric analysis of siCD147 (pink) or siControl (black) Jurkat IL-2-luciferase cells. The cells were cell surface stained with mAbs MEM-48 to CD18, MEM-101A to CD29, MEM-59 to CD43, MEM-156 to CD98 and MEM-M6/1 to CD147. As isotype control served mAb AFP-01 to AFP.

Observing the increased expression level upon CD147 silencing, we wanted to analyze the impact of CD28, CD43 and CD98 on IL-2 expression. To figure out this question, Jurkat IL-2-luciferase cells were silenced for CD28, CD43 or CD98 and were analyzed for IL-2 promoter activity by a luciferase reporter gene assay.

The silencing efficiency (Figure 13ag) differed for all three siRNA constructs. According to flow cytometric analysis of the cell surface staining, the expression level of CD98 could be decreased by 99%, CD43 by 75% and CD28 by 50%. CD98 silenced cells exhibited strong growth retardation, while CD28 and CD43 silencing did not affect growth efficiency. The impact of CD28, CD43 and CD98 silencing on IL-2 expression was analyzed by the luciferase reporter gene assay. The cells were stimulated for 20h with superantigen pulsed Raji B cells, as described earlier, and cell lysates were analyzed for luciferase activity (Figure 13b). CD28 and CD98, but not CD43, silencing significantly decreased the IL-2 promoter activity. The results for CD28 were expected as it is a known costimulator of TCR/CD3 mediated signaling, and thereby has an impact on the IL-2 expression[132-134]. Therefore, we suggest that the upregulated surface expression of CD28 or CD98 might be linked to the increased IL-2 production in CD147 silenced cells.

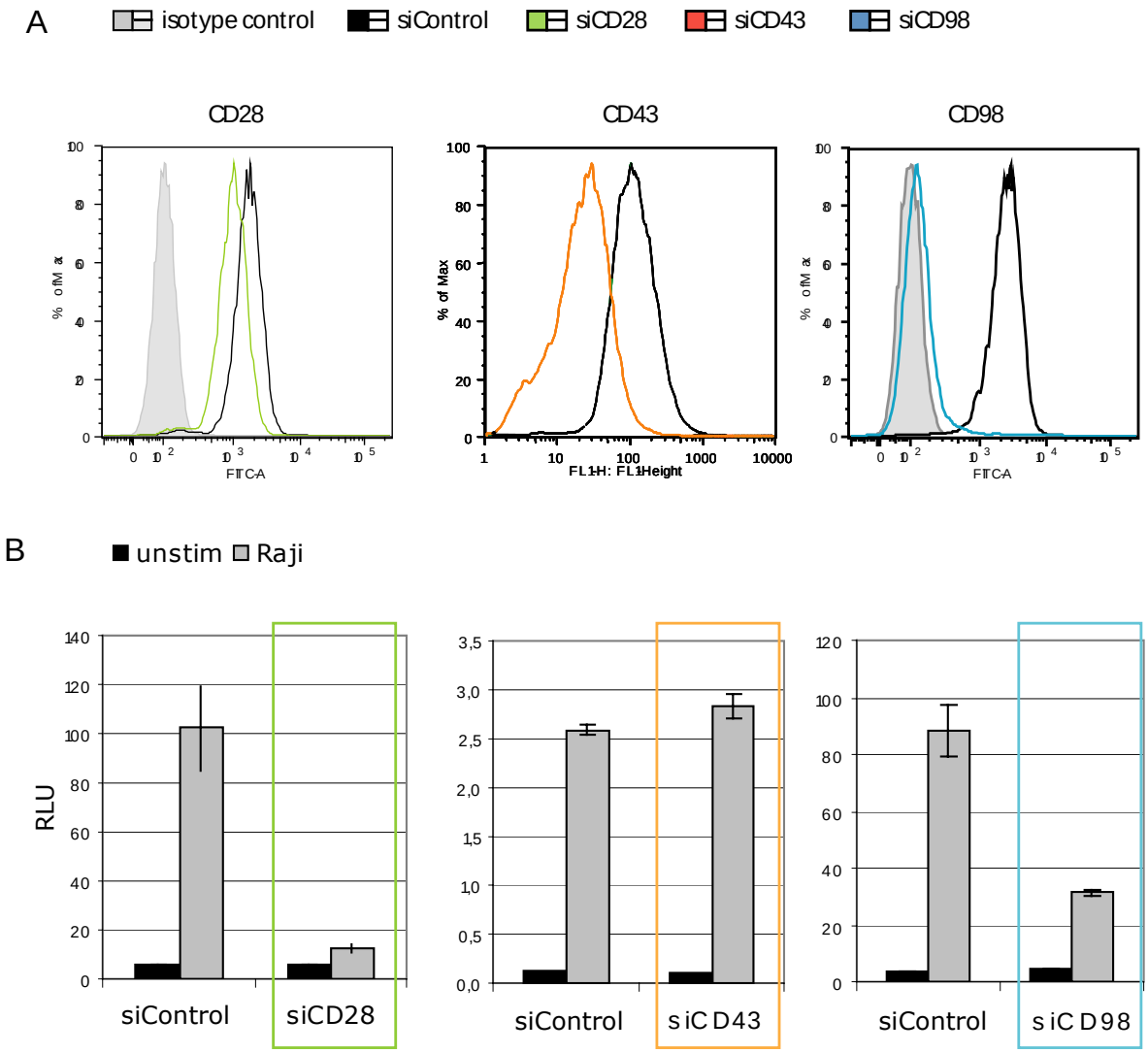


Figure 13: Effect of CD28, CD43 and CD98 silencing on IL-2 promoter activity. A) Jurkat IL-2 cells transduced with siCD28, siCD43, siCD98 siRNA or with non-target siRNA siControl were flow cytometrically analyzed at day 7 post infection for silencing efficiency. B) Silenced cells were stimulated for 20h with superantigen-pulsed (100ng/ml) Raji B cells. Luciferase activity was analyzed in the cell lysates.

5.2.2 Changes in phosphorylation patterns of signaling cascades leading to IL-2 expression upon CD147 silencing

Because CD28 was upregulated upon CD147 silencing, we assumed that a CD28 supported TCR/CD3 signaling pathway might be deregulated, leading to an increased IL-2 production. Therefore, components of the TCR/CD3 downstream signaling pathway were analyzed for their activity with the help of phospho-specific antibodies.

Jurkat IL-2-luciferase cells transduced with siCD147 and non-target siRNA control were stimulated with PMA/ionomycin (10ng/ml/1µM) for 0', 1', 5', 10', 30' and 60'. The lysates were electrophoretically separated on a 10% SDS-PAGEs and were blotted to membranes. Membranes were first analyzed for pRaf (Ser296), pRaf (Ser259), pRaf (Ser 338), pIκB (Ser32), pp44/42 (Thr202/Tyr204), pp38 (Thr38/Tyr182), pJNK (Thr183/ Tyr185), pAkt (Thr308), pAkt (Ser473), pGSK3β (Ser9), phospho-PDK1 (Ser241), phospho-IκB (Ser32), phospho-NF-κB (Ser536), pLck (Y416), pLck (Y505) and cNFAT. After stripping with pH 2.5, membranes were reprobed for ERK (p44/42), ZAP70, cRaf, Lck and actin as loading controls. Additionally the membranes were tested for CD147 (MEM-M6/1) and CD28 (Leu28). Because TCR signaling strongly depends on cytoskeleton, cytoskeletal and associated proteins, as pFAK (Tyr397), FAK, p-paxillin (Tyr118), annexin II and phospho-PAK1 (Thr423) PAK2 (Thr402) were analyzed in addition. Results are shown in (

Figure 14).

By Western blot analysis, we could observe a slight increase in the phospho-specific band for pNF-κB (Ser536) in CD147 silenced cells upon stimulation. Therefore, CD147 might exert its inhibitory function on the IL-2 promoter activity via the NF-κB pathway. Further, the CD147 knockdown cells displayed no immuno-reactive band of higher molecular weight for pRaf (Ser259), the specific band for inhibitory phosphorylation of cRaf mediated by Akt or the AMP-activated protein kinase AMPK[135].

