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„Investigating immunomodulatory properties of the
transferrin receptor (CD71) in human T cells“

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1 INTRODUCTION

1.1 Iron as an intrinsic factor in physiological processes

Elemental iron is a crucial micronutrient that is essential for mammals and is vital in principle for all living organisms. Fundamental cellular processes mediating proliferation and survival – like respiration, DNA synthesis and oxygen transportation need iron or heme containing enzymes ^[1, 2]. Therefore, iron homoeostasis is a rigorously regulated cycle, maintained by several proteins.

1.1.1 Non-heme iron

Generally, inorganic iron is found as iron-sulfur clusters (ISC) (Fe₂-S₂, Fe₃-S₄, and Fe₄-S₄ clusters) of metalloproteins, which exhibit multiple functions. In membranes of mitochondria and chloroplasts, they act as co-factors for respiratory enzyme complexes within the electron transport chains (ETC). Furthermore, the mitochondrial aconitase, which is a key player of the citric acid cycle, contains ISCs that directly interact with enzyme substrates and are crucial for energy production ^[3]. The bifunctional role of iron-regulatory protein-1 (IRP-1) in vertebrates is regulated by its Fe₄-S₄ clusters, depending of the cellular iron status serving as a cytosolic aconitase or as RNA-binding protein. In addition, IRP-1 also has an important role as sensor and responder to oxygen availability, reactive oxygen species (ROS) and reactive nitrogen species (RNS) ^[4].

The intrinsic biological function of non-heme iron can be also seen in the field of DNA repair and DNA damage detection, where eukaryotic primases and helicases contain strongly conserved iron-sulfur domains ^[3]. Iron is not only required for genomic integrity, but also for DNA synthesis where its bioavailability sets a limiting step due to the activity of ribonucleotide reductase enzyme that contains iron in its catalytic center ^[1]. Studies have shown that without the presence of iron, cells are trapped in G1 phase of the cell cycle ^[5].

Other non-heme proteins containing mono-nuclear iron centers are lipoxygenases and cyclo-oxygenases which are pro-inflammatory enzymes [6].

Considering iron as a crucial nutrient, homoeostasis is tightly regulated to ensure adequate iron levels for sufficient cellular maintenance and to avoid toxicity of iron in excess. Iron as a potent electron donor (Fe(II)) and acceptor (Fe(III)) is not always beneficial, as it also harbors negative effects, especially in an aerobic environment or in presence of unsaturated fatty acids. Harmful free radicals, called “reactive oxygen intermediates” (ROIs) are produced in redox reactions termed as iron-catalyzed Fenton and Haber-Weiss reactions. Hydroxyl radicals ($\text{OH}\cdot$) are formed due to reaction of ferrous iron with hydrogen peroxides (H_2O_2), and the oxidized metal can be reduced back to ferrous iron in the presence of superoxide (O_2^-). Both – superoxide and hydrogen peroxides – are ubiquitous byproducts of respiratory processes of the cell [7]. These highly active free radicals attack lipids and proteins, and further cause oxidative damage to DNA leading to base alterations, strand breaks and can result into mutations [8]. This chemical mechanism is also implemented in the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex of monocytes and macrophages, causing a “respiratory burst” upon microbial infection which is part of the defense machinery [9].

1.1.2 Heme-derived iron

A remarkable amount of cellular iron is contained in the centre of a prosthetic group termed as heme, which consists of a protoporphyrin ring and a Fe(II) ion [2, 7]. The most prominent hemoprotein is hemoglobin that is found in the red blood cells and is involved in the transport of oxygen and other gas ligands within the blood stream of vertebrates. Other important heme containing proteins are myoglobin as oxygen carrier in muscle tissues, ATP generating cytochromal proteins, ROS protective catalases and endothelial nitric oxide synthases, involved in regulation of blood pressure. Heme is mainly synthesized in liver and bone marrow, where iron is introduced to the protoporphyrin ring via the catalysator ferrochelatase in the mitochondria. Hence, heme is translocated to the cytosol for incorporation into hemoproteins. Heme

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breakdown takes place in the spleen, where the aging erythrocytes containing high heme amounts are degraded by macrophages. End-product of the degradation via microsomal heme oxygenases is bilirubin, which is enzymatically further processed in the liver and becomes excreted via feces [2, 7, 10].

1.1.3 Mechanisms of dietary iron uptake and subsequent delivery

To compensate the common iron loss, the adult human body absorbs a small amount of iron (1-3 mg) daily, whereas its total iron content average 3-5 g [7]. High concentrations of iron can be found in liver, muscle cells and bone marrow. The considerable part of iron is present as hemoglobin in erythrocytes. Heme-derived iron undergoes recycling after senescent erythrocytes commit phagocytosis [11].

The general iron uptake occurs in the duodenum by apical brush border enterocytes, where iron (Fe(III)) undergoes several steps until its cellular release [1].

The inorganic iron uptake is well described since the last years. The first step of non-heme dietary iron absorption involves its reduction from (Fe(III)) to (Fe(II)) by a surface ferrireductase

enzyme, so in order it can be transported by divalent metal transporter-1 DMT1 into the enterocytal cytosol [2, 4]. DMT1 also serves as a gateway for copper,

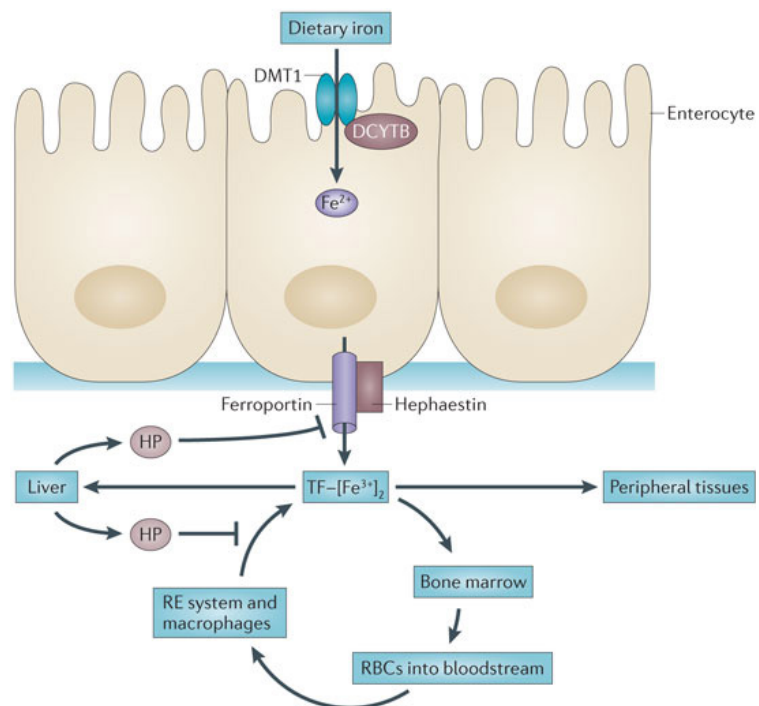


Figure 1 Iron metabolism. In the duodenum, nutritive ferric iron uptake occurs via enterocytic DMT-1 with simultaneous reduction to Fe(II) mediated presumably by Dcytb in the duodenum. Basolateral ferroportin releases iron after oxidation via hephaestin into the bloodstream, where it initially binds to the transport protein transferrin, from where iron gets delivered to the peripheral tissue or becomes part of the haemoglobin containing red blood cells (RBCs), of which the iron is recycled in the reticuloendothelial system (RE system). Figure adapted from Torti and Torti [a].

cadmium, zinc, manganese and other ions. Several studies suggest duodenal cytochrome-b (Dcytb) as a possible reducing agent ^[12]. In contrast, processing of dietary heme iron, arising from degraded hemo-globin and myoglobin, differs to inorganic iron due to its higher bioavailability compared to it. The identification of proteins responsible for duodenal heme import has not been successful yet and a few putative proteins are discussed like proton-coupled folate transporter/ haem carrier protein (PCFT/HCP1) ^[2]. However, iron release from heme is accomplished by the beforehand mentioned heme oxygenases in enterocytes.

Subsequently, the processed iron enters the poorly defined labile iron pool (LIP) ^[13], which consists of fluctuating, redox-active and chelatable iron associated with diverse proteins and chelates. Comprising only a minor fraction of total cellular iron, the LIP is a sensitive parameter for the cellular iron status, which is controlled by intracellular sensors within the homeostasis. Normally, avoidance of hazardous reactions is granted by keeping the iron content of the LIP on marginal levels. After its export into the bloodstream by ferroportin-1 (FP-1) ^[14] through the basolateral membrane of the enterocyte, it becomes oxidized by hephaestin ^[2]. It was shown that hephaestin activity besides its oxidative function is required for cell surface stability of FP-1 ^[15]. Released iron is either stored in ferritin ^[16] which is highly expressed in liver and spleen or is distributed via the iron-transport protein transferrin (Tf) to a compartment. Regulation of duodenal iron intake is orchestrated by the pivotal peptide hormone hepcidin (HP) ^[11]. Produced in the liver, it directly acts upon elevated iron levels by down-regulating ferroportin in enterocytes and also inhibiting further iron release of iron-storing hepatocytes and reticuloendothelial macrophages. Furthermore, hepcidin is regarded as an important anti-microbial peptide that is highly expressed during infection and inflammation, contributing to the innate host defense by limiting iron access of diverse pathogens in iron need, and results in hypoferremia ^[11, 17].

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1.1.4 Iron trafficking in mitochondria

Since mitochondria hold an important role as heme and ISC producer, they are indispensable within the cellular iron processing and also serve as iron storage platforms via mitochondrial ferritin (MtF) in a homologous manner to cytoplasmic ferritin [18]. While the delivery mechanism from the cytosolic iron compartment to the mitochondria is still obscure, promising candidates for export mechanism in humans like mitochondrial ABC protein 6 or ABCB7 for ISCs [18] or an isoform of feline leukemia virus subgroup C receptor (FLVCR1) for heme [19] in humans were recently found and are under investigation.

1.1.5 Intracellular iron storage

The structure of ferritin is highly conserved among eukaryotes [20]. In mammals, ferritin is an omnipresent iron storage protein which is mainly found intracellular and in serum. The ability to buffer and sequester iron highlights the role of ferritin to prevent iron overload or deficiency. High amounts of ferritin can be found in enterocytes, hepatocytes and reticuloendothelial macrophages. Each ferritin protein consists of 24 subunits and can retain up to 4000 iron molecules which are oxidized by the heavy-chain subunit before incorporation. Little is known about the iron release of ferritin, but it is hypothesized that iron is released after degradation of the iron storage protein [21].

Recently, studies provide strong evidence that ferritin has a direct immunomodulatory role within subsets of lymphocytes and myeloid cells, and may take part in the pathogenesis of autoimmunity [21].

1.2 The transferrin receptor system

1.2.1 Transferrin

Transferrin is a glycoprotein with an approximate molecular weight of 76.5 kDa and transports iron in a nonreactive state from intestine, reticuloendothelial system, and liver parenchymal cells to proliferating and transferrin receptor (TfR) bearing cells. In mammals, transferrin has two homologous N-terminal

and C-terminal iron-binding domains, each capable of binding one ferrous atom (Fe(II)). Reports refer a metabolic half-life of transferrin varying from 6 to 8 days [22].

Another member of the transferrin family is lactoferrin, a multifunctional protein with protective immunological function which is present in milk, saliva, tears, sweat and neutrophil granules [23]. In contrast, the Tf homologue melano-transferrin, which can bind only one iron atom, is involved in tumor genesis. Its explicit biological function remains still unclear [24].

1.2.2 The transferrin receptor

The membrane bound transferrin receptor CD71 (TfR1, here referred as TfR) is an important and well-characterized protagonist of the iron metabolism. The surface receptor is widely expressed on all nucleated and proliferating cells.

1.2.2.1 Structural properties of the transferrin receptor

1984, two different groups identified the primary structure of the TfR1 [25, 26]. TfR1 is a transmembrane type 2 glyco-protein that consists of a disulfide-bonded homodimer (Cys 89 and Cys 98) [27], of which each identical subunit of 90-95 kDa can bind one Tf molecule. The large C-terminal end interacts with the Tf complex, followed by a single trans-membrane region, whereas the short N-terminal tail stays cytoplasmic. The extracellular domain contains a trypsin-sensitive site (Arg 100), which can be attacked by an integral membrane metalloprotease [28, 29]. The released form – termed soluble TfR (sTfR) – dissociates into the serum. Concentration of sTfR in serum is used as clinical indicator for the functional iron status, which is independent of iron stores. An additional benefit is the fact that during chronic disease and inflammation sTfR levels are unaffected [30]. It is hypothesized that sTfR may signal reticuloendothelial cells or duodenal enterocytes to activate iron export [31]. A study has shown that TfR shedding is inhibited by Tf interaction [32], but still the biological significance remains a target of investigation. Recently the proteolytic fate of the remaining N-terminal domain has been revealed, indicating that signal peptide peptidase-like 2b as being responsible as the major driver of

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cleavage. Additionally, binding of human serum albumin (HSA) was found to be a binding partner of the c-terminal peptide fragment of TfR1 after its subsequent release [33].

After synthesis in the endoplasmatic reticulum, post-translational modification of the TfR via phosphorylation and fatty acylation occurs. The exodomain contains three N-linked glycosylations at asparagine residues (two high mannose, one complex oligosaccharide) and one O-linked site at Thr104. The absence of the N-linked glycans causes impaired Tf binding, hence implying that these sites are of importance. The functional role of the O-linked oligosaccharid is linked to TfR

shedding, which is promoted when the glycan is eliminated. The TfR ectodomain can be divided into three different subunits, namely in the apical, protease-like and helical domain [34].

1.2.2.2 The transferrin – transferrin receptor cycle

The detailed TfR-Tf interaction structure was revealed by cryo-electron microscopy in 1999 by Cheng Y. *et al.* [35] and shows a rather different image of the binding mode than reasoned from previous studies. The unorthodox laterally interaction of Tf is illustrated in figure 3.

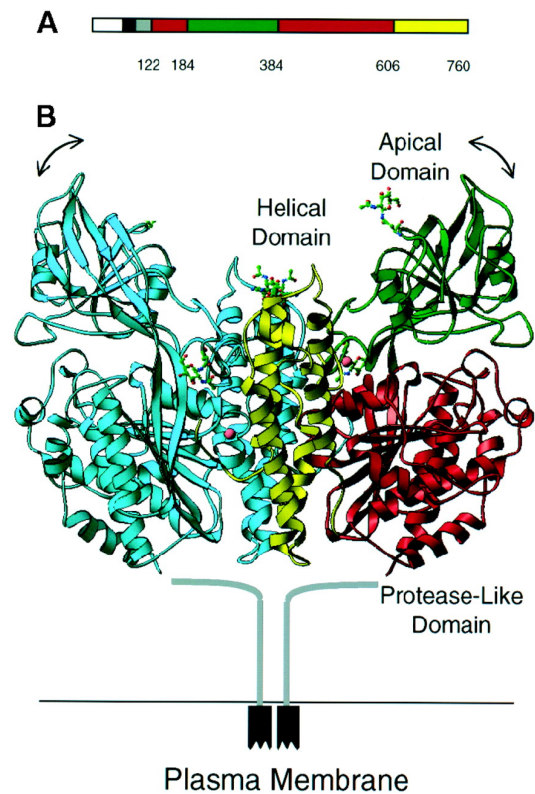


Figure 2 Domains of the extracellular TfR polypeptide, shown in crystal structure. (A) Linear domain organization of the TfR polypeptide chain. The cytoplasmic domain is white; the transmembrane segment is black; the stalk is gray; and the protease-like, apical, and helical domains are red, green, and yellow, respectively. Numbers indicate residues at domain boundaries. The TfR homodimer is organized in a butterfly-shape. (B) Schematic ribbon diagram. The right part is colored according to the previous illustration. Arrows indicate displacements observed in non-crystallographical models and pink spheres refer to Sm3+ ions in the crystal structure. The figure is obtained from Lawrence *et al* [b].

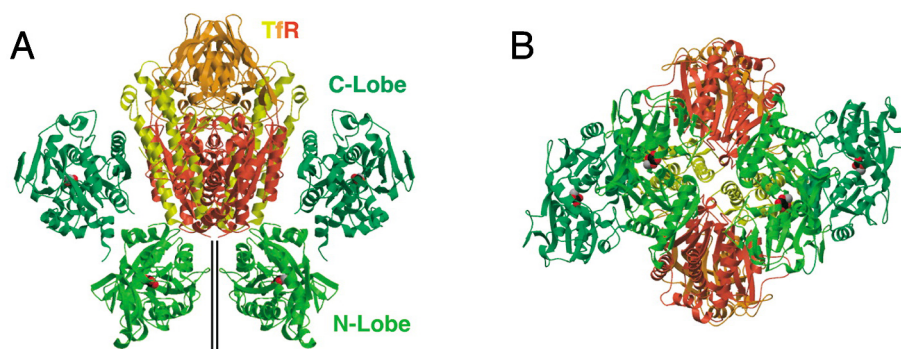


Figure 3 Atomic model of the TfR/holo-Tf shown as a ribbon diagram. (A) Face-on view and (B) bottom view. Colors indicate red: TfR protease-like domain; yellow: TfR helical domain; orange: TfR apical domain; dark green: Tf C-lobe; light green: Tf N-lobe; red sphere: iron ion. The TfR stalks are indicated by black lines in (A). Figure is adapted from Cheng *et al* [c].

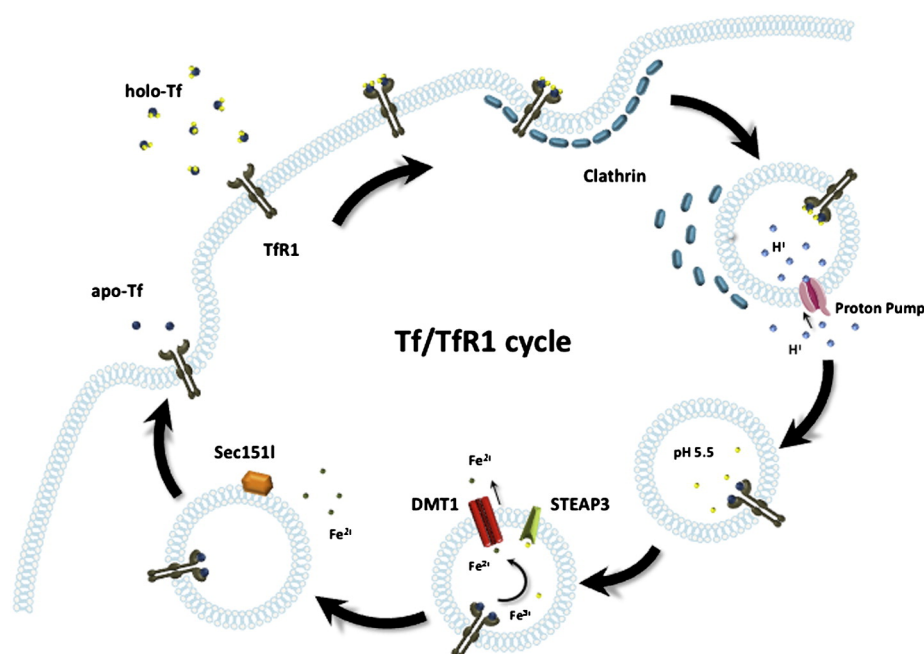


Figure 4 Overview of the cellular TfR/Tf cycle. Iron-loaded transferrin binds to its receptor, CD71, and the membrane starts to invaginate to build clathrin coated pits. After the internalization of TfR/Tf, the endosomal pH drops and causes a conformational structure change that leads to the release of iron from Tf. Reduced by STEAP3, iron enters via DMT-1 the cytoplasm and TfR/Tf is redirected to the surface, which involves the trafficking protein Sec151, from where Tf disassembles. Figure is obtained from Gkouvatsos *et al* [d].

Holo-Tf (Tf, loaded with two iron atoms) has a 30 to 500 fold higher affinity to the TfR in comparison to apo-Tf (Tf with no bound iron) or Tf bound only to one iron molecule. Interaction of Holo-Tf and TfR leads to internalization via clathrin-mediated endocytosis. Conformational changes in Tf and following dissociation of iron inside the endosome occurs due to acidification to pH 5,5, induced by a proton pump ATPase. Afterwards, released iron gets reduced by

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the ferric reductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and is then translocated via DMT1 across the endosomal membrane to the cytosol [11]. In the cytosol, TfR/apo-Tf is non-selectively delivered to two distinct endosomal populations: dynamic and static early endosomes. From both populations, the TfR can enter the slow recycling pathway via the perinuclear recycling compartment or can be quickly and directly trafficked to the surface. [36]. When conjugated with a monoclonal antibody, the antibody-TfR complex is directed to the degradative pathway and a general shift of the TfR trafficking occurs [37].

However, the apo-Tf/TfR1 complex gets recycled, remigrating to the cell membrane via the trafficking protein Sec15l1, where the ligand is released from its receptor and enters bloodstream in the end [11].

1.2.2.3 Alternative iron uptake system

In the early 90s of the 20th century, several evidence were found that also non-intestinal cells, in particularly cancer cells, exhibit transferrin independent iron uptake mechanisms [38, 39]. Studies have shown this observation in the cell lines K562, Raji, HeLa and HL-60 and many others. Ferric and ferrous iron are incorporated via different pathways. The alternative iron uptake system is linked to other metal uptake mechanisms and is strongly activated in an iron poor environment [40].

1.2.2.4 Regulation of transferrin receptor expression

The expression level of TfR1 correlates with the cellular metabolic requirements for iron. Rapidly proliferating cells such as activated peripheral blood mononuclear cells, intestine derived cells or malignant cells, express TfR at greater levels compared to slowly or nonproliferating cells [1]. The TFRC gene is located on chromosome 3 and its expression is post-transcriptionally regulated. The 3' untranslated region (3'UTR) of TfR1 mRNA contains iron-responsive elements (IREs) that form a series of five hairpin stem-loop structures to which cytosolic iron regulatory proteins (IRPs) bind. IRP1 and IRP2, two closely related *trans*-acting IRPs, are known to date. In vertebrates, this

regulatory mechanism encompasses all key players involved in iron uptake, export and sequestration to maintain the iron homeostasis. The iron sensitive IRPs permit a higher rate of mRNA translation and stability in case of cellular iron deficiency, while in iron excess, IRP1 has a minor RNA binding affinity and acts as aconitase whereas IRP2 is targeted for proteasomal degradation [4, 11].

Iron-independent signals from oxidative stress like ROS, RNS and hypoxia can also influence IRP activity and thus affect TfR expression as consequence [41].

1.2.2.5 Other ligands of the transferrin receptor

Besides the classical ligand Tf exist several other ligands that are revealed in the last two decades.

The human hemochromatosis protein HFE (High *iron* Fe) was found to be mutated in hemochromatosis, which is the most common form of iron overload disease. HFE is a transmembrane protein, mainly found on the surface of liver and intestinal cells that interacts with TfR1 and transferrin receptor 2 (TfR2). It competes with Tf for their overlapping binding sites and thus is involved in iron homeostasis. In the situation of increased hepatic serum iron levels, TfR1 binds predominantly Tf and TfR2 sequesters HFE, leading to expression of another iron regulatory protein called hepcidin via SMAD or mitogen-activated protein kinase (MAPK) pathway [42].

ACAP1, a GTPase-activating protein (GAP), binds to the cytoplasmic part of TfR and coordinates directed cargo sorting and recycling of internalized TfR [43].

In 2013, a study by Kenneth *et al* [44] identified TfR1 as binding partner of the IKK complex (inhibitor of NF- κ B kinase) which acts downstream as regulator of NF- κ B (nuclear factor κ B), a crucial transcription factor involved in immunological and stress responses. In the absence of TfR, the efficient assembly of IKK and nuclear translocation of NF- κ B are negatively altered. This data is consistent with previous results from studies [45, 46] with an exogenous ligand of TfR1, gambogic acid. Treatment of human cell lines with this potent organic compound induced an anti-proliferative and anti-inflammatory response in several cancer cells. Interaction of gambogic acid is directly

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mediated via TfR1, as downregulation of the receptor reverts the drug effect. Gambogic acid as characterized NF- κ B inhibitor was also shown to promote tumor necrosis factor α (TNF α)-mediated apoptosis, since NF- κ B as antagonist is functionally missing [45].

Overexpression of TfR has long been observed as accompanying characteristic with pathologic IgA complexes in IgA nephropathy, an inflammatory kidney disease. Moura *et al* identified TfR1 as IgA1 receptor in mesangial primary cells and B and T cell lines [47]. Next to the classical IgA receptor Fc α I (CD89), TfR1 was shown to be involved in the IgA nephropathy disease progress.

1.2.3 Transferrin receptor 2

In 1998 and 1999, Glöckner *et al* and Kawabata *et al* discovered independently a second type of transferrin receptor, TfR2. In contrast to TfR1, the α TfR2 transcript shares only 45% sequence identity and 66% similarity with the human TfR1 ectodomain, and also has a 25- to 30 fold lower binding affinity to Tf [48]. Another difference is the regulation of TfR2 expression as well as the tissue distribution, where TfR2 can be mainly found in liver, erythroid precursor cells and erythroloekaemic cells and to a far lesser amount to a special subset of cell and tissue types. The distinct biological function of TfR2 in iron homeostasis and other further cellular pathways is still under investigation [49].

1.3 The role of iron and transferrin receptors in immunity

1.3.1 Overview of the human immune system

Vertebrates possess two different kinds of immune defense systems. The evolutionary older and first responder against infectious pathogens is the innate immune system that acts non-specifically. Natural Killer cells (NK cells) and macrophages are typical effector cells of this type of immunity. In case of ineffective elimination of the infection, the adaptive immune system is activated after sufficient development of lymphocytes that can be subdivided into two major groups: B and T cells, which both arise from a common bone marrow-derived precursor. B cells produce antibodies that bind to extracellular

microbial antigens or toxins that lead to the clearance of the infection and are referred as humoral immunity. In contrast, only T cells, which are part of the cell-mediated immunity, can induce killing of cells that harbor intracellular pathogens like viruses that cannot be detected by antibodies. Dendritic cells (DCs) which belong to the group of antigen-presenting cells (APC), serve as link between the innate and adaptive immune system and act in the first line defense barrier [50].

1.3.1.1 T cell immunity

T cells can be functionally subdivided into two major groups: T-helper cells (CD4⁺) and cytotoxic T-cells (CD8⁺). Cytotoxic T-cells drive elimination of host cells that are infected with pathogens like viruses and bacteria via major histocompatibility complex (MHC) class I-associated peptide recognition and are also involved in immune responses against tumor cells. Among T-helper cells, two prominent subsets with specialized effector functions are described. When naïve CD4⁺ T cells are exposed to distinct antigens, costimulators and cytokines, they differentiate depending on the signalosome into T_H1 or T_H2 subsets and subsequently act as effector cells to activate B cells, macrophages and CD8⁺ cells, secreting lineage dependent cytokines [50, 51]. In general, cells of the T_H1 type mediate cellular immunity against intracellular pathogens and cancerous cells, while T_H2 cells contribute to humoral immunity in case of extracellular parasite invasion by inducing a strong antibody response. [50]. In the last decade, the definition of T cell subsets became more heterogeneous as well as the discrimination by their specific cytokine signature. Since other kinds of T-helper cells have emerged, e.g. T_H17 cells and T regulatory (Treg) cells, the additional subsets are setting new insights, nuances and limitations to the T_H1 or T_H2 hypothesis [52, 53].

After antigen exposure, most of the lymphocytes die, and memory cells remain usually circulating quiescent in the body for long periods to be quickly reactivated upon reinfection, promoting adaptive immunity [50].

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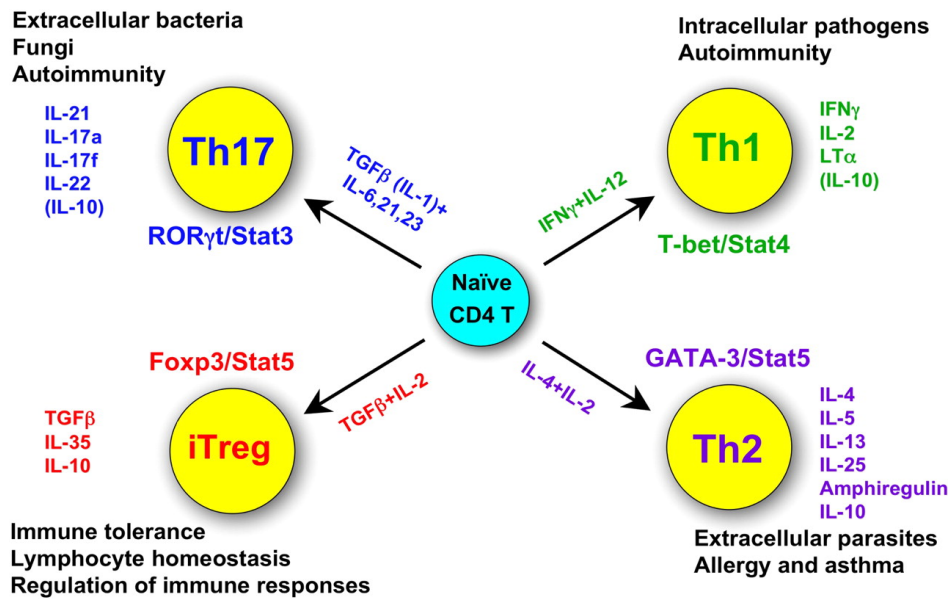


Figure 5 An Overview of the four major subsets of T cells. Shown are their signature cytokines and which are pivotal for their development from naïve T cells. In addition, their specific regulatory transcription factors are shown and for which immunological processes each T cell subset is generally responsible. Illustration obtained from Zhu and Paul, 2008 ^[e].

1.3.2 Different vulnerability of T cell subsets to iron depletion or overload

Noticeably, T_H1 and T_H2 subsets differ by their ability to deal with iron depletion via TfR blockade by an IgG antibody, the iron chelator deferoxamine or the combination of both agents. While both treated T helper cell lineages react with down-modulation of the TfR receptor, only T_H1 clones are inhibited in their proliferation because of absent iron molecules for the DNA synthesis. The T_H2 subset remains relatively unaffected and it is speculated that it is due to their larger and less labile iron storage pools ^[54]. This observation appears to explain the sensitivity of the immune system to iron homeostasis interferences, which may result in a shift of functional available effector T cells and becomes a pathological condition in iron-deficient patients with chronic diseases like auto-immunity or chronic infection ^[55]. In case of an iron-overload situation, research in rats indicates that it directs T cell differentiation towards the CD8⁺ compartment ^[56].

More recently, it was demonstrated that in mice that iron-overload suppresses Treg expansion and promotes T_H17 proliferation indirectly via secreted cytokines of macrophages ^[57].

1.3.3 T cells need the transferrin receptor for accurate maturation

T cell maturation is located in the thymus to which early T cell progenitors migrate from the bone marrow. There, the cells undergo thymopoiesis by selection and somatic rearrangement of the antigen receptor gene segments, which is the key mechanism to their antigen specificity. Naïve and mature T cells translocate to the peripheral lymphoid organs and circulate there. In case of antigen recognition and accessible second signals, the cells get activated and differentiate into effector and memory cells [50].

Iron is not only of great importance during the expansion of each cell independent of its origin, but is required in different amounts among the diverse subsets of T cell populations and affect its distinct function.

The transferrin receptor was shown to be crucial in T cell development. In chimeric mouse models, Ned *et al* (2003) have shown that TfR deficient T lymphocytes are arrested at an early stage of maturation (CD4⁺8⁻3⁻). All tissues except those of hematopoietic origin are capable to proliferate normally, which gave further evidence that there are alternative iron import mechanisms that maintain the cellular iron need [58]. A study with hypotransferrinemic (TfR ^{hpx/hpx}) and heterozygous mice (TfR ^{+/-}) came to the same conclusion [59]. An *ex vivo* study with fetal thymus organ cultures supports these findings that TfR requirement is stage-specific, due to the observation that in presence of CD71 antibodies T lymphocytes stagnate within the double negative state and conversion to double positive is reached only by the minority although TfR is expressed in all populations [60].

1.3.4 Requirement of the transferrin receptor in T cell activation

Resting, mature T cells exhibit insignificant or undetectable levels of TfR, but TfR surface expression is rigorously up-regulated upon mitogen-stimulated activation, which is linked to the increased need of iron during proliferation and can be used as marker[61].

Furthermore, CD71 is involved in the activation process of naïve, mature T-cells via direct interaction of the ζ chain with the T-cell receptor-CD3 complex and

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with the ζ chain-associated protein kinase 70 kDa (ZAP70), a T-cell-specific protein tyrosine kinase (PTK) [62]. Tyrosin-phosphorylation of proteins involved in the co-receptor dependent T cell activation cascade is achieved in a similar manner like TCR crosslinking via the TfR with a monoclonal antibody (FG1/6) of which induces proliferation and IL-2 production. Direct T cell receptor (TCR) stimulation causes TfR tyrosine-phosphorylation and activation of a TfR associated PTK [62]. Recently published mass spectrometry data of protein-protein interactions in activated CD4⁺ T cells has also shown that CD71 together with ZAP70 interacts with the adaptor protein Linker for Activation of T cells (LAT) [63].