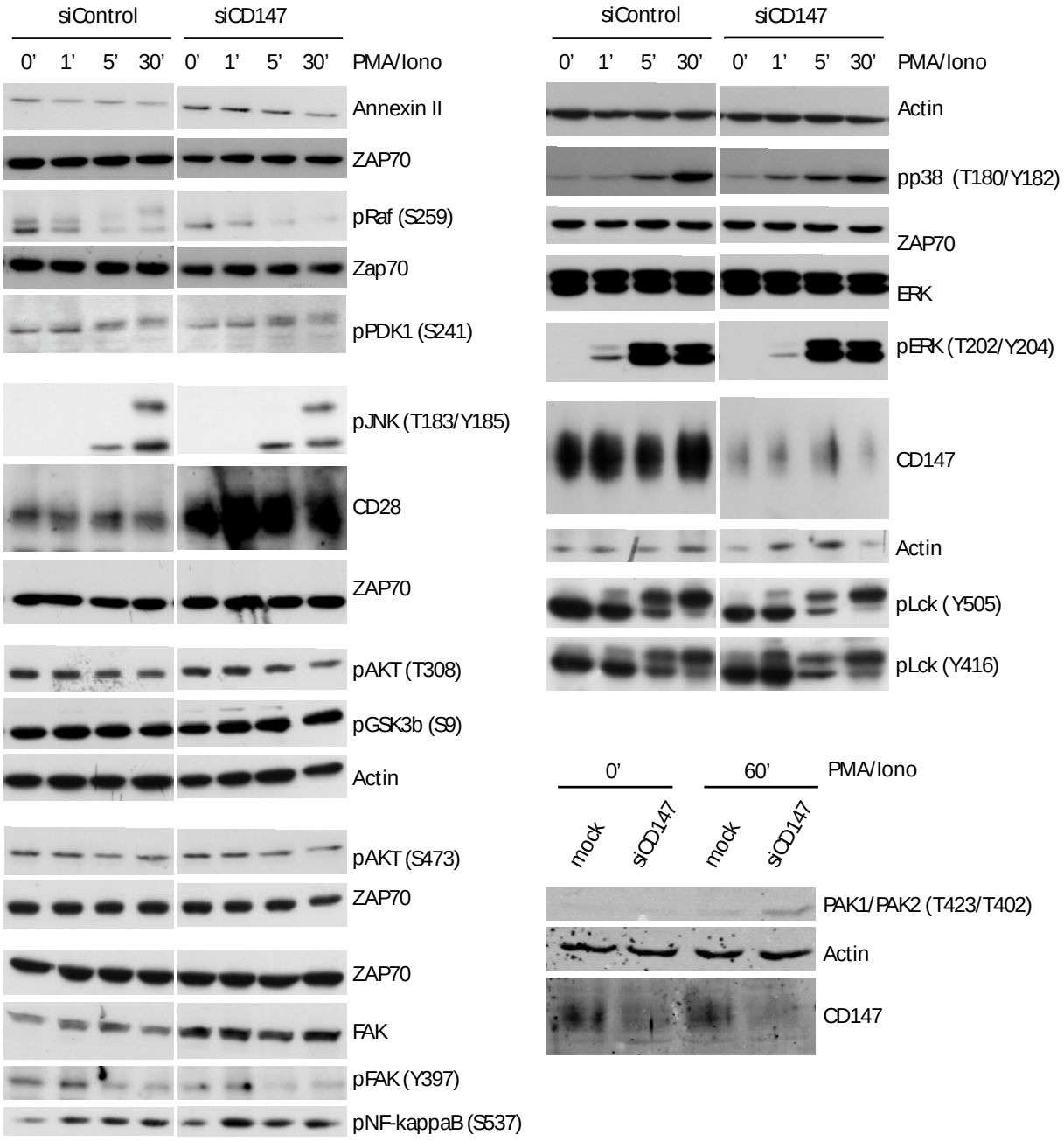


Figure 14: Biochemical analysis of TCR/CD3 and CD28 signaling pathways. Silenced and control Jurkat cells were stimulated for 0, 1, 5, 30 or 60 minutes with PMA/ionomycin (10ng/ml/1µM) at 37°C. Lysates were analyzed with the help of phospho-specific antibodies by Western blotting (antibodies are described in detail in the Materials & Methods section).

In general, the MAP kinase pathways, mediated by ERK, p38 or JNK, were not significantly affected upon CD147 silencing. Besides, a significant increase of annexin II and FAK expression upon CD147 silencing was found. Moreover, less FAK phosphorylation on the activating autophosphorylation site tyrosine 397 was observed. We could also verify, recently published results, that PAK was stronger phosphorylated in CD147 silenced T cells upon stimulation[99].

However, we could not find any changes in the Akt (also called PKB) phosphorylation pattern, the kinase that should be responsible for the affected phosphorylation sites of PAK and Raf. Moreover, an additional target for Akt mediated inhibitory phosphorylation, the glycogen synthase kinase 3 β (GSK3- β), exhibited no changes in the phosphorylation pattern. Thus, the component responsible for these changes in Raf and PAK phosphorylation could not be figured out with the help of available phospho-specific antibodies.

5.3 *Find interaction partners of CD147 that could mediate changes in IL-2 and IFN-γ production*

Due to the changes in FAK activation and, because CD147 was already shown to interact with β1-integrins[57], we hypothesized, that CD147 might modulate β1-integrin activity via its external or transmembrane domain in T cells. Thereby, CD147 might exert its inhibitory function on T cell stimulation by influencing cytoskeleton properties.

To shed light on this hypothesis, we wanted to see, if CD29, the heavy chain of β1-integrins is also interacting with CD147 in T cells. Further, we aimed to find out how FAK is associated in this hypothetical complex.

In a first approach, CD147, CD29 and FAK were immuno-precipitated and analyzed for co-precipitation. Therefore, as described in Materials and Methods, an ELISA plate functionalized with goat α-mouse antibody was indirectly coated with the following antibodies: AFP-01 to alpha-fetoprotein, Lck-01 to Lck, Clone2A7 to FAK, Clone HB1.1 and MEM-101A

to CD29 and MEM-M6/1, MEM-M6/3 and MEM-M6/4 to CD147. Further, an immunoprecipitation for Lck was done as a positive control, because this Lck antibody should precipitate Lck to a high degree under these conditions in lysates from Jurkat T cell line. As negative control an immunoprecipitation for alpha-fetoprotein – just expressed in fetal cells – was performed. As an additional negative control, wells coated just with goat α-mouse antibody were used. Jurkat IL-2-luciferase cells were lysed in 1%NP-40 and the lysate was added to coated plates for immunoprecipitation. The precipitates were eluted with hot Lämmli-buffer and separated electrophoretically on SDS-PAGE. Proteins were transferred to a Immobilin-P transfer membrane and analyzed for FAK, CD29, CD147 and Lck (Figure 15).

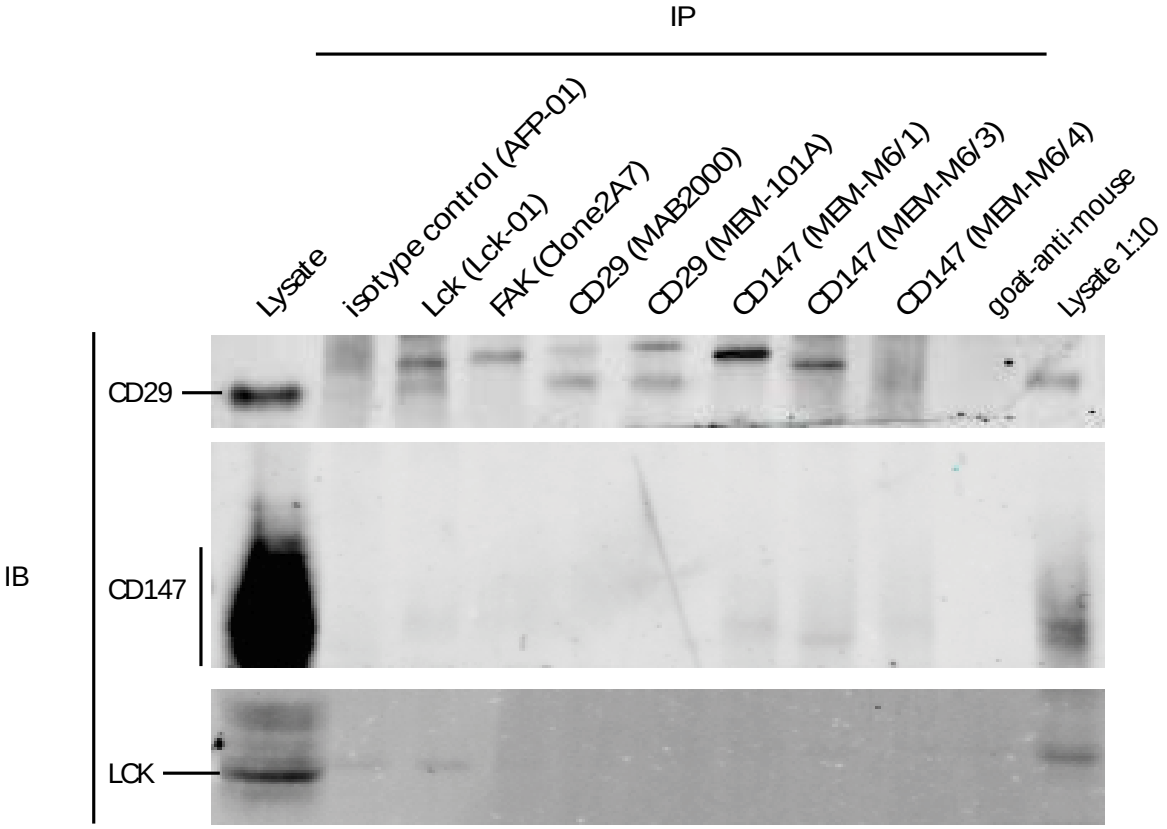


Figure 15: Co-Immunoprecipitation of CD147, CD29 and FAK. Jurkat IL-2-luciferase cells were lysed in 1% NP-40 and different antibodies were used for immunoprecipitation of CD147, CD29 and FAK. AFP and goat-anti mouse antibody served as negative control, Lck as positive control. Immunoprecipitations were analyzed by Western blot with following antibodies: MEM-101A to CD29, MEM-M6/1 to CD147 and Lck-01 to Lck.