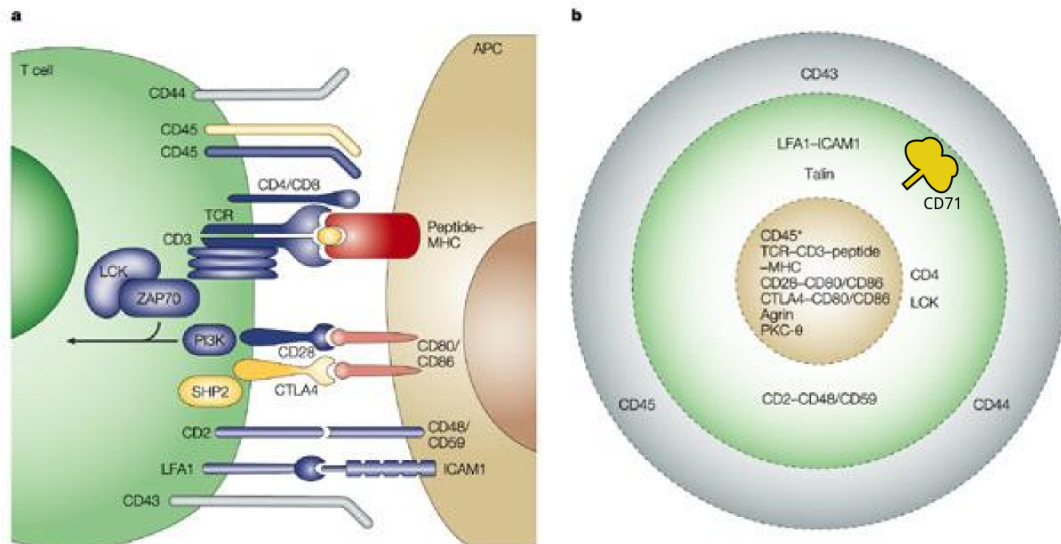


Figure 6 The immunological synapse. (A) A schematic front view illustrates involved receptors, ligands and signaling molecules of the T cell – APC recognition site. Blue color indicates activating/co-stimulatory, yellow inhibitory and grey otherwise function. The central peptide – MHC complex is marked red. (B) The simplified concentric structure of the immunological synapse includes the central region of the supra-molecular activation complex (cSMAC) (yellow), the peripheral ring surrounding the cSMAC (pSMAC, green) including CD71 and the distal area to the pSMAC (dSMAC, grey). The figure is adapted from Huppa and Davis, 2003 [f].

Moreover, it was shown that the TfR is needed for the development of immunological synapses (IS) between antigen presenting cells (APCs) and T cells, and is also involved in transduction function before IS formation [64]. The IS is a cellular structure, consisting of a MHC class II and TCR conjugate which form the central supramolecular activation cluster (c-SMAC) together with additional coreceptors and are surrounded by an area of integrins that is called

the peripheral supramolecular activation cluster (p-SMAC). The IS formation is accompanied by cytoskeleton rearrangement, redistribution of surface proteins and lipids and is followed by induction of intracellular signaling cascades [50, 65]. TfR being part of the IS are provided by T cells and can be found primarily in the p-SMAC. Intracellular pools of TfR are also observed to translocate to the site of IS during formation. T cells, which are preincubated with an anti TfR mAb (FG 2/12), are strongly impaired in their ability to form IS, where pretreated APCs stay unaffected. TfR blockade also results in reduction of CD3 ϵ subunits and Lck translocation to the c-SMAC [64].

1.3.5 The role of the TfR in B cells

B lymphocytes highly upregulate CD71 during stimulation with Cowan 1 *Staphylococcus aureus* (CSA) together with supernatants of phytohemagglutinin treated T cells, but not in presence of CSA alone [66]. T cell independent induction of CD71 can be also observed in B cells by crosslinking of membrane IgD [67]. While B cells require the TfR for cell growth, it is not necessary for accurate cytokine production [66].

B-cells lacking TfR during lymphocyte development appear to have a less severely affected phenotype when compared to thymocyte development in the Rag2^{-/-} mouse model of Ned *et al* [58]. Early differentiation events in TfR deficient B cells remain unaltered, although the number of matured cells is reduced. This may implicate that B lymphoid cells are not able to manage increased iron needs with alternative iron uptake systems during expansion like other tissues that developed normally [58].

1.3.6 Iron storage and TfR in dendritic cells and macrophages

Monocytes are the major progenitors of macrophages and DCs and originate from bone marrow. Monocyte derived macrophages belong to the iron storing immune cells and have multiple pathways to internalize iron. Besides the classical TfR/Tf internalization mechanism, several other alternative possibilities to acquire iron exist in macrophages: transmembrane uptake of ferrous iron by DMT-1, via lactoferrin or ferritin and phagocytosis of senescent

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erythrocytes [68]. Freshly isolated peripheral blood monocytes do not express TfR, but upregulate the expression during maturation although it is not characterized by cell growth. In contrast to other cell types, macrophages show enhanced expression of TfR in iron-rich environment, which contributes to their iron storage function [69]. *In vitro* experiments demonstrated that monocytic differentiation to macrophages or DCs is dependent to the iron restricted expression of cyclin-dependent kinase inhibitor p21 and iron deficiency results in an impaired phenotype [70, 71]. Once, fully differentiated, macrophages are resistant to TfR directed iron depletion. Stimuli like lipopolysaccharides (LPS) of microbes binding to the toll-like receptor -4 (TLR-4) or NK cell /T_H1 cell derived interferon- γ (IFN- γ) induce the production of reactive nitrogen intermediates or ROS via the formerly described Haber-Weiss reaction in order to kill the invasive pathogens [50]. A similar defense mechanism is provoked in activated neutrophils. As a counter strategy, bacteria sequester for example transferrin bound iron from the host environment, and therefore restrict access to iron for macrophages [72].

Dendritic cells are described as a heterogeneous cell type that exhibits the most potent APC function. Antigen recognition by patrolling DCs is followed by uptake, degradation and subsequent presentation of the fragmented antigen via MHC class I or II molecule on the surface. From the periphery, they migrate to secondary lymphoid organs like lymph nodes where an antigen-specific immune response is initiated after APC mediated T cell activation. Moreover, they also contribute to immunological tolerance by displaying self-antigens and promoting T cell anergy and are involved in B cell signaling [50, 73]. Resting (immature) DCs have a high antigen uptake rates and also phagocytose captured invaders or debris [74]. Similar to macrophages, TfR expression is upregulated in DCs during maturation in line with a second iron translocator DMT-1, and simultaneous down-regulation of the iron-exporter FP-1 [75]. Iron is an integral part of DC development: not only differentiation of DCs is stopped in the absence of iron as mention before [71], but also DC maturation is negatively altered in the presence of the iron chelator desferrioxamine [70]. Direct targeting of iron uptake via CD71 specific mAbs only marginally alters the proliferation

and function of monocyte derived DCs (mo-DCs) [76]. As CD71 is the latest discovered IgA receptor in B and T cells [47], its role in DC differentiation was in the focus in the study by Pasquier *et al* in 2004. This study indicates that TfR is upregulated along with the classical IgA receptor Fc α RI in immature mo-DCs contrary to other Fc receptors after stimulation (TNF- α , IL-1 β , LPS, CD40L). Fc α RI, together with the TfR, are shown to co-localize during polymeric IgA1 internalization in immature DCs, but not in activated DCs, where only Fc α RI seems to be responsible for endocytosis [77]. IgA complex internalization is known to induce allostimulatory activity of mo-DCs, accompanied by IL-10 production and elevated CD86 levels [78].

1.3.7 Cytokines in relationship to iron homoeostasis

Upon infection, cells of the adaptive and innate immune system secrete cytokines, which mediate and regulate the immune response. Since iron is a fundamental element needed for proliferation, differentiation and maturation of immune cells and therefore influences the effector function, multiple regulatory pathways exist to tune iron homoeostasis in regard of the immunological state. The TfR and other iron related proteins are usually post-transcriptionally modulated via the IRE/IRP regulation pathway, but also independent systems exist [55].

The major sources of cytokines of the innate immune system are mononuclear phagocytes, macrophages and NK cells to mediate local or systemic inflammation. They are produced in response to stimuli like LPS, bacterial peptidoglycans and viral RNA and function as link between the innate and adaptive immune system. Regulation and mediation of the adaptive immune response is generally accomplished by T cell derived cytokines [50].

IFN- γ

Interferon- γ is the only member of the type II interferon family and promotes the early host defense against intracellular pathogens by triggering phagocytosis activity and TNF- α secretion of macrophages. Acting among the innate and adapted immune system, IFN- γ is the major signature cytokine of the

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T_H1 cell population, shifting T cell differentiation in a positive feedback loop towards the T_H1 subset. Moreover, IFN- γ is secreted by cytotoxic T cells and NK cells and promotes isotype switching of B cells and upregulation of MHC expression of APCs [50, 79]. IFN- γ augments iron intake via upregulation of DMT-1 synthesis in monocytes and inhibition of FP-1 expression in macrophages [80]. Controversially, it reduces TfR expression in monocytes, an effect that is abolished with IL-10 administration [80]. Moreover, IFN- γ enhances ferritin synthesis, as well as NO formation [81]. Furthermore it was shown *in vivo* and *in vitro* that iron uptake via TfR negatively modulates the expression of the R2 signaling chain of the IFN- γ receptor in T cells. This receptor component is responsible for signal transducers and activators of transcription -1 (STAT-1) mediated apoptosis, and makes T_H1 cells resistant to the antiproliferative effect of IFN- γ [82].

IL-2

IL-2 is the principal promoter of proliferation, survival and differentiation of T lymphocytes and is one of the major signature cytokines of the T_H1 cell subset. In NK cells, it triggers proliferation and cytotoxic activity and antibody production in B cells, which is still only observed *in vitro*. Upon stimulation of naïve and effector T cells, IL-2 receptor (IL-2R) becomes upregulated, while in contrast regulatory T cells harbor constitutively expressed IL-2R for autocrine signaling of survival factor IL-2 [50]. IL-2 induces expression of TfR to maintain cellular growth, which is accomplished by a posttranscriptional mechanism [83].

IL-4

Mast cells and T_H2 cells, which are typically present in allergic diseases, are the main sources of the interleukin-4. IL-4 that is an autocrine factor of T_H2 cells, polarizes T cell development toward T_H2 cells and therefore acts as an antagonist of IFN- γ . Secreted IL-4 launches the protective eosinophil-driven defense against helminthic invaders via induction of B cell isotype class switching from IgM to IgE and IgG4 [50]. Another effect of IL-4, together with IL-13, is the alternative activation of macrophages, plus the upregulation of their TfRs to enhance iron uptake in the absence of IRP mechanisms. In addition, ferritin translation increases due to IRP inactivation [84].

IL-13

IL-13 is a prominent cytokine of the allergic phenotype and displays similar functions like IL-4, including IgE promotion. As a local inflammatory mediator, it drives fibrosis, mucus production by lung epithelial cells and expression of endothelial adhesion molecules and chemokines to direct monocytes and granulocytes to the inflamed tissue [50]. As mentioned before, IL-13 participates together with IL-4 in iron-regulatory signaling [84].

IL-17

Primarily produced by the functional subset of T_H17 cells, which are present in epithelial and mucosal tissue, IL-17 is like many other cytokines part of the host defense against microbes, especially against fungal and bacterial infections. Main targets of IL-17 are neutrophils and macrophages that leads to inflammatory migration [85].

IL-22

IL-22 is a pro-inflammatory member of the IL-10 family and is produced by various different immune cells of the innate and adaptive immune system, which are present in epithelial, intestinal and respiratory tissues. Its distinct role in autoimmune diseases is currently under investigation [86].

IL-10

IL-10 is an anti-inflammatory and immunosuppressive cytokine that downregulates the innate and cell-mediated immune response after eliminated infection. Mainly secreted by activated macrophages and Tregs, it acts as negative feedback regulator, inhibiting macrophagic actions by IL-12 suppression, and antagonizing IFN- γ production by T_H1 cells. Additionally, IL-10 inhibits T cell activation by downregulation of MHC II molecules and costimulators on DCs and macrophages [50]. IL-10 positively influences iron uptake via CD71 in activated monocytes [80]. It may be also involved in the iron internalization activity of macrophages by positively modulating CD163, the hemoglobin scavenger receptor, which increases the uptake of hemoglobin/haptoglobin complexes into monocytes [87].

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TNF- α

Mononuclear phagocytes and macrophages are the main producers of tumor necrosis factor- α (TNF- α), which has a broad spectrum of physiological functions. Upon infection with gram-negative bacteria or other pathogens, TNF- α mediates the recruitment of neutrophils and monocytes to the inflammatory site and activates endothelial cells [50]. It inhibits hepcidin formation and stimulates ferritin transcription in order to enhance iron storage in reticuloendothelial cells. Furthermore, it promotes erythrophagocytosis that results together with increased iron storage in case of chronic diseases of anemia and is characterized by hyperferritinemia and hypoferremia [88].

IL-1

IL-1, of which two minor homologous isoforms exist (IL1- α and IL1- β) shares similar characteristics with TNF- α regarding the secretion source and field of action, but has no impact upon apoptosis. This cytokine can be also found to be produced by neutrophils, endothelial and epidermal cells [50]. Moreover, it is involved in the signal cascade for NF- κ B activation and is responsible for ferritin transcription and translation via an acute phase box motif within the ferritin mRNA [88].

IL-6

IL-6 plays a role in the innate and acquired immune response. As homodimer produced by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells, it mediates the acute-phase response along with hepatocyte stimulation. It induces ferritin expression via interaction with the acute-phase box of ferritin mRNA and hepcidin formation in monocytes and macrophages [50, 88].

1.3.8 The alternative role of CD71 as IgA1 receptor

1.3.8.1 General functions of IgA

Antibodies are an integral part of humoral immunity and are produced in high amounts by plasma cells upon infection to trigger complement system and phagocytosis pathways. Antigen-specific immunoglobulins keep circulating at

constant levels in adult humans, of which immunoglobulin A (IgA) derived from bone marrow plasma cells comprises the second largest amount of antibodies after IgG in serum. Secretory IgA (SIgA) is provided by local B cells in extensive amounts and is present in mucous excretions like tears, sweat, saliva and colostrum. SIgA exerts its protective function at the surface of gastrointestinal, respiratory and urogenital tissues that are common entry sites of pathogenic microorganisms [89, 90].

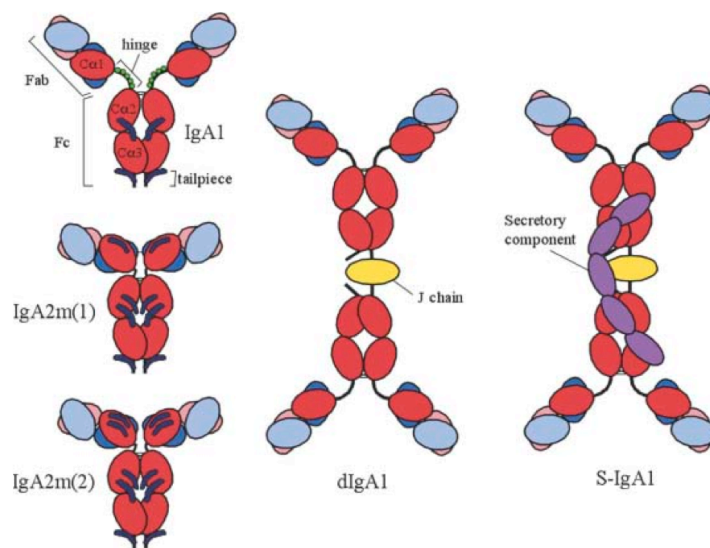


Figure 7 Different forms of human IgA. IgA1, IgA2m(1) and IgA2m(2), and the dimeric (dIgA1) and secretory (S-IgA1) forms of IgA1 are displayed. Red parts indicate constant and pink variable domains of the heavy chain. Constant regions of the light chains are illustrated in mid blue and variable regions in pale blue. The J chain on the dimeric form is shown in yellow and the secretory component in purple. Green circles on the monomeric IgA1 point out O-linked sugars and dark blue circles refer to N-linked oligosaccharides. The image is obtained from Woof and Kerr [h].

Structurally, human IgA comprises two heavy and two light chains and can be subdivided into two classes: IgA1 and IgA2. IgA1 has an extended hinge region of 13 amino acids that is a target of bacterial proteases, while IgA2 is resistant to them. Coherent to this property, IgA2 is predominantly present in mucosal fluid, where SIgA is secreted as polymeric complex with an J chain that consists at least of one IgA2 protein, while serum IgA circulates prevalently as monomeric IgA1 (mIgA1) [91].

The protective nature of SIgA as first line of defense is extensively studied and far better understood than the role of serum IgA that features opposed functional characteristics. Serum IgA facilitates immune suppression by

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inhibition of IgG-mediated phagocytosis, oxidative burst activity, inflammatory cytokine secretion and of immune cell migration. On the contrast to the anti-inflammatory property, abnormal activity of serum IgA is found to be directly associated with autoimmune diseases (celiac and Crohn's disease) and can be also observed in patients suffering of AIDS [89, 90].

Interestingly, *Mus musculus*, which is the most commonly used model organism within immune biology, its IgA composition significantly differs from the human IgA system due to expression of only one IgA subclass, which is predominantly polymeric in serum and as well as the absence of homolog Fc receptors [92].

1.3.8.2 The diversity of IgA receptors

IgA interacts via the Fc part with specific cell surface receptors, of which five are known to date. The first described and most important receptor is Fc α RI (CD89) that can bind all kind IgA subclasses upon ligation on monocytes, interstitial DCs, macrophages, Kupffer cells, platelets, neutrophils or eosinophils and transmits the anti-inflammatory signals of serum IgA [92]. CD89 signal transduction has an unique inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) [93]. Low valency binding of one monomeric serum IgA to dimeric CD89 molecules induces an inhibitory signal due to specific ligand-receptor configuration, where on the other hand polymeric serum IgA that plays a pathogenic role, generates an pro-inflammatory signal with higher avidity [90].

Other structurally unrelated receptors are the polymeric Ig receptor (pIgR), which traffics IgM and IgA across epithelial barriers and releases a dimeric SigA at the mucosal surface and the Fc α / μ receptor on B-cells and macrophages. The asialoglycoprotein receptor (ASGP)-R mediates the IgA metabolism prevalently in hepatic tissue [92].

Moura *et al* discovered in 2001 CD71 as alternative IgA receptor and therefore fifth known member of IgA receptor in a study of mesangial cells regarding IgA nephropathy and the prevalent overexpression of CD71 and polymeric colocalized IgA1 deposits in this cell type [47]. TfR binds exclusively IgA1 antibodies with a significant preference for monomeric IgA1 over polymeric

IgA1 or SIgA, which was confirmed *in vitro* in Daudi B cell line in the absence of CD89. Further, it was shown that IgA1 binding is inhibited by transferrin in a dose-dependent manner. The study hypothesized that the unique IgA1 hinge region where 3 to 5 O-linked glycosylation sites are located, may mediate the selective binding. A subsequent study ^[94] contradicts that assumption and indicates that the specific binding is especially promoted by O- and to a minor fraction by N-linked glycans and not by the presence of IgA1 hinge region alone in primary human mesangial cells. Both types of transferrin receptors, TfR1 and TfR2, which have 45% sequence identity, are capable of IgA1 binding, which suggests that they share the same IgA binding site. In addition, they demonstrated that hypoglycosylation of IgA1 leads to a formation of polymeric IgA1 and increased binding to CD71, which therefore also explains the pathologic complexes in IgA nephropathy ^[94]. Aberrant, polymeric IgA1 expression induces upregulation of TfR and proliferation of mesangial cells and results in increased IL-6 and transforming growth factor β (TGF- β) production ^[95].

More recently, polymeric IgA1 was shown to be involved as a positive modulator via CD71 in erythropoietin dependent erythropoiesis ^[96].

1.4 The TfR as target of pathogens

Recently, a study from Danyelle *et al* in 2013 revealed the transferrin receptor as a virus entry factor of the hepatitis virus C (HCV) in humans. Results show that TfR depleted cells by blocking or siRNA knockdown showed notable decreased infection rates. Furthermore, the results indicate that CD71 acts after CD81 that is also an entry factor and may be involved in HCV internalization process ^[97]. The possible connection between hepatic iron overload in HCV infected patients and the role of CD71 is an aspect that is still under investigation.

In South America, rodents are the source of five zoonotic strains of New World arenaviruses, which are responsible for hemorrhagic fevers in humans and use CD71 as entry factor ^[98]. Interestingly, the viral entry mechanism in humans is analog to the investigated host-virus interaction found in rodents, indicating

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that the TfR of the two species is orthologous enough. Secreted interleukin 6 (IL-6) during the acute-phase response of infection stimulates hepcidin expression and thus leads to a decreased serum iron level due to iron uptake inhibition. As a consequence, TfR expression is upregulated and advances further virus binding and entry, but a direct proof of this correlation is still not evident, although several other studies strengthen the relationship of IL-6, hepcidin and TfR expression [98].

Furthermore, the murine transferrin receptor is also used as an entry factor of a virus, namely the mouse mammary tumor virus (MMTV). Hypothesized for decades that MMTV may be responsible for human breast cancer, several studies exist that argue for or against it, supporting the contra evidence that a rather similar virus potentially contributes to tumor induction [99]. Additionally, other reports identified the transferrin receptor as entry factor in different animal pathogenic viruses [100].

1.5 The transferrin receptor as therapeutic approach

Increased cellular metabolism is directly linked with high transferrin receptor expression to accomplish the enhanced iron need of growth related processes. In common, CD71 is strongly upregulated in malignant tissues compared to a healthy sample. A variety of cancer studies have shown that TfR overexpression is frequently correlated with the malignancy of tumor progression and cancer prognosis as well as the risk of relapse e.g. in bladder cancer [101, 102]. The oncogen C-myc is prevalently overexpressed in human tumors and regulates metabolic and cell cycle related proteins [103]. In 2006, a study gave evidence that the transcription factor C-myc directly acts downstream of CD71 in neoplastic B cells and therefore upregulates its expression and subsequent iron intake, facilitating cell proliferation and growth [104].

Thus, CD71 is used as a potential therapeutic target in anti-cancer drug research. Easy accessible via the cell surface, the TfR can be targeted efficiently and directly with neutralizing or single-chain antibodies, antibodies conjugated with chemotherapeutical or other conjugated compounds or either indirectly via transferrin conjugated compounds [101, 102, 105]. By using the physiological

uptake mechanism, the delivery of drugs is selectively enhanced in malignant cells and also results in the advantage of overcoming multi-drug resistances (MDR), which develop frequently during chemotherapy [102]. Additionally, the difficult to overcome blood-brain-barrier (BBB) can be bypassed via transcytosis by such antibody or Tf conjugated drugs for cancer treatment or other therapies of brain disorders [106]. In the field of Alzheimer's disease research, a bifunctional antibody with low affinity to TfR and with high affinity to the enzyme β -secretase (BACE1) was developed in 2011, which enhanced the distribution of the mAb, leading to an increased decline of the amyloid- β (A β) peptides and their pathologic sedimentation in the brain [107].

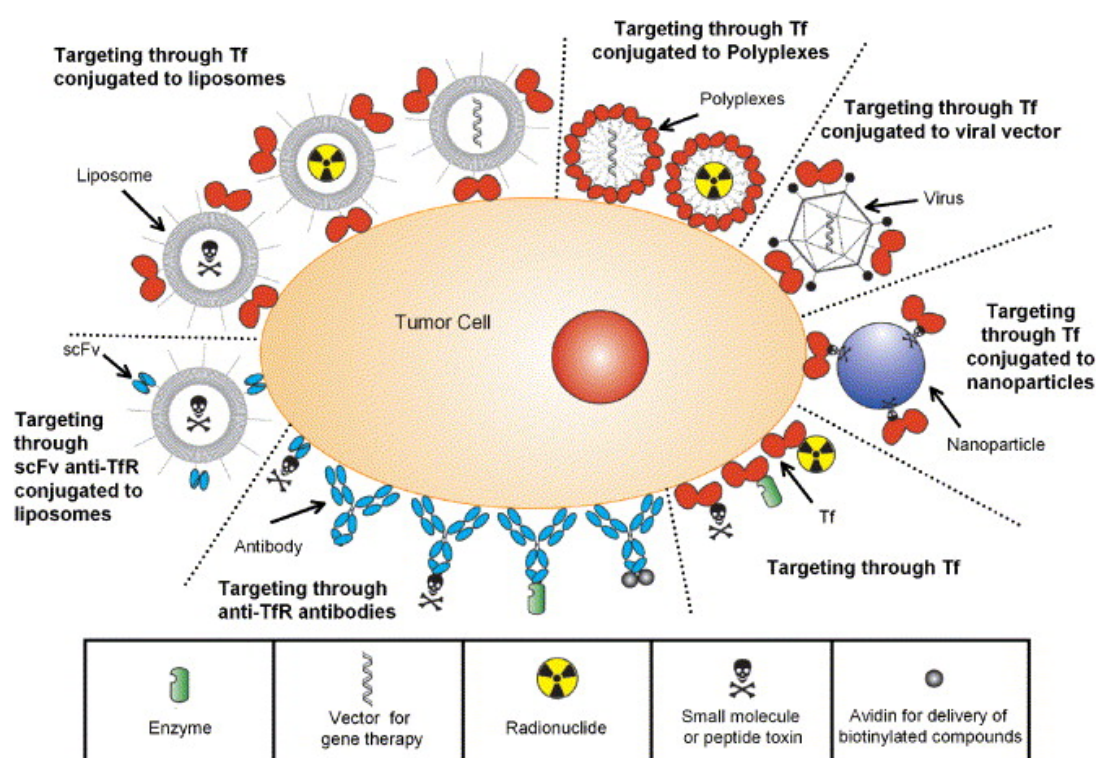


Figure 8 Overview about various different approaches of distributing therapeutical agents into malignant cells. Figure adapted from Daniels *et al* [g].

Gene therapy is another therapeutic approach in cancer therapy with the aim to restore aberrant gene function in order to stop further facilitation of neoplasm and metastasis. Promising results in a phase I clinical trial are obtained in case of the commonly dysregulated, oncogenic transcription factor p53 by using liposomal nanodelivery complex carrier via CD71 [108].

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Noninvasive detection of malignant cells *in vivo* is a preferred diagnostic option. Targeting the highly expressed TfR via iron-oxide nanoparticle linked Tf enables a promising molecular imaging tool in order to detect even small tumors in early cancer stages via magnetic resonance imaging (MRI) [109]. Further, a related method was established by using ^{99m}Technetium-radiolabeled transferrin molecules in a mouse model of rheumatoid arthritis to identify inflammatory cells in synovial fluid or the increased expression of TfR in inflamed synovium via scintigraphy [110].

1.6 Investigating the transferrin receptor function via antibodies

With the mouse anti human IgA mAb 42/6 and IgG mAb B3/25 as tool, Taetle *et al* investigated the mechanism behind the CD71 related growth deprivation *in vitro*. The mAb 42/6 was observed to inhibit Tf binding at 4°C while B3/25 did not, and turned out to only have minimal effects on Tf binding at the physiological temperature of 37°C. First, this circumstance was explained by the steric hindrance of Tf/TfR interaction in presence of large monomeric or multimeric IgA molecules and IgG differs in size [111]. The counterevidence for this hypothesis was performed by Guerrero *et al*, when they showed that the CD71 mAb FG 2/12, which belongs to the IgG1 subgroup, blocked efficiently transferrin binding [112]. Furthermore, mAb 42/6 and B3/25 were shown to decrease TfR expression over time, but only mAb 42/6 inhibits Tf uptake and results in growth deficiency [111]. Additionally, other studies indicate that treatment with mAb 42/6 results in S-phase arrest [113]. In another way acts the IgG CD71 mAb A24, which induces apoptosis in tumor T lymphocytes and competes with Tf for the binding sites and further inhibits Tf uptake [114]. On contrary, there are also CD71 mAbs like FG 1/6 (IgG1) available, an antibody that together with phorbol esters at submitogenic concentrations promotes T cell activation. Moreover, this antibody stimulates IL-2 secretion, which points out a possible complementary signal of the mAb FG 1/6 via CD71 to protein kinase C in T lymphocytes [115]. Induction of tyrosine phosphorylation in T cells by mAb FG 1/6 was reported in a later study and correlates with the kinetics of TCR stimulation [62].

The mAb FG 2/12 that was mentioned before was used together with FG 1/6 and FG 1/5 in the study, which examined the role of CD71 within the immunological synapse. Only FG 2/12 significantly reduced the contact between T cell and APC, and indicates CD71 as factor in synapse formation ^[64].

Furthermore, the previously used CD71 mAbs seem to cover another functional epitope, which was revealed in a natural killer (NK) cytotoxicity assay. FG 1/5 and FG 2/12 successfully inhibited NK lysis of HeLa cells, and only FG 2/12 against Molt-4 cells. From this and other results, the authors suggest that the recognition of cellular surface structures is blocked and no other processes.

2 AIM OF STUDY

The well defined transferrin receptor system serves as crucial iron uptake mechanism that is critical for cellular growth and survival. While in the field of cancer research CD71 is a prominent protein preferentially used as prognostic tool or as therapeutic target in several medical trials, its modulatory role in T lymphocytes is far less studied. Despite accruing evidence indicate that TfR is involved in important T cell events, still little is known about its significance. Several studies used T cell lines rather than human primary T cells, which may not reflect the original situation as cancerous cells permanently express high quantities of CD71.

In order to shed light on the potential immuno-modulatory role of TfR in T cell activation in more detail, like proliferation and cytokine secretion, four murine monoclonal antibodies directed against human CD71 were used. VIP-1, 15-221, 13-344 and 5-528 were produced in our laboratory and cover three different epitopes. Additionally, the role of serum IgA in regard to CD71 and its function is examined in this study.

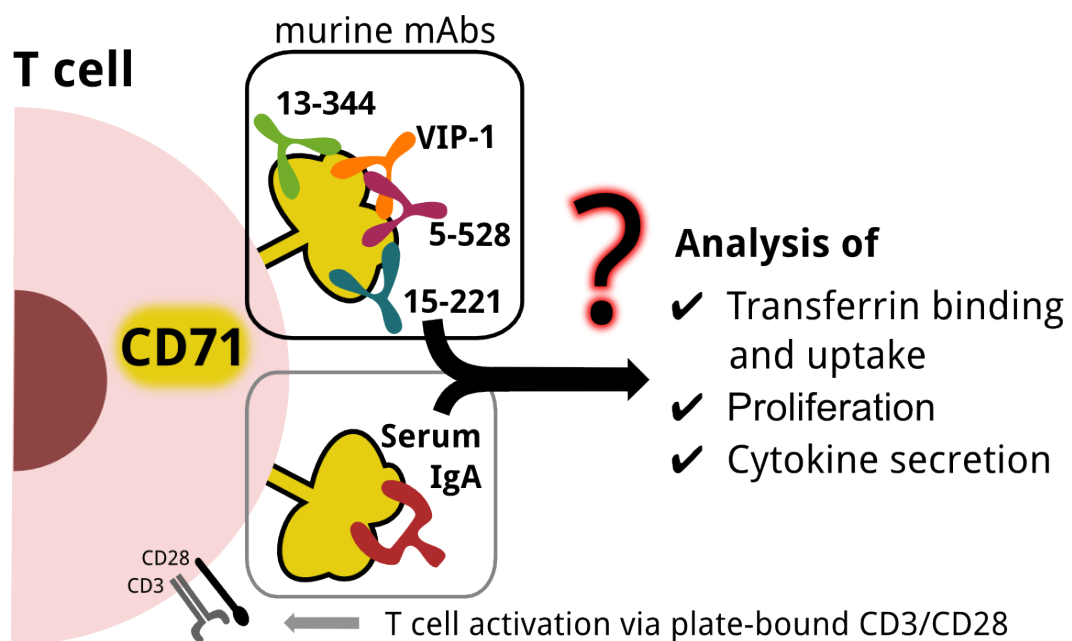


Figure 9 Overview of the aim of the study

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Antibodies

Specificity	Clone	Isotype	Species	Source
T cell stimulation				
CD3	OKT3	IgG2a	Mouse	Jansen-Cilag, Vienna, AT
CD28	15E8	IgG1	Mouse	Caltag Laboratories, Burlingham, CA
CD63	CD63-11C9	IgG3	Mouse	Otto Majdic, IFI, Vienna, AT
Primary antibodies				
Calf intestine alkaline phosphatase	ViAP-2D5	IgG1	Mouse	Otto Majdic, IFI, Vienna, AT
CD71	VIP-1	IgG1	Mouse	Otto Majdic, IFI, Vienna, AT
CD71	15-221	IgG2b	Mouse	Otto Majdic, IFI, Vienna, AT
CD71	13-344	IgG1	Mouse	Otto Majdic, IFI, Vienna, AT
CD71	5-528	IgG1	Mouse	Otto Majdic, IFI, Vienna, AT
Plasma-derived serum IgA		Polyclonal	Human	Kindly provided by Baxter BioScience, Vienna AT
CD89	A59	IgG1, κ	Mouse	Kindly provided by Stockinger Lab, Vienna AT
Secondary antibodies				
Mouse	Oregon Green 488	IgG	Goat	Molecular Probes, Invitrogen, Eugene, US
Human	Anti IgA (α)	IgG	Goat	Molecular Probes, Invitrogen, Eugene, US
Phycoerythrin conjugated Streptavidin (Strep.PE)				Strep.: Sigma-Aldrich, St. Lois, US PE: Cyanotech, Kailua-Kona, US

Table 1 List of antibodies

MATERIALS AND METHODS

3.1.2 Media, Reagents and chemicals

Name	Working dilution	Source
Beriglobin	20 mg/mL (1:8 in 1% BSA-PBS)	Aventis Behring, Vienna, AT
IL-2	50 U/mL	R&D Systems, Inc., Minneapolis, US
Ionomycin	100 nM	Sigma, St. Louis, US
PMA	100 nM	Sigma, St. Louis, US
Penicillin	100 U/mL	GIBCO, Invitrogen, Vienna, AT
Streptomycin	100 U/mL	GIBCO, Invitrogen, Vienna, AT
L-glutamine	2 mM	GIBCO, Invitrogen, Vienna, AT
Fetal calf serum (FCS)	10%	HyClone, Utah, US
Thymidine (methyl-³H)	1 mCi/mL	Perkin Elmer, Waltham, MA
Holotransferrin, FITC conjugate	Varies	Molecular Probes, Invitrogen, Eugene, US

Table 2 List of reagents

3.1.2.1 Other reagents:

RPMI 1640 (GIBCO, Invitrogen, Vienna, AT)

DMEM (GIBCO Ltd., Paisley, UK)

IMDM (Lonza, Verviers, BE)

PBS, calcium and magnesium free (Lonza, Verviers, BE)

FACS fluid (Becton Dickinson, New Jersey, US)

Ficoll-Paque (GE Healthcare, Uppsala, SE)

Medium for cell culture

IMDM and RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 g/mL streptomycin was used.