Generally, the immunoprecipitation was not efficient, but still we could find a small amount of immunoprecipitated CD29, CD147 and Lck. Interestingly, the immuno-reactive bands for CD147 upon CD147 immunoprecipitation differed slightly in size. These slight differences in size of CD147 precipitate could indicate that these antibodies bind different glycosylation variants of CD147. Unfortunately, FAK could not be precipitated. We found CD29 to co-immunoprecipitate with Lck and with CD147, when precipitated with mAb MEM-M6/4. However, CD147 or Lck did not co-immunoprecipitate with CD29, possibly due to the weak immunoprecipitation efficiency.

To increase immunoprecipitation and co-immunoprecipitation efficiency, we employed affinity tagging on CD147. Therefore, two variants of Flag-tagged CD147 fusion proteins, generated by Dr. Paster, were used. The two constructs, where the Flag-tag (DYKDDDDK) was attached to the C- or N-terminus of CD147, were transduced into Jurkat IL-2-luciferase cells. By flow cytometric and Western blot analysis, we found that just the N-terminally Flag-tagged CD147 fusion protein could be expressed properly. Therefore, Jurkat IL-2-luciferase cells expressing N-terminally Flag-tagged CD147, were stimulated for 0’ and 5’ with PMA/ionomycin (10ng/ml, 1µM), lysed in 1% NP-40 and subjected to immunoprecipitation with Flag-specific antibodies coupled to agarose beads. The pull-down experiment was also performed with Jurkat IL-2-luciferase cells, containing no Flag-tagged protein and Jurkat J14 SLP76-Flag cells, expressing Flag-tagged SLP76, as control. Precipitated proteins were eluted under low pH and analyzed on SDS-PAGE by silver stain and on Western blot for CD29, CD147, Flag-tag and actin (Figure 16).
The N-terminal Flag-tagged CD147, as well as SLP76-Flag, could be efficiently precipitated (Figure 16). The Western blot results exhibited no co-immunoprecipitation of CD29 with Flag-tagged CD147. Further Flag-CD147 precipitation did not reveal any specific, significant and reproducible co-immunoprecipitation on the silver stained gel.

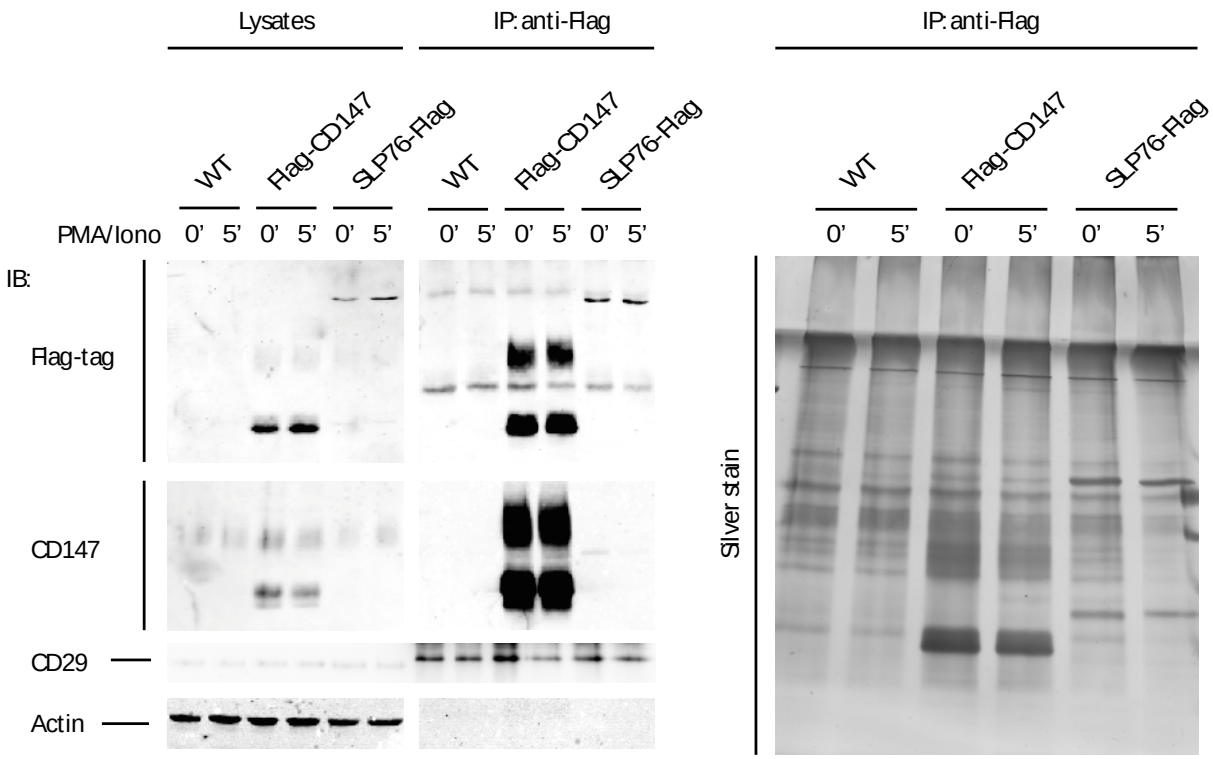


Figure 16: Immunoprecipitation of Flag-tagged CD147. Jurkat IL-2-luciferase (WT), Jurkat IL-2-luciferase Flag-CD147 (Flag-CD147) and Jurkat J14 SLP76-Flag (SLP76-Flag) were stimulated for 0 and 5 minutes with PMA/ionomycin (10ng/ml/1µM). The cells were lysed with 1% NP-40 and subjected to precipitation by α-Flag-coated beads. Precipitates were analyzed with M2 to Flag, MEM-M6/1 to CD147, MEM-101A to CD29 and α-actin antibody on a Western blot. One SDS-PAGE was silver stained.

6 Discussion

During an immune response, T cells have to get activated to defend the body from pathogens. Upon successful defense, the T cells have to be inactivated again to stop the production of cytotoxic products during an inflammation. This tight regulation of T cells is conducted through costimulatory molecules such as CD28 or ICOS, and inhibitory molecules as PD-1 or VLA-4. As CD147 is upregulated upon T cell activation[1] and as a reduction in CD147 expression resulted in hyperproliferation[2-4] of human T cells and to an increased IL-2 and IFN-γ cytokine response, it was hypothesized, that CD147 serves as inhibitory molecule upon T cell stimulation.

To find the immunomodulatory domain within CD147, several CD147 deletion and swap mutants were investigated for their impact on T cell stimulation. By overexpression and reconstitution studies in Jurkat T cell line, we could show that the combination of CD147's extracellular and transmembrane domain was necessary to downregulate IL-2 and IFN-γ expression by reducing the NFAT activity. These results are contradictory to the recently published data from Ruiz et al. (2008)[99]. In this study it was shown, that the myristoylated fusion protein of CD147's cytoplasmic domain and GFP could suppress NFAT activity. Ruiz et al. observed this effect when they overexpressed Vav-1 to boost the NFAT activity. However, the use of a myristoyl residue to anchor the cytoplasmic domain to the plasma membrane might have led to dyslocalization of their construct. We think, that the myristoyl residue did not target this construct to the typical localization of CD147, which is rather outside of lipid rafts[4]. To avoid this problem we substituted the CD147 transmembrane domain with the transmembrane domain of CD7, a protein, which was also found to localize outside of lipid rafts. Thereby we wanted to achieve a proper localization of our constructs into non-lipid raft regions of the plasma membrane. Further support for the

immunomodulatory function of CD147's transmembrane and extracellular domain comes from the interaction partners of CD147. Most of CD147's interactions rely on CD147's extracellular and transmembrane domain, as for instance the interaction with β1-integrins, CD98 or with cyclophilins. All these interaction partners were shown to have an impact on TCR signaling, especially on cytoskeletal mechanisms. Therefore, it is highly probable, that CD147 transduces signals through a lateral interaction partner, which affects cytoskeleton and thereby decreases IL-2 promoter activity upon T cell stimulation.

Next, we analyzed known lateral interaction partners of CD147 and TCR costimulatory surface molecules for their surface expression upon CD147 silencing by flow cytometric analysis. We found an upregulated surface expression of CD28, CD43 and CD98 upon CD147 silencing. The enhanced surface staining observed by flow cytometry could result from either enhanced gene expression, increased epitope accessibility by changes in complex assembly[4] or lowered turnover rates. As we also found a higher expression level by Western blot analysis (data shown for CD28 in Figure 14), it is likely, that CD147 affects the gene expression or the turnover rates. On the one hand, CD147 signaling might cause general changes in gene expression by induction of chromatin remodeling processes, as will be discussed later. On the other hand, the increased surface expression could also be linked to lower turnover rates in the CD147 knockdown cells. This turnover could be mediated by endocytotic and exocytotic[136] mechanisms by e.g. annexin. Additionally proteolytic cleavage of surface marker by proteinases could also affect the surface expression of proteins. For instance, MMPs were found to act in concert with the y-secretase to proteolytically degrade CD43[80]. Interestingly, a cytoplasmic fragment of CD43 generated by proteolysis was shown to act in a Notch-like manner influencing gene expression[85]. By stimulating the MMP[48] and y-secretase activity[122] CD147 could mediate the turnover of plasma membrane molecules and additionally affect gene expression

To investigate the impact of CD28, CD43 and CD98 surface expression on the IL-2 expression, stable knockdown cells for each of these proteins were generated. Reporter gene assays revealed that the expression of CD28 and CD98, but not CD43, highly correlated with the IL-2 promoter activity. Because CD28 and CD98 expression positively correlated with the IL-2 expression, they are major candidates leading to increased IL-2 and IFN-γ Th1 cytokine response in the CD147 knockdown T cells.

To elucidate the underlying pathway of CD147's signal transduction, components downstream of the T cell receptor and of integrins, possibly affecting IL-2 promoter activity, were investigated. Therefore, lysates of CD147 silenced cells were analyzed by phospho-specific antibodies on the Western blot. As shown in Figure 14 we found significant changes in the phosphorylation patterns of NF-κB, FAK, PAK and Raf and also changes in the total FAK and annexin II expression.

We observed enhanced phosphorylation of NF-κB at serine residue 536 upon CD147 silencing. There are contradictory studies about the function of this major phosphorylation site of NF-κB upon T cell activation. Wang et al. (2006)[137] found increased nuclear import and transcriptional activity of NF-κB upon serine 536 phosphorylation, while Mattioli et al. (2004)[138] observed a decreased nuclear import of pNF-κB. The study from Wang and colleagues suggests that CD147 inhibits transcriptional activity of NF-κB, in addition to NFAT, to negatively regulate IL-2 promoter activity. However, the enhanced NF-κB phosphorylation might result also from increase activation stimulus without any effect on the IL-2 promoter activity. The intrinsic autophosphorylation site tyrosine 397 of the FAK showed a reduced phosphorylation in CD147 knock down cells, however, the total FAK expression level was increased upon CD147 silencing. This points to a lower β1-integrin activity, monitored by lower FAK activation. Preliminary data investigating β1-integrin activity by an activation epitope specific antibody are supporting this hypothesis. As CD147 was reported

to associate with CD98 and β1-integrins to form an adhesive complex[7], the CD147 silencing could impair the assembly or function of this complex. We hypothesize that the loss of CD147 impairs β1 integrin mediated signaling. How this is related to the IL-2 promoter activity, has to be further elucidated.

Additionally we confirmed higher PAK1 phosphorylation in CD147 silenced cells, as was stated by Ruiz et al. (2008)[99]. PAK1 is an important effector of Rac G-protein mediated cytokine and stress responses induced motility and apoptosis[139]. The activation of PAK1 via Rac takes place at plasma membrane microdomains and relies on induction of PAK1's autophosphorylation on several sites[140,141]. Threonine 423 is a target site for autophosphorylation as well as phosphorylation by PDK1[140,142]. Because we could not observe any changes in the PDK1 phosphorylation pattern, we conclude, that the increased PAK phosphorylation was PDK1 independent. Possibly, microdomain composition in the absence of CD147 favors PAK localization and activation at the plasma membrane. Additionally, PAK-1 was shown to activate Raf by phosphorylation on threonine 338[143]. However, this phospho-specific site was not investigated yet in CD147 silenced cells.

Because CD147 was previously shown to affect MAP kinase pathways[56,99] the phosphorylation patterns of p38, JNK, cRaf and ERK upon CD147 silencing were investigated. Concerning the MAP kinase Raf we found, that the inhibitory phosphorylation on serine 259 disappeared in the CD147 knockdown cells. Surprisingly we did not find any further deregulations in the MAP kinase pathways, and especially no effect on Raf's downstream target ERK. The phosphorylation on serine 259 by Akt or AMPK leads to the binding of the adapter protein 14-3-3, which inhibits Raf's function[135]. Akt was obviously unaffected by CD147, as the phosphorylation status of Akt on threonine 308 and on serine 473 and thereby its activity did not change upon CD147 silencing. Therefore, Raf is more likely to be phosphorylated at serine 259 by AMPK. The activation state of AMPK after

CD147 silencing was not tested yet. Furthermore, the 14-3-3 proteins should be analyzed more closely, because they might affect other pathways, when not bound to Raf. CD43 mediated PKC activation, for instance, induces Cbl-b phosphorylation and binding to 14-3-3. By this mechanism CD43 downregulates Cbl-b's negative regulatory function on T cell receptor signaling. Additionally CD43 was shown to induce Vav tyrosine phosphorylation[81,82]. Yet, CD147 was shown to inhibit CD43's effect on β2-integrin activity[5]. Therefore it might be possible, that CD43's inhibitory function on Cbl-b can be also blocked by CD147, leading to increased 14-3-3 association with Raf instead of Cbl-b. Additionally CD147 might reduce the capacity of CD43 to stimulate Vav, resulting in a decreased NFAT activity. This hypothesis needs further enlightenment.

Next, we found annexin II, a Ca^{2+} and acidic phospholipid- binding protein to be increased in CD147 knockdown cells in contrast to control cells. The immunoreactive signal of annexin II decreases upon stimulation with PMA/ionomycin. Annexin II is known to be responsible for Ca^{2+} induced cortical actin skeleton remodeling. By that it plays an important role for cortical cytoskeleton architecture, membrane trafficking and can further influence ion fluxes across the membrane[101-103]. Annexin II was shown to interact with prohibitin[144]. Prohibitin affects chromatin accessibility by interaction with the retinoblastoma (Rb) protein, transcription factor E2F, a histone deacetylase and a nuclear receptor corepressor[145]. Thus, one could hypothesize, that increased annexin II upon CD147 silencing might induce epigenetic changes leading to a higher IL-2 expression level. This hypothesis would also explain the general upregulation of many other genes as CD28, CD43 and CD98 in CD147 silenced cells. On the other hand annexin II is known to be essential for enhanced pinocytosis induced upon hyperosmotic shock conditions[146]. Because our knockdown cells might suffer from hyperosmotic shock, due to delocalized MCT, annexin II might be activated and stabilized under these conditions without a direct interaction with CD147. This hypothesis needs further analysis by a pH-

sensitive fluorescent dye (e.g. BCECF) and real time PCR of annexin II mRNA.

To sum up, the investigation of TCR and integrin mediated pathways led to the idea, that CD147 signaling is crucially influencing cytoskeleton organizing components and possibly the NF-κB pathway.

Finally, we wanted to enforce our hypothesis by showing direct interactions of CD147 with integrins or FAK. Therefore, CD147, CD29 and FAK were immunoprecipitated and investigated for co-immunoprecipitations. This immunoprecipitations were not very efficient, but supported the idea of CD29 interaction with CD147 in T cells. To increase the efficiency of the IP, C- and N-terminal Flag-tagged CD147 were expressed in Jurkat T cells for subsequent immunoprecipitation. Unfortunately, C-terminally tagged CD147 was not expressed properly, but N-terminally tagged CD147 could be highly expressed and immunoprecipitated. However, β1 integrin CD29 did not co-precipitate with the N-terminally Flag-tagged CD147. We suppose that the Flag-tag sterically hindered any interactions at the N-terminal Ig-domain.