Freezing medium

RPMI 1640 supplemented with 25% FCS and 10% DMSO

10x PBS stock solution

5.8 g KH_2PO_4 , 16.6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 72 g NaCl were solubilized in aqua bidest (=ddH₂O), filled up to 10 liters and adjusted pH to 7.2.

PBS/BSA stock solution (20%):

100 g BSA and 10 g NaN₃ were dissolved in 500 mL PBS buffer, stirred for 1 hour and filtrated. For the PBS/BSA wash buffer, a 1:20 dilution with PBS buffer was prepared.

MACS-buffer

1000 mL PBS def. + 25mL human serum albumin (stock: 20%, Biotest, Dreieich, DE) + 10 mL EDTA (stock 0,5 M); sterile filtration, stored at 4°C

3.1.3 Cell lines

Name	Type	Source
BW 5417	Murine thymoma	Steinberger, IFI Vienna, AT
BW CD71	Murine thymoma, transduced with human CD71	Steinberger, IFI Vienna, AT
Capan-1	Human epithelial, pancreas	ATTC, Manassas, US
Daudi	Human lymphoblast, B-cell	ATTC, Manassas, US
HCT-116	Human epithelial, colon	ATTC, Manassas, US
HeLa Ohio	Human epithelial, cervix	ATTC, Manassas, US
Jurkat	Human lymphoblast, T-cell	ATTC, Manassas, US
K-562/7618	Human myleoblast, erythroid	ATTC, Manassas, US
KG-1/27024	Human myleoblast, macrophage	ATTC, Manassas, US
MCF-7	Human epithelial, mammary gland	ATTC, Manassas, US
OTMA	Human lymphoblast, B-cell	Otto Majdic, IFI Vienna, AT
Raji	Human lymphoblast, B-cell	ATTC, Manassas US

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RL-5	Rabbit lymphoblast, T-cell	Steinberger, IFI Vienna, AT
SK-N-BE(2)	Human neuroblast	ATTC, Manassas US
SW-480	Human epithelial, colon	ATTC, Manassas US
THP-1	Human monocyte	ATTC, Manassas US
Multi-channel reporter (MCR) Jurkat (clone JE6)	Human lymphoblast, T-cell	Steinberger, IFI Vienna, AT

Table 3 List of cell lines

Cells were cultured in IMDM/RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

3.2 Methods

3.2.1 Freezing and thawing cells

Mammalian cells can be long-term cryopreserved in liquid nitrogen with minimal loss of viability. For that storage method, cells were spun down, counted and resuspended in freezing medium cell type dependent to a concentration between 5 to 100 x 10⁶cells/mL depending on the cell type. 1 ml aliquots were filled into cryotubes (Nalgene Nunc International, Roskilde, DK) and kept overnight at -80°C in a freezing box filled with isopropanol before being transferred to the liquid nitrogen vessel.

Cryotubes were first thawed in a water bath at 37°C, followed by slow resuspension in a drop-by-drop method. Subsequently, the cells were washed twice, counted and cultured in supplemented RPMI1640 with FCS.

3.2.2 Isolation of peripheral blood mononuclear cells (PBMNCs)

PBMNCs were isolated from buffy coats purchased from the Red Cross in Austria and the Department of Blood Group Serology and Transfusion Medicine of the Medical University of Vienna. PBMNCs were separated from whole blood of healthy donors by density gradient centrifugation using Ficoll-Paque. Blood

was diluted 1:2 to 1:3 with heparin-medium. For density gradient centrifugation 15 mL of Ficoll-Paque were prepared in 50 mL tubes and a layer of heparinized blood was carefully pipetted onto. The cells were spun 30 minutes at 900 g without brake. After centrifugation, granulocytes and erythrocytes gathered at the bottom of the tube, peripheral blood mononuclear cells in the interphase. The white interphase ring was transferred into a new tube and spun down (5 minutes at 900g). The supernatant was discarded. The cells were washed twice with MACS-buffer: The pellet was resuspended in a few mL of buffer, filled up, spun down. The supernatant was discarded, and the cells again resuspended. The number of PBMNCs was determined.

3.2.3 Magnetic cell sorting (MACS)

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

3.2.3.1 T cell isolation

To isolate T cells from up to 1×10^9 PBMNCs the MACS technique was used. T cells were isolated by collecting the flow through of PBMNCs depleted by using different mAbs at a concentration of $10 \mu\text{g/mL}$ containing anti-CD14 (MEM18) for collecting monocytes, anti-CD16 (3G8) for binding granulocytes and NK

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cells, anti-CD19 for B cells (BU 12) and anti-CD33 (4D3) for monocytes, thrombocytes and myeloid progenitors (negative selection). Freshly isolated PBMCs were resuspended in 750 μ L MACS buffer and incubated with 250 μ L biotinylated antibodies for 15 min at 4°C. To remove unbound antibodies, the cells were washed twice with MACS buffer and again resuspended in 750 μ L buffer. Then 250 μ L streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, DE) were added to the suspension and incubated for 15 min at 4°C. In the meantime a CS column (Miltenyi Biotec) that can be loaded with a maximum of 1×10^9 cells, was placed onto a VarioMACS apparatus and equilibrated with 40 mL MACS buffer. PBMCs were loaded onto the column, T cells were eluted and washed until a volume of 50 mL was reached. The collected T cells were centrifuged for 5 minutes at 500g, resuspended and the number of cells was determined. Before measuring the cell number two drops of Zap-Oglobin (Beckman Coulter, Miami, US) are added to remove residual erythrocytes.

3.2.4 T cell proliferation assay

96-well plates (Costar, Sigma Aldrich) were coated with CD3 (OKT3), CD3+CD28 (OKT3/15E3) or CD3+CD63 (OKT3/11C9) mAbs at a concentration 5 μ g/mL. The plates were coated with a volume of 50 μ L of antibody-solution overnight at 4°C or for 4 hours at 37°C. Unbound antibodies were removed by washing with PBS. 2×10^5 T cells in RPMI1640 medium supplemented with FCS were added into each well and incubated in combination with soluble CD71 mAbs and/or serum derived IgA. Proliferation of T cells was monitored by measuring (methyl- ^{3} H)-Thymidine (Perkin Elmer, Waltham, US) incorporation on day 3 of the culture. Cells were harvested 17 hours later, and cell proliferation was measured as counts per minute (cpm) on a microplate scintillation counter (Perkin Elmer, Waltham, US). Assays were performed in triplicates.

3.2.5 Restimulation of T cells

T cells were stimulated as in the chapter described before for 72 hours. On the third day, the cells were harvested, washed twice, counted and seeded at a density of 1×10^6 /mL in a 24 well plate in supplemented medium. After 3 days, resting cells were harvested, counted and restimulated with either CD3 alone/

CD3+CD28 or CD3+CD63 plate-bound mAb, supplemented with or without 10 U/mL IL-2, but in the absence of soluble CD71 mAbs in 96 well plates with 2×10^5 T cells per condition. Cell proliferation was monitored by thymidine incorporation assay.

3.2.6 Proliferation assay

Cells were seeded at a density of 2×10^4 / mL in RPMI1640 medium supplemented with FCS in 24-well plates. Soluble CD71 mAbs were added at a final concentration of 10 µg/mL. On the seventh day, cells were counted with CASY cell counter in triplicates.

3.2.7 T cell cytotoxicity assay

Human primary T lymphocytes, which were activated via CD3 or CD3+CD28 in presence or absence of VIP-1, 13-344 or serum IgA for three days, were used as effector cells. Briefly, 2×10^6 target cells (THP-1) were resuspended in 100 µL PBS and labeled with ^{51}Cr (50 µCi) for 1 hour at 37°C. After washing two times with medium, 5×10^3 target cells/well were added to triplicates with titrating numbers of T cells in 96-well U bottom plates. To determine the spontaneous release, only medium was added to the target cells in triplicate and to define the maximal release, 100 µL of a Triton X-100 solution (2%) was added to a separate triplicate of target cells. The cells were then centrifuged and incubated at 37°C for 4 h for induction of redirected killing of target cells THP-1. By adding the CD3 mAb OKT-3 at 1 µg/mL. THP-1 cells were selected, because they express FcγR and facilitate cross-linking of CD3/TCR complexes of the cytotoxic T lymphocyte (CTL) by OKT-3. Released ^{51}Cr was then harvested with a filter harvesting system (Skatron Ltd., Oslo, NO) and measured on a γ-counter (Topcount Model 5000 from Packard). The percentage of specific lysis was calculated by the formula: $(\text{value of probe} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release}) \times 100$.

3.2.8 Analysis of cytokine secretion

The Fluorokine MAP system, using LUMINEX xMAP technology (R&D Systems Inc., Minneapolis, US), provides a tool to simultaneously measure multiple

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cytokines in a single sample. Analyte-specific antibodies are pre-coated onto color-coded beads. Standards and samples are pipetted into the wells and analytes of interest are bound by the immobilized antibodies. After washing away any unbound substances, labeled antibodies specific to the analytes of interest are added. Following a wash to remove any unbound labeled antibodies, the beads are resuspended in buffer and read-out was performed by using the Luminex¹⁰⁰ analyzer. Advanced optics capture the color signals and digital signal processing sorts and translates the signal into real-time, quantitative data for each reaction. Supernatants of activated T lymphocytes were harvested on day three of primary stimulation and IL-2, IL-4, IL-10, IL-13, IL-17, IL-22, IFN- γ and TFN- α were measured via the Luminex¹⁰⁰ System (R&D Systems Inc., Minneapolis, US) as described. Assays were performed in duplicates.

3.2.9 Flow cytometry

In a flow cytometer, single cells move past the excitation source and the light hitting the cells is either scattered or absorbed and then re-emitted (fluorescence). This scattered or re-emitted light is collected by a detector. Scattered light is the consequence of a light beam making contact with a cell, resulting in either reflected or refracted light reaching the detector. The pattern of light scattering is dependent on cell size

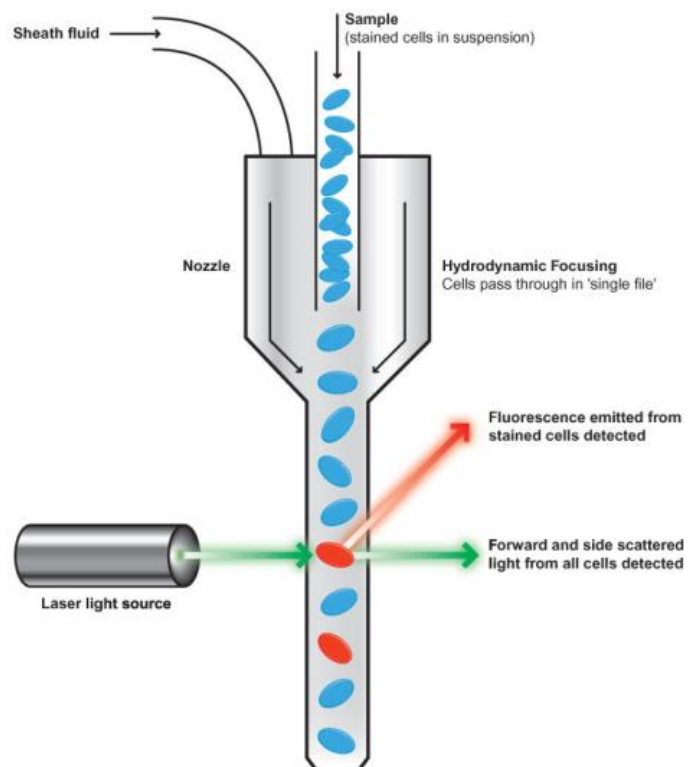


Figure 10 The schematic principle of flow cytometry analysis. A sample moves as single cell stream through the nozzle, where it passes a focused laser light beam. The then scattered light and emitted fluorescence of the cells gives informations about several cell properties like cell size and granularity as well as is fluorescence intensity [i].

and shape, giving relative measures of these cellular characteristics as cells flow through the beam. This can be quite useful, as cells can be sorted on the basis of size or shape into different collection tubes using a technique called electrostatic deflection which employs charged plates to change the path of the cell. Fluorescence-based detection depends on the absorption of light by the cell and the subsequent re-emission of this light at a different wave-length. Flow cytometers make use of this technology by employing filters to block the original light source from reaching the detector, while the fluorescent emission is allowed through for detection, which allows only a very low background of stray light to reach a detector. In flow cytometry experiments, fluorescence is often achieved by the deliberate labeling of a cellular component using a fluorescent marker covalently bound to an antibody, usually a type of dye. These dyes fluoresce only when light of the appropriate wavelength (specified by the frequency of the laser) hits them, causing the emission of secondary light at a different wavelength. Detection of the second wavelength is used as a measure of the presence of the antigen recognized by the antibody on the cell.

3.2.9.1 Membrane staining with unconjugated and biotinylated mAbs

The cell suspension (2×10^5 /condition) was centrifuged 5 minutes at 300g and the pellet was resuspended with 50 μ L Beriglobin in order to block Fc receptors. 20 μ L of the respective antibody at 20 μ g/mL was added to the cells, mixed and incubated 30 minutes at 4°C. In case of biotinylated mAbs, 20 μ L of VIP-1 diluted 1:20, 15-221 diluted 1:180, 13-344 diluted 1:60 and 5-528 diluted 1:15 was used. Each assay was washed twice with 1%-BSA/PBS, centrifuged (5 minutes at 300g, without break), the supernatant was discarded. Then the cells were resuspended in 20 μ L of Oregon green (20 μ g/mL,) or Streptavidin-PE and were kept at 4°C for 30 minutes. Afterwards, they were washed twice again as described before and were resuspended in 100 μ L FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The cell suspension was kept at 4°C until analysis by flow cytometry, using a FACScalibur Flow cytometer (Becton Dickinson, Palo Alto, CA) and Cell Quest Pro Software for data analysis.

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3.2.9.2 *IgA membrane staining*

Cells at a density of 1×10^6 /mL were rotated for one hour at 4°C in presence of 1 mg/mL serum IgA, diluted in RPMI 1640 without FCS. Afterwards the cells were washed twice with 1% BSA/PBS and 2×10^5 cells were resuspended in 20 µL of polyclonal FITC conjugated goat anti-human IgA (α chain) and incubated for 30 minutes at 4°C. The following procedure is analog to described steps after secondary antibody incubation in the section described before.

3.2.9.3 *Receptor down-modulation*

Cells at a density of 5×10^5 /mL were incubated with 20 µg/mL CD71 antibodies or 1 mg/mL serum IgA in a 24-well plate for 17 to 18 hours. Afterwards they were harvested, washed twice, stained and finally analyzed via flow cytometry.

3.2.9.4 *Transferrin binding uptake assay*

The cells (2×10^5 /condition) were centrifuged 5 minutes at 300g, the pellet was resuspended in 25 µL DMEM and transferred in FACS tubes. Afterwards, the cells were incubated with 25 µL of holo-transferrin conjugate (final concentration

10 µg/mL) for 30 minutes at 4°C for binding or 37°C in the incubator for uptake analysis. Each assay was washed twice with 1%-PBS/BSA, resuspending the cells in 100 µL FACS fluid in the end. The tubes were kept on ice until they were analyzed by flow cytometry.

3.2.9.5 *IgA cross inhibition assay*

Cells (2×10^5 /condition) were resuspended in 25 µL PBS to avoid additional Beriglobin derived IgA. Subsequently, serum IgA at 1 mg/mL or CD71 mAbs at 10 µg/mL were added, and incubated at 4°C for 30 minutes. Without washing, the respective antibodies were added: CD71 mAbs at 10 µg/mL or 100 µg/mL biotinylated serum IgA, and again the cells were incubated for 30 minutes at 4°C. Afterwards, the cells were washed twice, and resuspended in 20 µL Oregon Green for unconjugated antibodies or Streptavidin-PE for biotin labeled antibodies. After incubation time of half an hour at 4°C, the cells were washed

twice again, resuspended in FACS buffer and stayed on ice upon flow cytometry analysis.