The Model:

To summarize the results, we found, that CD147's immunomodulatory structure in T cells consists of CD147's transmembrane and extracellular domain. We hypothesize that CD147 interacts laterally with other transmembrane proteins, to transduce it's negative regulatory signals into the cell. Yet, we did not define this interaction partner. In this study, we further show that CD98 enhances T cell stimulation and is upregulated upon CD147 silencing. As CD98 was shown to interact with CD147's extracellular domain[6], CD98 might be a promising lateral transducer of immunomodulatory signals. Furthermore, CD98 and CD147 were shown to form a complex with β_1-integrins[7]. This might explain the observed CD147 dependent phosphorylations of the cytoskeleton associated signaling components FAK and PAK. However, cyclophilins or MCTs might serve as signal transducer for CD147 too and should therefore be included in subsequent experiments.

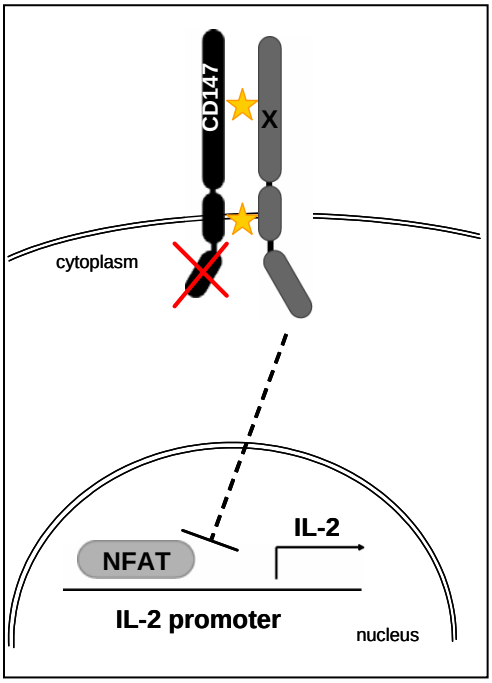


Figure 17: Model of CD147 immunomodulatory signaling. CD147 transduces its signals via lateral interaction partner X into the cell. By reducing NFAT activity, CD147 regulates the IL-2 promoter activity.

Additionally, we discovered that CD147 negatively regulates NFAT to inhibit IL-2 promoter activity. As our results point to a p38 and JNK MAP kinase independent regulation of NFAT activity, the expression levels and activity of calcineurin of calmodulin should be analyzed in future studies. Furthermore, our study shows CD147 dependent phosphorylation of NF-κB, which probably affects NF-κB transcriptional activity on the IL-2 promoter. With the help of a reporter gene assay one might proof this indication.

7 Abbreviations

AFP	alpha feto protein
AMPK	AMP activated protein kinase
AP-1	activator protein 1
APC	allophycocyanine
ASCT2	ASC-System (astroglia-rich primary cultures) amino acid transporter-1
ATF-2	activating transcription factor 2
BAD	Bcl associated death promoter
Bim	Bcl-2 interacting mediator
Bcl10	B cell lymphoma 10
Brg	Brahma/SW12-related gene
Carma1	caspase recruitment domain membrane-associated guanylate kinase
CasL	Crk associated substrate like protein
Cbl-b	Casitas B-lineage lymphoma b
CBP	CREB binding protein
CCL3	chemokine (C-C motif) ligand 3
CD147	cluster of differentiation 147
COX	cytochrome oxidase
CRAC channels	calcium release activated calcium channels
CRE	cAMP response element
CREB	CRE binding protein
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCR3	chemokine (CXC motif) receptor 3
DAG	Diacylglycerol
EpCam	epithelial cell adhesion molecule
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
ERM	Ets related molecule
FAK	focal adhesion kinase
FITC	fluorescein iso-thiocyanate
GATA-3	GATA binding protein 3
GFP	green fluorescent protein
Grb-2	growth factor receptor-bound protein 2
GTP	guanidine trisphosphate
HG	high glycosylated
HIF-1	hypoxia-inducible factor 1

HRPO	horse radish peroxidase
ICOS	inducible T cell costimulator
IFN-γ	interferon-γ
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL-2	interleukin-2
IL-12	interleukin-12
IP	immunoprecipitation
IP3	inositol 1,4,5-trisphosphate
IRES	internal ribosome entry site
IRF1	interferon regulatory factor
ITAMs	immunoreceptro tyrosine-based activation motifs
Jak	janus kinase
JNK	cJun N-terminal kinase
KO	Knockout
Lat-1	large amino acid transporter type 1 light chain
LAT	linker for activation of T cells
Lck	lymphocyte specific protein tyrosine kinase
LFA-1	lymphocyte function-associated antigen-1
LG	low glycosylated
LIM	LIN-11, Isl1, MEC-3
mAb	monoclonal antibody
MALT1	mucosa associated lymphoid tissue lymphoma translocation protein 1
MAP kinase	mitogen activated protein kinase
MCT	monocarboxylate transporter
MHC	major histocompatibility complex
MKK	mitogen activated kinase kinase
MMP	matrix metallo proteinase
NEMO	NF-kappaB essential modifier
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor k B
NK	natural killer
NLS	nuclear localization signal
Oct-1	octamer binding transcription factor
PAK	p21 activated kinase
PBMCs	Peripheral blood mononuclear cells
PD-1	programmed death 1

PDK-1	phosphoinosite-dependent protein kinase-1
PDZ	postsynaptic density 95, discs large, zonula occludens-1
PI3K	phosphoinositol-3-kinases
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
Pnn	pinin
Rb	retinoblastoma
SAPK	stress activated protein kinase
SEE	staphylococcal enterotoxin E
SH2	src homology 2
shRNA	small/short hairpin RNA
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
SOS	son of sevenless
SP1	selective promoter factor 1
STAT	signal transducer and activator of transcription
T-bet	T box expressed in T cellsTCR T cell receptor
Th1	T helper 1
TNF	tumor necrosis factor
TRAF-6	TNF receptor-associated factor 6
Treg cells	regulatory T cells
Tyk	tyrosine kinase
VEGF	vascular endothelial growth factor
VLA-4	very late antigen 4
ZAP70	zeta-chain-associated protein kinase 70
ZASP	Z-band alternatively spliced PDZ motif

8 References

1. Kasinrerk, W.*, et al.* Human leukocyte activation antigen M6, a member of the Ig superfamily, is the species homologue of rat OX-47, mouse basigin, and chicken HT7 molecule. *J Immunol* **149**, 847-854 (1992).

2. Igakura, T.*, et al.* Roles of basigin, a member of the immunoglobulin superfamily, in behavior as to an irritating odor, lymphocyte response, and blood-brain barrier. *Biochemical and biophysical research communications* **224**, 33-36 (1996).

3. Koch, C.*, et al.* T cell activation-associated epitopes of CD147 in regulation of the T cell response, and their definition by antibody affinity and antigen density. *International immunology* **11**, 777-786 (1999).

4. Staffler, G.*, et al.* Selective inhibition of T cell activation via CD147 through novel modulation of lipid rafts. *J Immunol* **171**, 1707-1714 (2003).

5. Khunkaewla, P.*, et al.* LFA-1-mediated leukocyte adhesion regulated by interaction of CD43 with LFA-1 and CD147. *Molecular immunology* **45**, 1703-1711 (2008).

6. Xu, D. & Hemler, M.E. Metabolic activation-related CD147-CD98 complex. *Mol Cell Proteomics* **4**, 1061-1071 (2005).

7. Melchior, A., Denys, A., Deligny, A., Mazurier, J. & Allain, F. Cyclophilin B induces integrin-mediated cell adhesion by a mechanism involving CD98-dependent activation of protein kinase C-delta and p44/42 mitogen-activated protein kinases. *Experimental cell research* **314**, 616-628 (2008).

8. Glimcher, L.H. & Murphy, K.M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes & development* **14**, 1693-1711 (2000).

9. Leitenberg, D. & Bottomly, K. Regulation of naive T cell differentiation by varying the potency of TCR signal transduction. *Seminars in immunology* **11**, 283-292 (1999).

10. Bacon, C.M.*, et al.* Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *The Journal of experimental medicine* **181**, 399-404 (1995).

11. Macian, F. NFAT proteins: key regulators of T-cell development and function. *Nature reviews* **5**, 472-484 (2005).

12. Bacon, C.M.*, et al.* Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7307-7311 (1995).

13. Farrar, J.D.*, et al.* Selective loss of type I interferon-induced STAT4 activation caused by a minisatellite insertion in mouse Stat2. *Nature immunology* **1**, 65-69 (2000).

14. Farrar, J.D., Smith, J.D., Murphy, T.L. & Murphy, K.M. Recruitment of Stat4 to the human interferon-alpha/beta receptor requires activated Stat2. *The Journal of biological chemistry* **275**, 2693-2697 (2000).

15. Visconti, R.*, et al.* Importance of the MKK6/p38 pathway for interleukin-12-induced STAT4 serine phosphorylation and transcriptional activity. *Blood* **96**, 1844-1852 (2000).

16. Morinobu, A.*, et al.* STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12281-12286 (2002).

17. Ouyang, W.*, et al.* The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3888-3893 (1999).

18. Ni, Z., Hassan, M.A., Xu, Z., Yu, T. & Bremner, R. The chromatin-remodeling enzyme BRG1 coordinates CIITA induction through many interdependent distal enhancers. *Nature immunology* (2008).

19. Pattenden, S.G., Klose, R., Karaskov, E. & Bremner, R. Interferon-gamma-induced chromatin remodeling at the CIITA locus is BRG1 dependent. *The EMBO journal* **21**, 1978-1986 (2002).

20. Avni, O.*, et al.* T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature immunology* **3**, 643-651 (2002).

21. Afkarian, M.*, et al.* T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nature immunology* **3**, 549-557 (2002).

22. Beima, K.M.*, et al.* T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-

dependent functional effects. *The Journal of biological chemistry* **281**, 11992-12000 (2006).

23. Usui, T.*, et al.* T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *The Journal of experimental medicine* **203**, 755-766 (2006).

24. Grogan, J.L. & Locksley, R.M. T helper cell differentiation: on again, off again. *Current opinion in immunology* **14**, 366-372 (2002).

25. Fehervari, Z., Yamaguchi, T. & Sakaguchi, S. The dichotomous role of IL-2: tolerance versus immunity. *Trends in immunology* **27**, 109-111 (2006).

26. Murayama, A.*, et al.* A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. *The EMBO journal* **25**, 1081-1092 (2006).

27. Nel, A.E. T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse. *The Journal of allergy and clinical immunology* **109**, 758-770 (2002).

28. Nel, A.E. & Slaughter, N. T-cell activation through the antigen receptor. Part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy. *The Journal of allergy and clinical immunology* **109**, 901-915 (2002).

29. Nandiwada, S.L., Li, W., Zhang, R. & Mueller, D.L. p300/Cyclic AMP-responsive element binding-binding protein mediates transcriptional coactivation by the CD28 T cell costimulatory receptor. *J Immunol* **177**, 401-413 (2006).

30. Yokosuka, T.*, et al.* Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nature immunology* **6**, 1253-1262 (2005).

31. Leitenberg, D., Falahati, R., Lu, D.D. & Takeda, A. CD45-associated protein promotes the response of primary CD4 T cells to low-potency T-cell receptor (TCR) stimulation and facilitates CD45 association with CD3/TCR and lck. *Immunology* **121**, 545-554 (2007).

32. Falahati, R. & Leitenberg, D. Changes in the role of the CD45 protein tyrosine phosphatase in regulating Lck tyrosine phosphorylation during thymic development. *J Immunol* **178**, 2056-2064 (2007).

33. Mor, A.*, et al.* The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nature cell biology* **9**, 713-719 (2007).

34. Mor, A., Dustin, M.L. & Philips, M.R. Small GTPases and LFA-1 reciprocally modulate adhesion and signaling. *Immunological reviews* **218**, 114-125 (2007).

35. Klasen, S., Pages, F., Peyron, J.F., Cantrell, D.A. & Olive, D. Two distinct regions of the CD28 intracytoplasmic domain are involved in the tyrosine phosphorylation of Vav and GTPase activating protein-associated p62 protein. *International immunology* **10**, 481-489 (1998).

36. Pages, F.*, et al.* Two distinct intracytoplasmic regions of the T-cell adhesion molecule CD28 participate in phosphatidylinositol 3-kinase association. *The Journal of biological chemistry* **271**, 9403-9409 (1996).

37. Gupta, S., Campbell, D., Derijard, B. & Davis, R.J. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science (New York, N.Y* **267**, 389-393 (1995).

38. Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. & Davis, R.J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Molecular and cellular biology* **16**, 1247-1255 (1996).

39. Wagner, S. & Green, M.R. DNA-binding domains: targets for viral and cellular regulators. *Current opinion in cell biology* **6**, 410-414 (1994).

40. August, A. & Dupont, B. Activation of src family kinase lck following CD28 crosslinking in the Jurkat leukemic cell line. *Biochemical and biophysical research communications* **199**, 1466-1473 (1994).

41. Lee, K.Y., D'Acquisto, F., Hayden, M.S., Shim, J.H. & Ghosh, S. PDK1 nucleates T cell receptor-induced signaling complex for NF-kappaB activation. *Science (New York, N.Y* **308**, 114-118 (2005).

42. Sun, L., Deng, L., Ea, C.K., Xia, Z.P. & Chen, Z.J. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Molecular cell* **14**, 289-301 (2004).

43. Bottex-Gauthier, C., Pollet, S., Favier, A. & Vidal, D.R. [The Rel/NF-kappa-B transcription factors: complex role in cell regulation]. *Pathologie-biologie* **50**, 204-211 (2002).

44. Schmitz, M.L. & Krappmann, D. Controlling NF-kappaB activation in T cells by costimulatory receptors. *Cell death and differentiation* **13**, 834-842 (2006).

45. Jiang, J.L. & Tang, J. CD147 and its interacting proteins in cellular functions. *Sheng Li Xue Bao* **59**, 517-523 (2007).

46. Li, R., Huang, L., Guo, H. & Toole, B.P. Basigin (murine EMMPRIN) stimulates matrix metalloproteinase production by fibroblasts. *Journal of cellular physiology* **186**, 371-379 (2001).

47. Lim, M.*, et al.* Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. *FEBS letters* **441**, 88-92 (1998).

48. Sun, J. & Hemler, M.E. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer research* **61**, 2276-2281 (2001).

49. Tang, W. & Hemler, M.E. Caveolin-1 regulates matrix metalloproteinases-1 induction and CD147/EMMPRIN cell surface clustering. *The Journal of biological chemistry* **279**, 11112-11118 (2004).

50. Taylor, P.M.*, et al.* Breast cancer cell-derived EMMPRIN stimulates fibroblast MMP2 release through a phospholipase A(2) and 5-lipoxygenase catalyzed pathway. *Oncogene* **21**, 5765-5772 (2002).

51. Guo, H., Zucker, S., Gordon, M.K., Toole, B.P. & Biswas, C. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. *The Journal of biological chemistry* **272**, 24-27 (1997).

52. Pushkarsky, T.*, et al.* CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 6360-6365 (2001).

53. Pakula, R.*, et al.* Syndecan-1/CD147 association is essential for cyclophilin B-induced activation of p44/42 mitogen-activated protein kinases and promotion of cell adhesion and chemotaxis. *Glycobiology* **17**, 492-503 (2007).

54. Pushkarsky, T.*, et al.* Cell surface expression of CD147/EMMPRIN is regulated by cyclophilin 60. *The Journal of biological chemistry* **280**, 27866-27871 (2005).

55. Yurchenko, V.*, et al.* Regulation of CD147 cell surface expression: involvement of the proline residue in the CD147 transmembrane domain. *The Journal of biological chemistry* **280**, 17013-17019 (2005).

56. Yurchenko, V.*, et al.* Active site residues of cyclophilin A are crucial for its signaling activity via CD147. *The Journal of biological chemistry* **277**, 22959-22965 (2002).

57. Berditchevski, F., Chang, S., Bodorova, J. & Hemler, M.E. Generation of monoclonal antibodies to integrin-associated proteins. Evidence that alpha3beta1 complexes with EMMPRIN/basigin/OX47/M6. *The Journal of biological chemistry* **272**, 29174-29180 (1997).

58. Cho, J.Y.*, et al.* The functional interactions between CD98, beta1-integrins, and CD147 in the induction of U937 homotypic aggregation. *Blood* **98**, 374-382 (2001).

59. Curtin, K.D., Meinertzhagen, I.A. & Wyman, R.J. Basigin (EMMPRIN/CD147) interacts with integrin to affect cellular architecture. *Journal of cell science* **118**, 2649-2660 (2005).

60. Poole, R.C. & Halestrap, A.P. Interaction of the erythrocyte lactate transporter (monocarboxylate transporter 1) with an integral 70-kDa membrane glycoprotein of the immunoglobulin superfamily. *The Journal of biological chemistry* **272**, 14624-14628 (1997).

61. Kirk, P.*, et al.* CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *The EMBO journal* **19**, 3896-3904 (2000).

62. Hashimoto, T., Hussien, R. & Brooks, G.A. Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex. *American journal of physiology* **290**, E1237-1244 (2006).

63. Wilson, M.C., Meredith, D. & Halestrap, A.P. Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex in situ. *The Journal of biological chemistry* **277**, 3666-3672 (2002).

64. Zhang, X., Chen, Z., Huang, H., Gordon, J.R. & Xiang, J. DNA microarray analysis of the gene expression profiles of naive versus activated tumor-specific T cells. *Life sciences* **71**, 3005-3017 (2002).

65. Guo, H.*, et al.* Characterization of the gene for human EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases. *Gene* **220**, 99-108 (1998).