3.2.10 Fluorescence microscopy

Cells were treated as described in chapter 3.2.7.1 and were resuspended in RPMI1640 medium supplemented with FCS after incubation with the respective antibodies and were kept on ice. For a period of 30 minutes, the cells were transferred in the 37°C incubator with 5% CO₂. Afterwards, the cells were plated on chilled microscope slides and kept in dark and on ice until analysis.

3.2.11 Proteome analysis

In cooperation with Rupert Mayer and Christopher Gerner from the Institute of Analytical Chemistry of Vienna, proteome analysis of untreated and with 10 µg/mL VIP-1 treated Jurkat cells was performed as subsequently described .

Cell lysis & protein extraction. 1 mL of each cell suspension were transferred to Falcon tubes and centrifuged for 5 min at 1800 rpm. Supernatant was removed and 100 µL of sample buffer (7.5M urea, 1.5M thiourea, 4% CHAPS, 0.05% SDS, 100mM DDT) added to each cell pellet and the pellet dissolved on ice by use of an ultrasonic homogenizer (Bandelin electronic, DE). Cell homogenates were transferred to fresh Eppendorf tubes and protein concentrations determined via Bradford assay (Bio-Rad-Laboratories, DE).

Filter aided protein digestion. Sample processing was performed on the basis of the filter aided sample preparation method. 20 µg of whole cell lysate samples prepared previously were loaded onto 3 kDa molecular weight cut off spin columns (Pall GmbH, AT) reduced and alkylated using dithiothreitol and iodacetamide respectively followed by on-filter digestion applying trypsin (Roche Diagnostics, DE) at 37°C over night. Tryptic peptides were purified by means of C18 spin columns (Pierce, Thermo Scientific) and samples dried by vacuum centrifugation. Peptide samples were stored at -20°C for subsequent LC-MS/MS analysis.

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LC-MS/MS analysis. Peptide samples were dissolved in 5 μL of 30% formic acid (FA) in which 10 fmol/ μL of 4 synthetic peptides were spiked for LC-MS quality control. 40 μL of eluent A (97.9% H_2O , 2% ACN, 0.1% FA) was added to the dissolved peptides and 10 μL thereof injected for LC-MS analysis via Dionex Ultimate 3000 RSLCnano LC system coupled to a Q Exactive orbitrap mass spectrometer (both Thermo Fisher Scientific, AT). Coupling between LC and MS was realized using a nanospray ion source (Thermo Fisher Scientific, AT). Measurement of each sample was carried out in duplicates. Pre-concentration was carried out on a 2 cm x75 μm C18 Pepmap100 pre-column (Thermo Fisher Scientific, AT) applying a flow rate of eluent A of 10 $\mu\text{L}/\text{min}$. Peptides were eluted for separation onto a 50 cm x75 μm Pepmap100 analytical column (Thermo Fisher Scientific, AT) by applying a gradient from 8% to 40% of eluent B (79.9% ACN, 20% H_2O , 0.1% FA) at a flow rate of 300 nL/min for 235 min. Mass spectrometer scan range for intact peptides was set from 400 m/z to 1400 m/z at a resolution of 70 000 (at m/z = 200). Recording of MS/MS spectra of the 10 most abundant peptide ions per 1.5 s was achieved by applying HCD fragmentation at 30% normalized collision energy followed by analysis in the orbitrap mass analyzer at a resolution of 17 500 (at m/z = 200).

MS data analysis. Protein identification was realized by using Proteome Discoverer 1.4.0.288 (Thermo Scientific, AT) applying Mascot 2.4 (Matrix Science, UK) as search engine. The search was performed against the human SwissProt Database (v 012013 containing 20 264 protein species entries), allowing 5 ppm mass tolerance for MS spectra and 20 ppm for MS/MS spectra and a maximum of 2 missed cleavages. Other search setting included carbamidomethylation of cysteines as fixed modification and N-terminal acetylation and methionine oxidation as variable modifications. False discovery rates were set to p-values of 0.05 and 0.01 for modest and high confidence peptides respectively. Only peptides identified with high confidence (p-value < 0.01) were used for protein identification.

4 RESULTS

4.1 CD71 epitope recognition of mAbs VIP-1, 15-221, 13-344 and 5-528 on various cell lines

The murine anti human CD71 mAbs VIP-1, 15-221, 13-344 and 5-528 are produced in our laboratory. VIP-1 is a well analyzed and used antibody in various studies, since it was first described in two publications in 1989 by Knapp *et al* [116]. Previous studies characterized and confirmed epitope specificity of our CD71 mAbs by cross inhibition assays, immunoprecipitation, Western Blot in Raji B cell lymphoma cell line and via binding study in human TfR transduced murine (BW) cell line [76].

4.1.1 CD71 expression is upregulated in activated T cells

Resting T lymphocytes do not express CD71, but strongly upregulate TfR expression upon mitogenic stimulation to accommodate the increased iron demand of the cells. The acquired data confirmed elevated CD71 expression in activated T cell as expected and also showed that the four CD71 mAbs detect CD71 at similar levels.

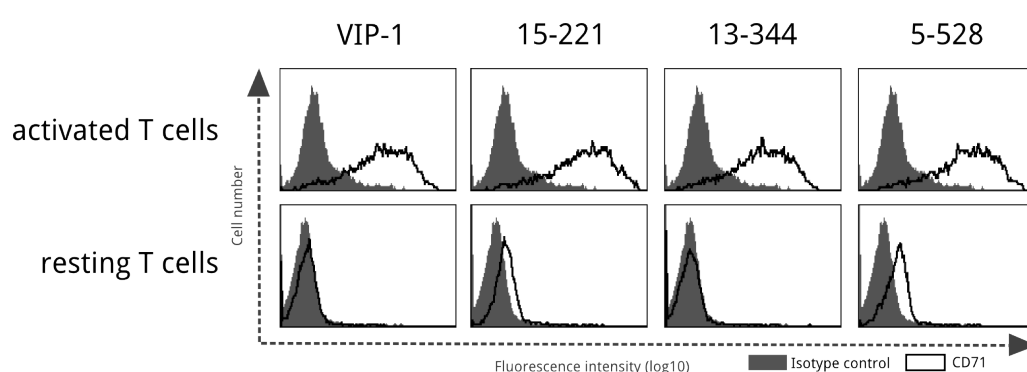


Figure 11 Increased TfR surface epitope expression on activated, primary T cells. Primary human T cells isolated from healthy donors were cultivated for 48 hours in the presence of 100 nM PMA and ionomycin, and were stained for CD71 surface expression. ViAP was used as isotype control.

RESULTS

4.1.2 Reactivity profile of CD71 mAbs are consistent in different cell lines

Human cell lines of various tissue origins and the human CD71 transfected murine BW cell line were tested via membrane staining for their reactivity profile of VIP-1, 15-221, 13-344 and 5-528 (fig. 12a). All cell lines showed a similar binding pattern of each CD71 mAb and further confirmed their specificity against the TfR. Generally, the binding of mAb 15-221 was characterized as partially stronger and 13-344 by a weaker fluorescence signal

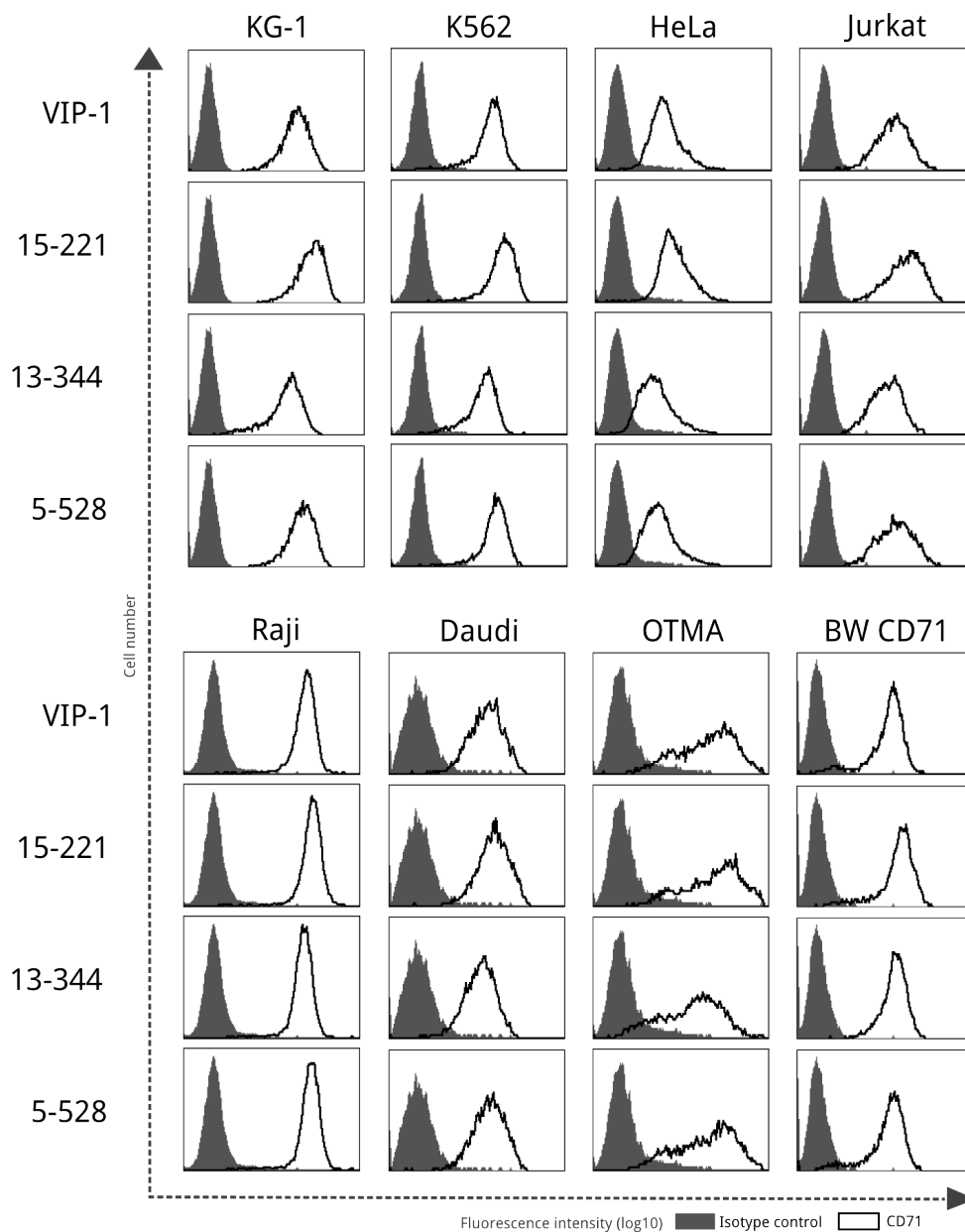


Figure 12 a Comparance of CD71 epitope recognition by VIP-1, 15-221, 13-344 and 5-528 on various cell lines showed similar results. Lymphoid (Daudi, Raji, OTMA and Jurkat), myelogenous (K562, KG-1) and epithelial (HeLa Ohio) cell lines, as well as the CD71 transfected murine cell line BW were immuno-stained at 4°C. ViAP was used as isotype control.

compared to VIP-1 and signals of all four mAbs were coherent. The fluorescence intensity of CD71 mAb 5-528 ranged at the same level like VIP-1. In addition to the HeLa cell line, non-hematopoietic human cell lines were immuno-stained with VIP-1 and 13-344 and represented consistent results (fig. 12b).

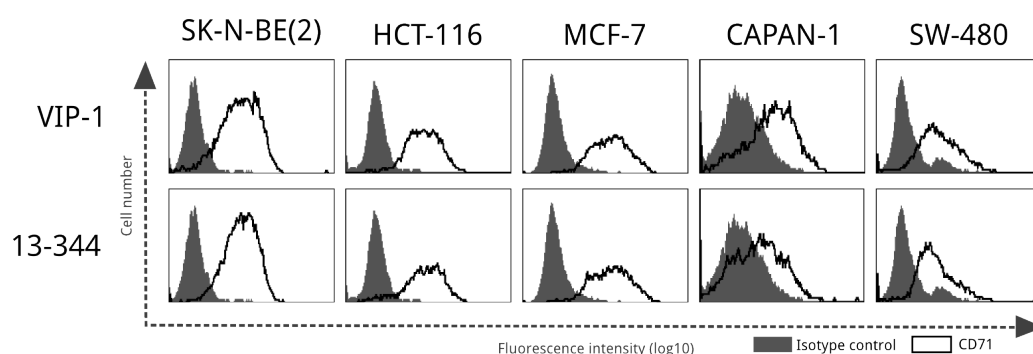


Figure 12 b Reactivity profile of mAbs VIP-1 and 13-344 in SK-N-BE(2) (neuroblastoma), HCT-116 (colorectal carcinoma) and SW-480 (colorectal carcinoma), MCF-7 (breast carcinoma) and CAPAN-1 (pancreatic ductal adenocarcinoma) cell lines. ViAP was used as isotype control.

4.1.3 Cross-reactivity of mAb 13-344 in rabbit cell line RL-5 and murine cell line BW5414

Cross-reactivity of mAb 13-344 with surface proteins of murine T cell and B cell lymphoma (BW5414, BW 1C8), but none with insect cells of *Spodoptera litura* in which a similar TfR/Tf system exists, was observed in previous work [76]. To analyze if the cross-reactivity of mAb 13-344 is restricted to murine cells or not, a rabbit T lymphoma cell line (RL-5) together with murine cell line BW5414 were tested. Equivalent to the former experiment with murine cells, 13-344 cross-reacted with rabbit and murine cells to a similar extent (fig. 13). The specificity of other CD71 mAbs to human tissue remained unaltered and showed no reactivity in the rabbit or murine cell line.

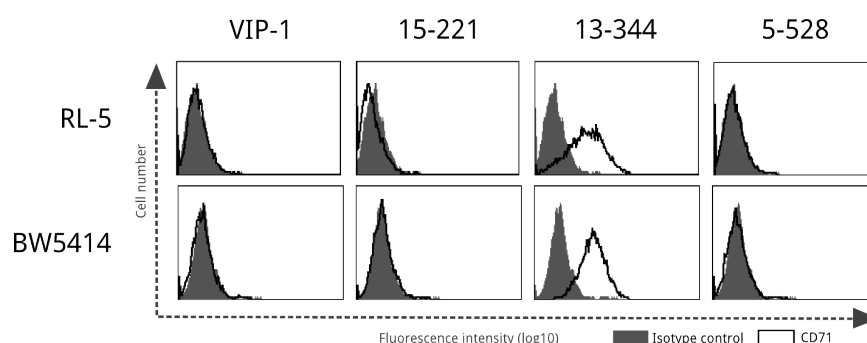


Figure 13 Cross-reactivity of 13-344 defined in BW5414 and RL-5 cell lines via membrane staining under the same culture and staining conditions used in human cell lines.

RESULTS

4.2 VIP-1 and 15-221 inhibit T cell proliferation

Several studies of CD71 mAbs that inhibit *in vitro* and *in vivo* cellular growth of malignant and normal cells are known, like studies with the mAb 42/6 (IgA) [111] or the mAb A24 (IgG) [114]. To determine the impact the four CD71 mAbs on T cells, a T cell proliferation assay was performed. Human primary T lymphocytes were activated via different plate-bound stimuli in the presence of soluble VIP-1, 15-221, 13-344 or 5-528. Significant growth-inhibitory effects were observed of VIP-1 and 15-221, which was reflected by a reduced cpm by more of 85% in presence of a co-stimulatory and by more of 66% in presence of a single TCR signal compared to untreated cells (fig. 14).

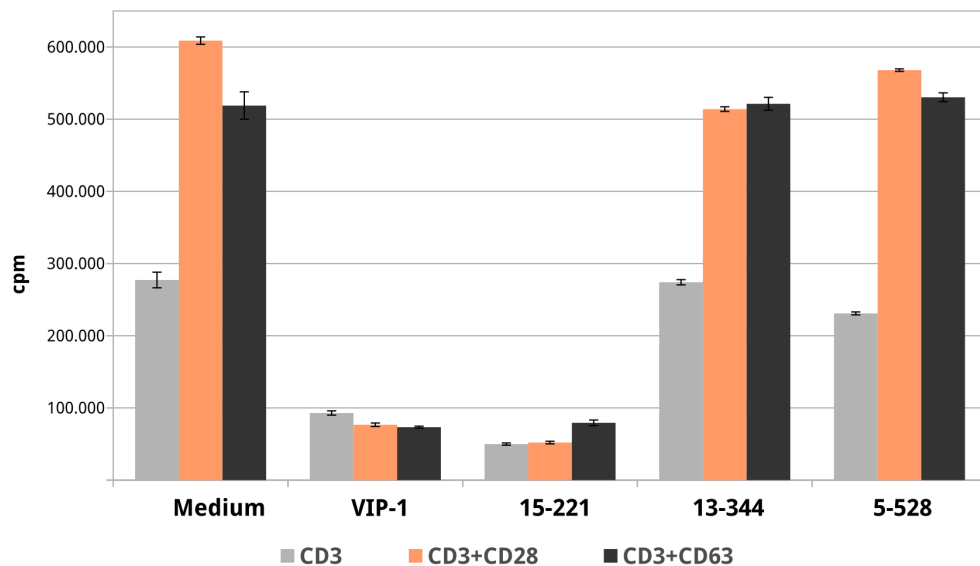


Figure 14 Inhibitory effect of VIP-1 and 15-221 on T cell proliferation. Primary human T lymphocytes were activated via immobilized CD3, CD3+CD28 or CD3+CD63 mAbs with and without soluble CD1 mAbs for three days. Thymidine incorporation was measured on day three after 17 hours. Results represent the mean ($\pm$ standard deviation) of triplicates from one representative experiment of three independent experiments.

4.2.1 Impact of CD71 mAbs on cytokine secretion is stimuli dependent in T cells

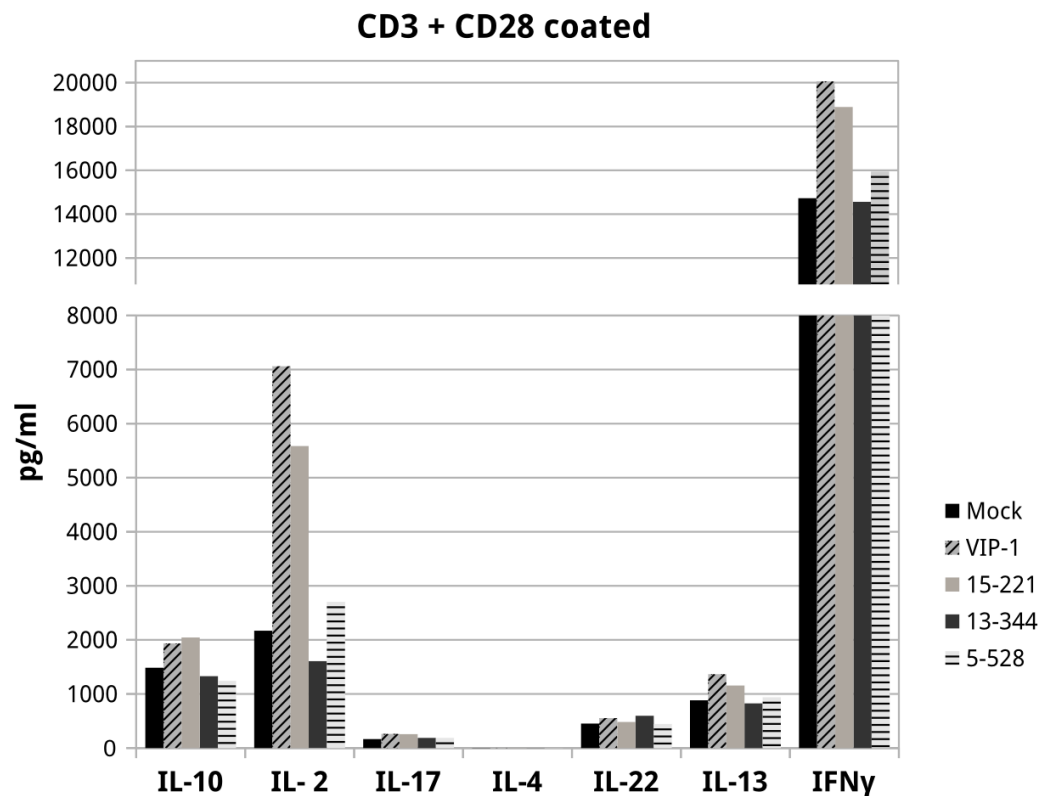


Figure 15 Enhanced IL-2 and IFN- γ production of CD3+CD28 stimulated T cells upon VIP-1 and 15-221 treatment. Primary human T lymphocytes were activated via immobilized CD3+CD28 with and without soluble CD71 mAbs. Supernatants were analyzed on day three of T cell activation. Data of one representative experiment out of two is shown. VIP-1 and 15-221 strongly induce IL-2 production, which is accompanied with elevated levels of IFN- γ , and to a moderate extent of IL-10, IL-13. IL-4 concentrations were measured at a low single-digit rate.

To the T cell stimulation in presence of CD71 mAbs, cytokine production was measured from the supernatant. Cytokine secretion was contradictory modulated, dependent on the stimulation signals (CD3+CD28 or CD3+CD63). Distinct cytokine levels of CD3+CD28 stimulated T lymphocytes in presence of VIP-1 or 15-221 were increased, especially IL-2 up to 2,6 to 3,5 fold induction, followed by a moderate IFN- γ and tendentious IL-10 and IL-13 augmentation (fig. 15). Inverted results were found in CD3+CD63 activated T cells where VIP-1 and 15-221 suppressed the cytokine production. Concentrations of IL-2, IL-22 and IL-13 were reduced down to between 1,6 and 2,7 fold. A moderate reduction of IFN- γ was observed (fig. 16). 13-344 and 5-528 had no significant effect on cytokine secretion, irrespective of co-stimulation.

RESULTS

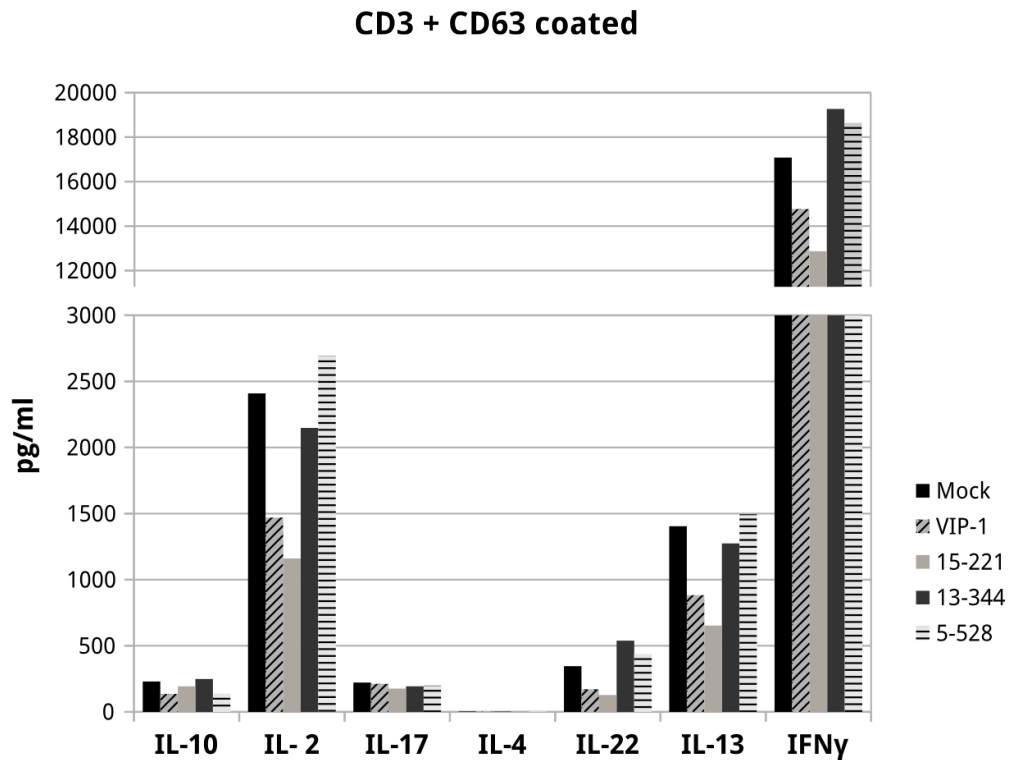


Figure 16 Suppressed IL-2, IFN- γ , IL-13 and IL-22 secretion of CD3+CD63 activated T cells. Primary human T lymphocytes were activated via immobilized CD3+CD63 with and without soluble CD71 mAbs. Supernatants were analyzed on day three of T cell activation. Data of one representative experiment out of two is shown. VIP-1 and 15-221 significantly reduce IL-2, IFN- γ , IL-13 and IL-22 production, and to a minor extent IL-10. IL-4 concentrations were measured at a low single-digit rate.

4.2.2 CD71 mAbs are effective down-modulators

Cell surface receptor down-modulation like with CD71 and other receptors is an often described physiological process that can occur due to permanent receptor engagement by receptor specific antibodies or ligands that induce internalization. To determine the potential down-modulatory abilities of CD71 mAbs, with PMA/Ionomycin preactivated lymphocytes were treated for 18 hours with CD71 mAbs and were immuno-stained only with OG alone to determine the remaining CD71 mAbs on the surface after 18 hours or with additionally added CD71 mAbs to test the level of remaining CD71 epitopes. VIP-1, 15-221, 13-344 and 5-528 induced downregulation of CD71, of which VIP-1 was the strongest and 13-344 was the weakest down-modulator. Remarkably, 13-344 treatment left no detectable mAbs on the cellular surface. 15-221, 13-344 and 5-528 treated cells showed nearly similar remaining,

antibody reactivities, which were marginal and correlated with their down-modulation intensities. (fig. 17).

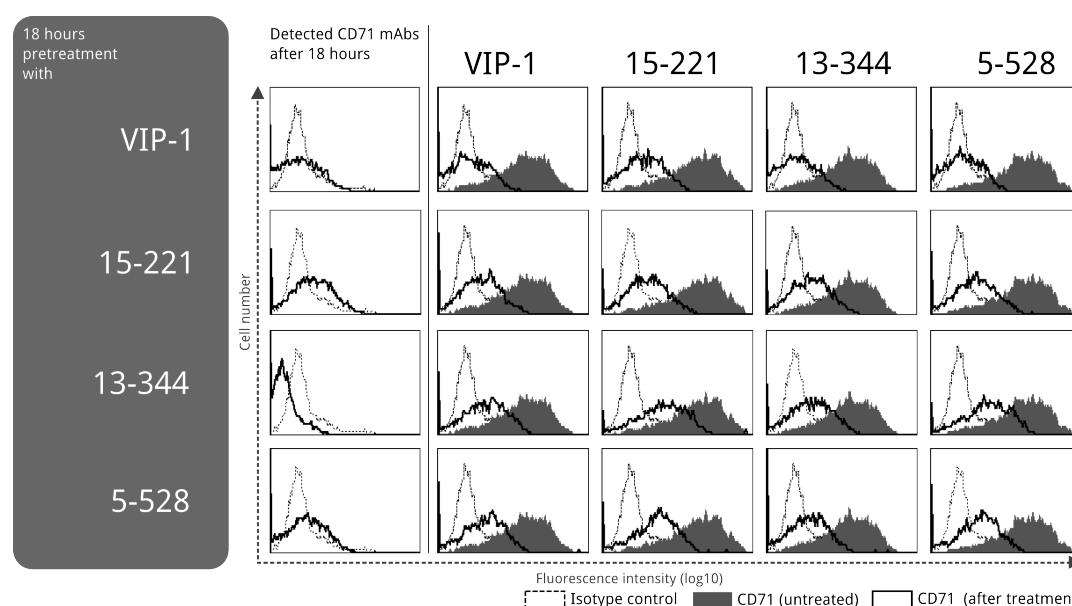


Figure 17 CD71 downmodulation after 18h with PMA/ionomycin activated lymphocytes. Isolated, human lymphocytes were preactivated with 100 nM PMA/ionomycin for 30h and were treated for additional 18 hours with 10 µg/ml CD71 mAbs at 37°C. Membrane staining was either accomplished with OG alone to or with CD71 mAbs. No VIP-1 and 13-344 mAbs are detected on the cellular surface after 18h, and only marginal levels of 15-221 and 5-528 mAbs. VIP-1 induces strong down-modulation of CD71. 15-221, 13-344 and 5-528 are also effective down-modulators. Comparing the percentage of down-modulation by fluorescence intensity shift, 13-344 has the lowest efficiency. Data represent one of three independent experiments.

4.2.3 Transferrin binding and uptake is reduced by VIP-1 and 15-221

In addition to the previously described experiment, lymphocytes treated with CD71 mAbs were analyzed in which measure their transferrin binding and uptake capability is affected by down-modulation. At 4°C, transferrin interaction was hardly detectable in VIP-1 and 15-221 down-modulated T cells, while cells that are cultured in the presence of 13-344 and 5-528 showed unaltered transferrin binding properties. At the physiological temperature of 37°C where transferrin uptake occurs, the inhibitory mAbs VIP-1 and 15-221 reduced the transferrin internalization by 50% in activated lymphocytes after down-modulation. Although 13-344 and 5-528 were previously confirmed to be effective down-modulators, transferrin binding and internalization remained at constant levels (fig.18.).

RESULTS

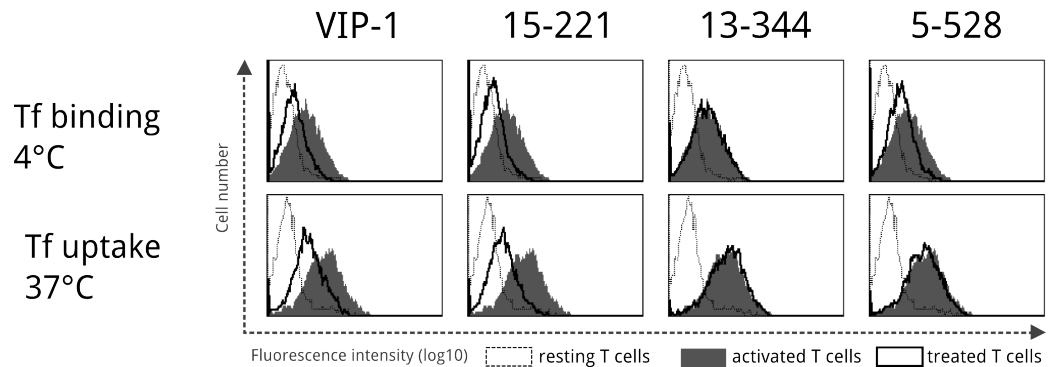


Figure 18 Reduction of transferrin binding and uptake due to VIP-1 or 15-221 down-modulation. Isolated, human lymphocytes were preactivated with 100 nM PMA/ionomycin for 30h and were treated for additional 18 hours with 20 µg/ml CD71 mAbs. At 4°, binding of FITC labelled transferrin was significantly reduced as well as the uptake at 37°C, which was inhibited by 50% in VIP-1 and 15-221 treated cells. 13-344 and 5-528 did not reduce transferrin binding or internalization. One representative experiment from three independent experiments is shown.

4.2.4 VIP-1 internalizes along with the transferrin receptor

To investigate if the down-modulatory CD71 mAb VIP-1 remains on the surface or enters the cell, VIP-1 was fluorophore tagged and its fate was visualized by fluorescence microscopy in Jurkat cells. At the physiological temperature of 37°C, VIP-1 internalized along with the TfR in condensed structures (fig. 19a), but stayed on the surface at 4°C (fig. 19b).

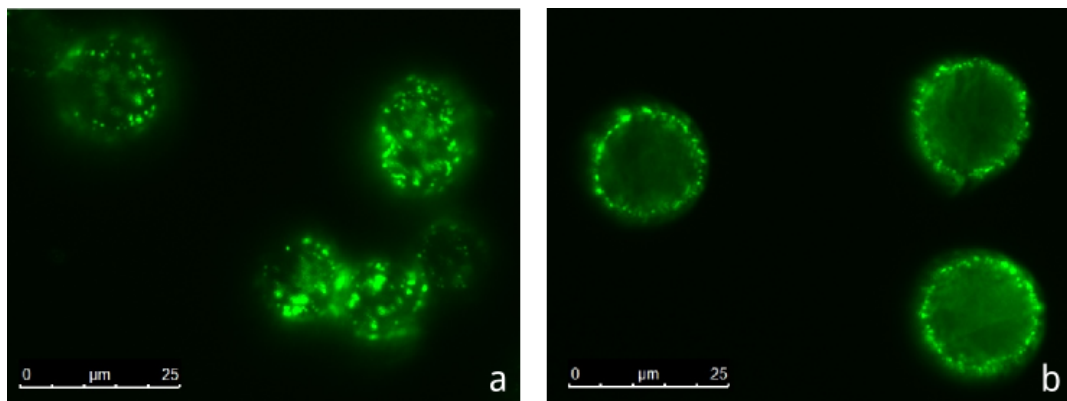


Figure 19 Internalization of VIP-1 at 37°C after engagement on cell surface at 4°C. Jurkat cells were incubated for 30 min at 4°C with 10 µg/ml VIP-1 and were subsequently fluorophore tagged for additional 30 min. Afterwards, the cells were cultured for 30 minutes at 37°C (a) or 4°C (b) and were analyzed via fluorescence microscope (Leica DMI4000 B). Representative pictures from three independent experiments

4.2.5 NFAT, AP-1 and NF-κB are upregulated in pretreated VIP-1 and 15-221 Jurkat cells during activation

A Jurkat T cell line (clone JE6) that was transduced with NF-κB_CFP, nuclear factor of activated T cells (NFAT)_eGFP and activator protein 1 (AP-1)_mCherry fluorescent protein based reporter constructs was used to assess the possible effects of the inhibitory mAbs VIP-1 and 15-221

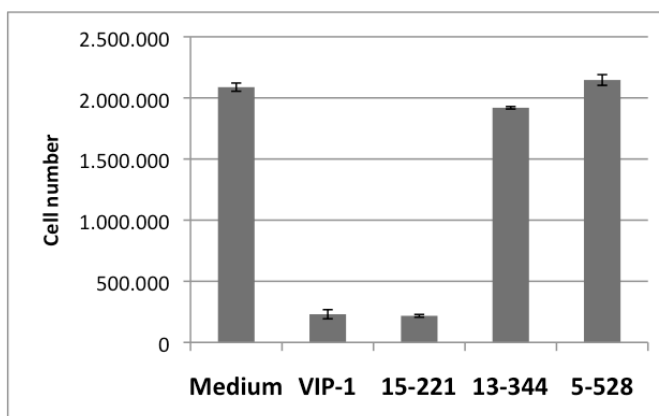


Figure 20 Proliferation of the multi-channel reporter cell line is reduced by 90% on day 7 in presence of VIP-1 and 15-221. 2.5×10^4 cells per condition are seeded in the beginning. Results represent the mean ($\pm$ standard deviation) of a triple analysis.

upon the simultaneous activation of these crucial transcription factors involved in T cell activation. Significant growth inhibitory effects of VIP-1 and 15-221 were monitored in the reporter cell line (fig. 20) and comparable to the obtained results from primary human T cells. These pretreated cells were then activated via plate-bound CD3 and CD28 mAbs in absence of CD71 mAbs for 20h.