66. Liang, L., Major, T. & Bocan, T. Characterization of the promoter of human extracellular matrix metalloproteinase inducer (EMMPRIN). *Gene* **282**, 75-86 (2002).

67. Shi, Y., Simmons, M.N., Seki, T., Oh, S.P. & Sugrue, S.P. Change in gene expression subsequent to induction of Pnn/DRS/memA: increase in p21(cip1/waf1). *Oncogene* **20**, 4007-4018 (2001).

68. Hanna, S.M.*, et al.* A novel form of the membrane protein CD147 that contains an extra Ig-like domain and interacts homophilically. *BMC biochemistry* **4**, 17 (2003).

69. Yu, X.L.*, et al.* Crystal structure of HAb18G/CD147: implications for immunoglobulin superfamily homophilic adhesion. *The Journal of biological chemistry* (2008).

70. Marchler-Bauer, A.*, et al.* CDD: a conserved domain database for interactive domain family analysis. *Nucleic acids research* **35**, D237-240 (2007).

71. Seulberger, H., Unger, C.M. & Risau, W. HT7, Neurothelin, Basigin, gp42 and OX-47--many names for one developmentally regulated immuno-globulin-like surface glycoprotein on blood-brain barrier endothelium, epithelial tissue barriers and neurons. *Neuroscience letters* **140**, 93-97 (1992).

72. Miyauchi, T., Masuzawa, Y. & Muramatsu, T. The basigin group of the immunoglobulin superfamily: complete conservation of a segment in and around transmembrane domains of human and mouse basigin and chicken HT7 antigen. *Journal of biochemistry* **110**, 770-774 (1991).

73. Tang, W., Chang, S.B. & Hemler, M.E. Links between CD147 function, glycosylation, and caveolin-1. *Molecular biology of the cell* **15**, 4043-4050 (2004).

74. Yurchenko, V., Constant, S. & Bukrinsky, M. Dealing with the family: CD147 interactions with cyclophilins. *Immunology* **117**, 301-309 (2006).

75. Tang, Y., Kesavan, P., Nakada, M.T. & Yan, L. Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. *Mol Cancer Res* **2**, 73-80 (2004).

76. Sidhu, S.S., Mengistab, A.T., Tauscher, A.N., LaVail, J. & Basbaum, C. The microvesicle as a vehicle for EMMPRIN in tumor-stromal interactions. *Oncogene* **23**, 956-963 (2004).

77. Fadool, J.M. & Linser, P.J. Evidence for the formation of multimeric forms of the 5A11/HT7 antigen. *Biochemical and biophysical research communications* **229**, 280-286 (1996).

78. Matias-Roman, S.*, et al.* Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. *Blood* **105**, 3956-3964 (2005).

79. Zhou, S., Zhou, H., Walian, P.J. & Jap, B.K. CD147 is a regulatory subunit of the gamma-secretase complex in Alzheimer's disease amyloid beta-peptide production. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 7499-7504 (2005).

80. Andersson, C.X.*, et al.* Shedding and gamma-secretase-mediated intramembrane proteolysis of the mucin-type molecule CD43. *The Biochemical journal* **387**, 377-384 (2005).

81. Pedraza-Alva, G.*, et al.* Regulation of Cbl molecular interactions by the co-receptor molecule CD43 in human T cells. *The Journal of biological chemistry* **276**, 729-737 (2001).

82. Pedraza-Alva, G., Merida, L.B., Burakoff, S.J. & Rosenstein, Y. T cell activation through the CD43 molecule leads to Vav tyrosine phosphorylation and mitogen-activated protein kinase pathway activation. *The Journal of biological chemistry* **273**, 14218-14224 (1998).

83. Pedraza-Alva, G., Merida, L.B., Burakoff, S.J. & Rosenstein, Y. CD43-specific activation of T cells induces association of CD43 to Fyn kinase. *The Journal of biological chemistry* **271**, 27564-27568 (1996).

84. Wong, R.C.*, et al.* Signal transduction via leukocyte antigen CD43 (sialophorin). Feedback regulation by protein kinase C. *J Immunol* **144**, 1455-1460 (1990).

85. Andersson, C.X.*, et al.* CD43 has a functional NLS, interacts with beta-catenin, and affects gene expression. *Biochemical and biophysical research communications* **316**, 12-17 (2004).

86. Xu, Q., Leiva, M.C., Fischkoff, S.A., Handschumacher, R.E. & Lyttle, C.R. Leukocyte chemotactic activity of cyclophilin. *The Journal of biological chemistry* **267**, 11968-11971 (1992).

87. Allain, F.*, et al.* Interaction with glycosaminoglycans is required for cyclophilin B to trigger integrin-mediated adhesion of peripheral blood T lymphocytes to extracellular matrix. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 2714-2719 (2002).

88. Pushkarsky, T., Yurchenko, V., Laborico, A. & Bukrinsky, M. CD147 stimulates HIV-1 infection in a signal-independent fashion. *Biochemical and biophysical research communications* **363**, 495-499 (2007).

89. Poole, R.C. & Halestrap, A.P. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *The American journal of physiology* **264**, C761-782 (1993).

90. Halestrap, A.P. & Price, N.T. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *The Biochemical journal* **343 Pt 2**, 281-299 (1999).

91. Fischer, K.*, et al.* Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* **109**, 3812-3819 (2007).

92. Feral, C.C.*, et al.* CD98hc (SLC3A2) participates in fibronectin matrix assembly by mediating integrin signaling. *The Journal of cell biology* **178**, 701-711 (2007).

93. Armulik, A., Velling, T. & Johansson, S. The integrin beta1 subunit transmembrane domain regulates phosphatidylinositol 3-kinase-dependent tyrosine phosphorylation of Crk-associated substrate. *Molecular biology of the cell* **15**, 2558-2567 (2004).

94. Rintoul, R.C.*, et al.* Cross-linking CD98 promotes integrin-like signaling and anchorage-independent growth. *Molecular biology of the cell* **13**, 2841-2852 (2002).

95. Wennerberg, K.*, et al.* The cytoplasmic tyrosines of integrin subunit beta1 are involved in focal adhesion kinase activation. *Molecular and cellular biology* **20**, 5758-5765 (2000).

96. Iwata, S., Ohashi, Y., Kamiguchi, K. & Morimoto, C. Beta 1-integrin-mediated cell signaling in T lymphocytes. *Journal of dermatological science* **23**, 75-86 (2000).

97. Kamiguchi, K., Tachibana, K., Iwata, S., Ohashi, Y. & Morimoto, C. Cas-L is required for beta 1 integrin-mediated costimulation in human Tcells. *J Immunol* **163**, 563-568 (1999).

98. Kasinrerk, W., Tokrasinwit, N. & Phunpae, P. CD147 monoclonal antibodies induce homotypic cell aggregation of monocytic cell line U937 via LFA-1/ICAM-1 pathway. *Immunology* **96**, 184-192 (1999).

99. Ruiz, S., Castro-Castro, A. & Bustelo, X.R. CD147 inhibits the nuclear factor of activated T-cells by impairing Vav1 and Rac1 downstream signaling. *The Journal of biological chemistry* **283**, 5554-5566 (2008).

100. Yan, L., Zucker, S. & Toole, B.P. Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. *Thrombosis and haemostasis* **93**, 199-204 (2005).

101. Gerke, V., Creutz, C.E. & Moss, S.E. Annexins: linking Ca2+ signalling to membrane dynamics. *Nat Rev Mol Cell Biol* **6**, 449-461 (2005).

102. Gerke, V. & Moss, S.E. Annexins: from structure to function. *Physiological reviews* **82**, 331-371 (2002).

103. Hayes, M.J., Rescher, U., Gerke, V. & Moss, S.E. Annexin-actin interactions. *Traffic (Copenhagen, Denmark)* **5**, 571-576 (2004).

104. Guy, P.M., Kenny, D.A. & Gill, G.N. The PDZ domain of the LIM protein enigma binds to beta-tropomyosin. *Molecular biology of the cell* **10**, 1973-1984 (1999).

105. Durick, K., Wu, R.Y., Gill, G.N. & Taylor, S.S. Mitogenic signaling by Ret/ptc2 requires association with enigma via a LIM domain. *The Journal of biological chemistry* **271**, 12691-12694 (1996).

106. Wu, R.*, et al.* Specificity of LIM domain interactions with receptor tyrosine kinases. *The Journal of biological chemistry* **271**, 15934-15941 (1996).

107. Jani, K. & Schock, F. Zasp is required for the assembly of functional integrin adhesion sites. *The Journal of cell biology* **179**, 1583-1597 (2007).

108. Intasai, N., Mai, S., Kasinrerk, W. & Tayapiwatana, C. Binding of multivalent CD147 phage induces apoptosis of U937 cells. *International immunology* **18**, 1159-1169 (2006).

109. Marieb, E.A.*, et al.* Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production. *Cancer research* **64**, 1229-1232 (2004).

110. Toole, B.P. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* **4**, 528-539 (2004).

111. Yang, J.M.*, et al.* Extracellular matrix metalloproteinase inducer (CD147) confers resistance of breast cancer cells to Anoikis through inhibition of Bim. *The Journal of biological chemistry* **281**, 9719-9727 (2006).

112. Pugh, C.W. & Ratcliffe, P.J. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine* **9**, 677-684 (2003).

113. Davidson, B., Goldberg, I., Berner, A., Kristensen, G.B. & Reich, R. EMMPRIN (extracellular matrix metalloproteinase inducer) is a novel marker of poor outcome in serous ovarian carcinoma. *Clinical & experimental metastasis* **20**, 161-169 (2003).

114. Rosenthal, E.L.*, et al.* Expression of extracellular matrix metalloprotease inducer in laryngeal squamous cell carcinoma. *The Laryngoscope* **113**, 1406-1410 (2003).

115. Tang, Y.*, et al.* Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. *Cancer research* **65**, 3193-3199 (2005).

116. Kanekura, T., Chen, X. & Kanzaki, T. Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. *International journal of cancer* **99**, 520-528 (2002).

117. Cohen, P.L. & Maldonado, M.A. Animal models for SLE. *Current protocols in immunology / edited by John E. Coligan ... [et al* **Chapter 15**, Unit 15 20 (2003).

118. Pistol, G.*, et al.* Roles of CD147 on T lymphocytes activation and MMP-9 secretion in systemic lupus erythematosus. *Journal of cellular and molecular medicine* **11**, 339-348 (2007).

119. Higgins, G.A. & Jacobsen, H. Transgenic mouse models of Alzheimer's disease: phenotype and application. *Behavioural pharmacology* **14**, 419-438 (2003).

120. Richardson, J.C.*, et al.* Ultrastructural and behavioural changes precede amyloid deposition in a transgenic model of Alzheimer's disease. *Neuroscience* **122**, 213-228 (2003).

121. Hersh, L.B. Peptidases, proteases and amyloid beta-peptide catabolism. *Current pharmaceutical design* **9**, 449-454 (2003).

122. Zhou, S., Zhou, H., Walian, P.J. & Jap, B.K. The discovery and role of CD147 as a subunit of gamma-secretase complex. *Drug news & perspectives* **19**, 133-138 (2006).

123. Zhou, S., Zhou, H., Walian, P.J. & Jap, B.K. Regulation of gamma-secretase activity in Alzheimer's disease. *Biochemistry* **46**, 2553-2563 (2007).

124. Yin, K.J.*, et al.* Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism. *J Neurosci* **26**, 10939-10948 (2006).

125. Vetrivel, K.S.*, et al.* Evidence that CD147 modulation of Abeta levels is mediated by extracellular degradation of secreted Abeta. *The Journal of biological chemistry* (2008).

126. Bamberger, M.E., Harris, M.E., McDonald, D.R., Husemann, J. & Landreth, G.E. A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. *J Neurosci* **23**, 2665-2674 (2003).

127. Spinale, F.G. Novel approaches to retard ventricular remodeling in heart failure. *Eur J Heart Fail* **1**, 17-23 (1999).

128. Schmidt, R.*, et al.* Extracellular matrix metalloproteinase inducer regulates matrix metalloproteinase activity in cardiovascular cells: implications in acute myocardial infarction. *Circulation* **113**, 834-841 (2006).

129. Burggraf, D., Martens, H.K., Dichgans, M. & Hamann, G.F. Matrix metalloproteinase (MMP) induction and inhibition at different doses of recombinant tissue plasminogen activator following experimental stroke. *Thrombosis and haemostasis* **98**, 963-969 (2007).

130. Burggraf, D.*, et al.* Matrix metalloproteinase induction by EMMPRIN in experimental focal cerebral ischemia. *The European journal of neuroscience* **22**, 273-277 (2005).

131. Kabouridis, P.S., Magee, A.I. & Ley, S.C. S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes. *The EMBO journal* **16**, 4983-4998 (1997).

132. Fraser, J.D., Irving, B.A., Crabtree, G.R. & Weiss, A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science (New York, N.Y* **251**, 313-316 (1991).

133. Shapiro, V.S., Truitt, K.E., Imboden, J.B. & Weiss, A. CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Molecular and cellular biology* **17**, 4051-4058 (1997).

134. Verweij, C.L., Geerts, M. & Aarden, L.A. Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated

through an NF-kB-like response element. *The Journal of biological chemistry* **266**, 14179-14182 (1991).

135. Rajalingam, K. & Rudel, T. Ras-Raf signaling needs prohibitin. *Cell cycle (Georgetown, Tex* **4**, 1503-1505 (2005).

136. Besse, F.*, et al.* The Ig cell adhesion molecule Basigin controls compartmentalization and vesicle release at Drosophila melanogaster synapses. *The Journal of cell biology* **177**, 843-855 (2007).

137. Wang, Y., Xiang, G.S., Kourouma, F. & Umar, S. Citrobacter rodentium-induced NF-kappaB activation in hyperproliferating colonic epithelia: role of p65 (Ser536) phosphorylation. *British journal of pharmacology* **148**, 814-824 (2006).

138. Mattioli, I.*, et al.* Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. *J Immunol* **172**, 6336-6344 (2004).

139. Ke, Y., Wang, L., Pyle, W.G., de Tombe, P.P. & Solaro, R.J. Intracellular localization and functional effects of P21-activated kinase-1 (Pak1) in cardiac myocytes. *Circulation research* **94**, 194-200 (2004).

140. Knaus, U.G. & Bokoch, G.M. The p21Rac/Cdc42-activated kinases (PAKs). *The international journal of biochemistry & cell biology* **30**, 857-862 (1998).

141. Symons, M. Adhesion signaling: PAK meets Rac on solid ground. *Curr Biol* **10**, R535-537 (2000).

142. King, C.C.*, et al.* p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1). *The Journal of biological chemistry* **275**, 41201-41209 (2000).

143. Chaudhary, A.*, et al.* Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. *Curr Biol* **10**, 551-554 (2000).

144. Bacher, S., Achatz, G., Schmitz, M.L. & Lamers, M.C. Prohibitin and prohibitone are contained in high-molecular weight complexes and interact with alpha-actinin and annexin A2. *Biochimie* **84**, 1207-1220 (2002).

145. Kinkade, R.*, et al.* A small molecule disruptor of Rb/Raf-1 interaction inhibits cell proliferation, angiogenesis, and growth of human tumor xenografts in nude mice. *Cancer research* **68**, 3810-3818 (2008).

146. Merrifield, C.J.*, et al.* Annexin 2 has an essential role in actin-based macropinocytic rocketing. *Curr Biol* **11**, 1136-1141 (2001).

9 Curriculum Vitae

Personal data

Name:	Verena Zojer
Date and place of birth:	24. September 1984 in Vienna, Austria
Nationality:	Austria
Address:	Meidlgasse 25/25 1110 Vienna Austria
Telephone:	+43/650/5617369
E-mail:	verena_zojer@gmx.at

Education

July 2007-July 2008	Diploma work within the scope of the study molecular biology at the Department of Molecular Immunology, Medical University of Vienna, in Vienna Supervisor: Univ. Prof. Dr. Hannes Stockinger
since 2004	study: Teacher training program for chemistry and physics at University of Vienna
since 2003	study: Molecular biology at University of Vienna
June 2003	Matura (A-levels: general qualification for university entrance)
1995 – 2003	Bundesgymnasium Geringergasse, Vienna

Congress presentations

Zojer V., Forster F., Paster W., Schiller H. B., Stockinger H. The human Ig-family member CD147 in T cell regulation. 5th GEN-AU Summer School Ultra-Sensitive Proteomics and Genomics. Litschau, Austria, September 13. – 14., 2007. Poster 37.

Zojer V., Forster F., Paster W., Schiller H. B., Stockinger H. Ig-family member CD147 in regulation of human T cell. Annual Meeting of the Austrian Society for Allergology and Immunology. Alpbach, Austria, December 12-15, 2007. 2007. December 12.-15., 2007. Poster TaA_4.

Schiller H.B., Zojer V., Paster W., Forster F., and Stockinger H. The human Ig-family member CD147 in regulation of T cell activation. Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI; DGfI). Vienna, Austria, September 3. – 6., 2008. Poster 76

Zojer V., Schiller H. B., Paster W., Forster F., Stockinger H. The human Ig-family member CD147 in regulation of T cell activation. 6th GEN-AU Summer School Ultra-Sensitive Proteomics and Genomics. Litschau, Austria, September 17. – 19., 2008. Poster 16.