Clearly, 13-344 and 5-528 pretreatment did not alter the expression of NFAT, AP-1 and NF-κB during T cell activation, whereas VIP-1 and 15-221 pretreated cells upregulate the reporter activity of all three transcription factors in a similar manner that is reflected by fluorescence peak shifts (fig. 21). In both conditions, by far the strongest increase of expression had NFAT whose mean fluorescence intensity (MFI) quintupled. Further, MFI of AP-1 was 2 times and NF-κB 1,7 times stronger compared to the medium control.

RESULTS

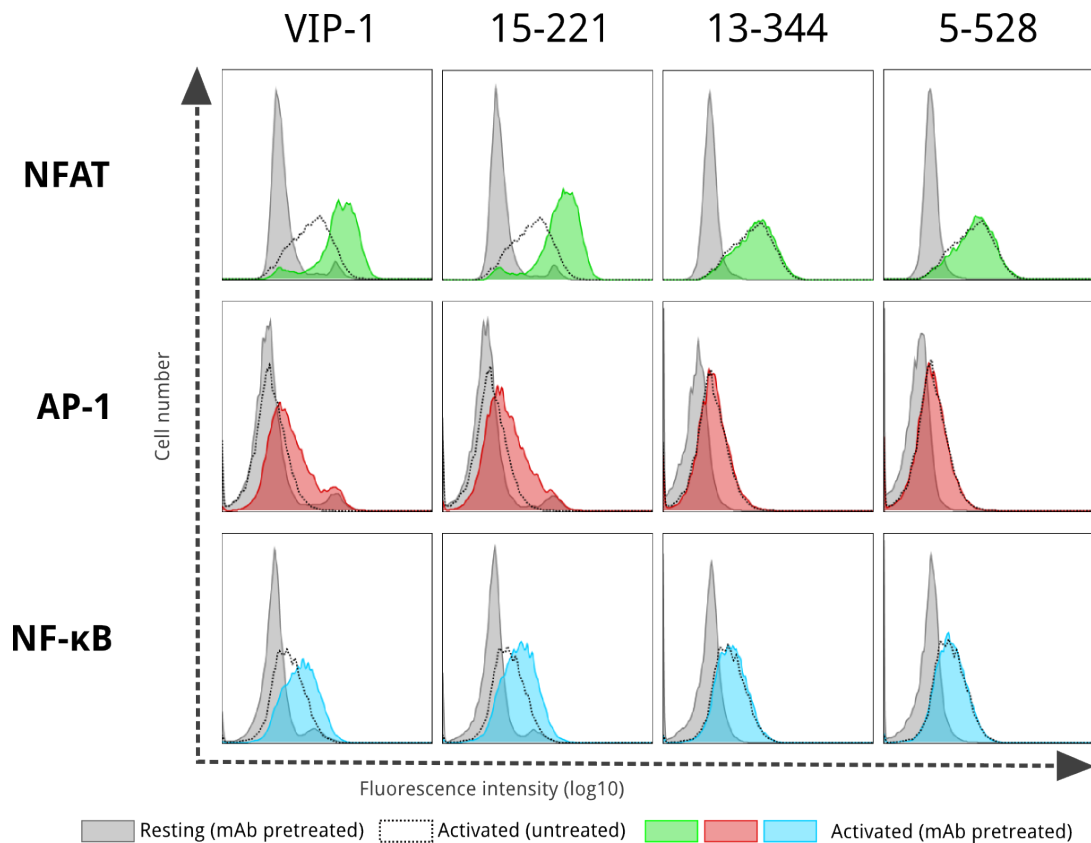


Figure 21 Enhanced activation of NFAT, AP-1 and NF-κB detected in VIP-1 and 15-221 pretreated Jurkat cells during activation via CD3 and CD28. 2.5×10^4 cells/well were seeded in a 24 well plate and cultivated for 7 days in absence or presence of 10 $\mu\text{g/mL}$ CD71 mAbs. Subsequently, the cells were extensively washed and seeded at 5×10^4 cells/well in triplicates. After 20h, CD3 and CD28 stimulated cells were harvested, washed and analyzed via flow cytometry.

4.2.6 Strong inhibitory effect is restricted to lymphoid lineage

In order to test the antiproliferative effect of VIP-1 and 15-221 in malignant cells, four different cell lines, namely HeLa Ohio, K562, Jurkat and Raji, were cultured for 7 days in the presence of one respective CD71 mAb. B and T lymphoma cells were observed to be remarkably sensitive to the treatment. Application of VIP-1 and 15-211 resulted in a reduced cell number of at least 81% to 93%, while 13-344 and 5-528 had no effect on proliferation. HeLa and K562 cells, which are derived from different tissues, had compared to Raji and Jurkat cells only a partially decreased proliferation rate by a maximum of 37% in case of VIP-1 treated K562 cells (fig. 22).

Visual examination via microscopy of VIP-1 or 15-221 treated Jurkat and Raji cells on the day of harvest showed in addition to the reduced cell growth a

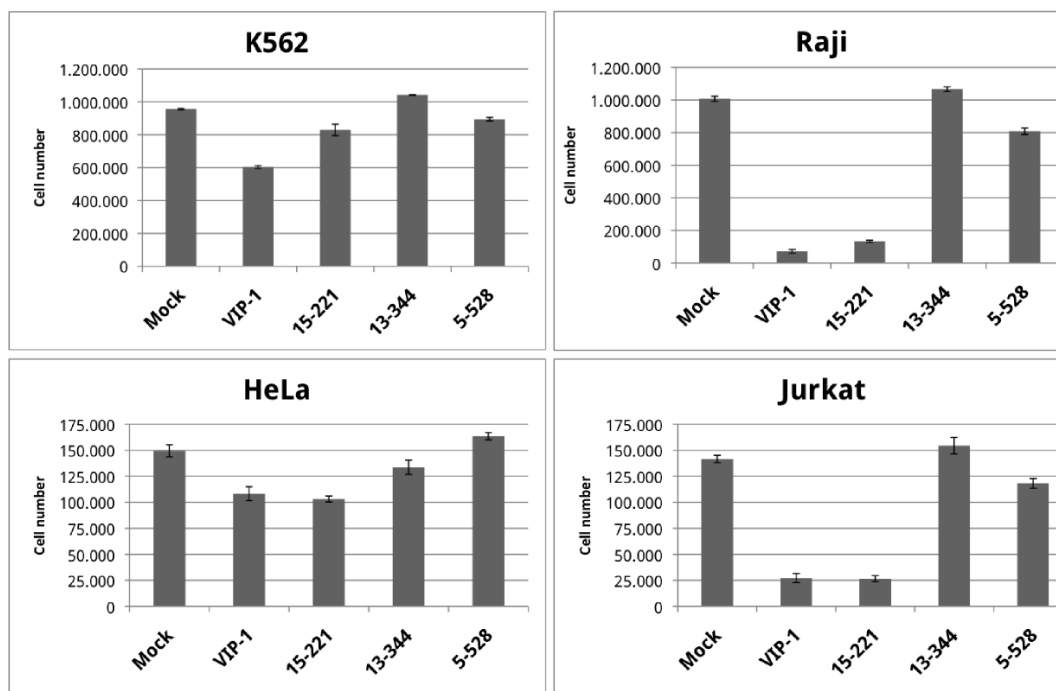


Figure 22 A more than 80% lower proliferation rate is observed in presence of VIP-1 and 15-221 grown Raji and Jurkat cells on day 7 of cell culture. Seeded at 2×10^4 cells per well, K562, HeLa, Jurkat and Raji cells were cultured in medium supplemented with or without CD71 mAbs for 7 days, after which the cell number was determined. The non-lymphoid cell line K562 shows a maximum of 37% cell number reduction in presence of VIP-1 and HeLa a maximum of 31% in presence of 15-221. Proliferation of Jurkat stagnates at a total number of about 2.7×10^4 cells per well. The strong proliferating Raji cell line has an inhibited cell number of 93% in case of VIP-1 and 87% in case of 15-221 treatment. Results represent the mean ($\pm$ standard deviation) of triple analysis from one representative experiment of three independent experiments.

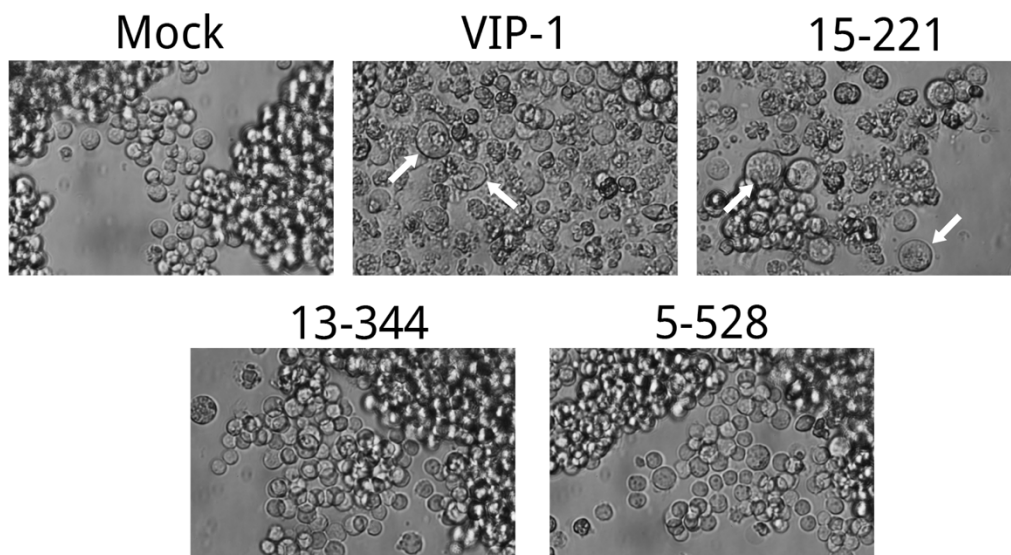


Figure 23 Cell morphology of Jurkat cells after seven days in presence of CD71 mAbs. Pictures were taken at 40x magnification using an inverted Nikon Diaphot microscope. Arrows highlight cells with increased cell size and granularity.

frequently aberrant cell morphology, which is characterized by an enlarged cell size and an increased granular segmentation within the cells (fig.23).

RESULTS

4.2.7 Inhibitory CD71 mAb VIP-1 has a sustainable effect in Raji and Jurkat cell lines

K562, Jurkat and Raji cells were cultivated for 7 days in the absence or presence of VIP-1 and 13-344 and were subsequently cultured for additional 14 days in medium without CD71 mAbs after washing. The cell number, which was determined at the end of each week before transfer to the fresh medium, showed that the proliferation of Jurkat and Raji cells was inhibited at 2 and 3 weeks in the absence of VIP-1. It is mentionable that 13-344 pretreated Jurkat showed an enhanced growth rate in week 2, which became insignificant in week 3 (fig.24).

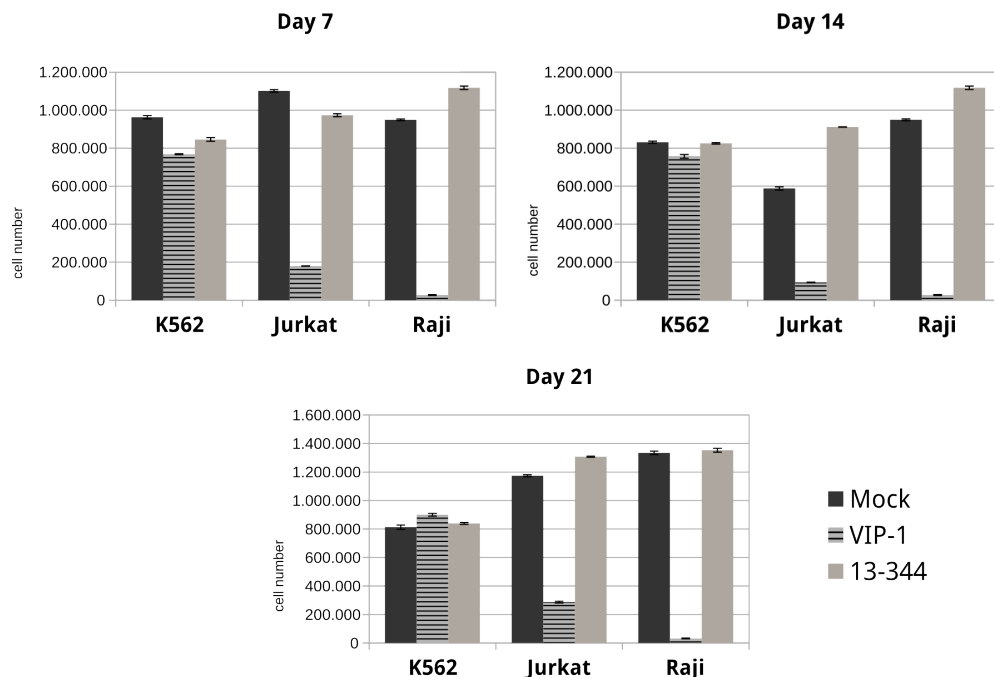


Figure 24 Sustained inhibitory effect on proliferation on lymphoid cell lines of VIP-1. 1.5×10^4 K562 cells, 2.5×10^4 Jurkat cells and 1×10^4 Raji cells were cultured w/ or w/o mAbs VIP-1 and 13-344 for one week. After washing, 2×10^4 cells were transferred in fresh medium w/o CD71 mAbs and cultured for another 7 days. This step was repeated once. Cell number was analyzed on the last day of one week. A sustained effect is observed with VIP-1, in lymphoid cell lines. 13-344 induced an enhanced proliferation of Jurkat and Raji cells in week 2. Representative results from one out of two experiments are shown. Bars represent the mean ($\pm$ standard deviation) of triplicate measurement.

In addition, the surface expression of CD71 was controlled in all three cell lines after the third week. The VIP-1 epitope was found to range at the same levels

like in the untreated cells and therefore showed that the TfR expression is fully restored (fig. 25).

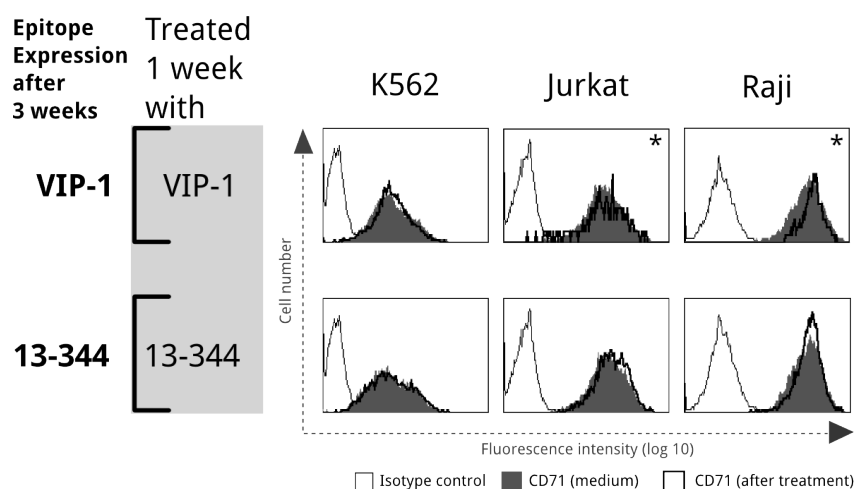


Figure 25 VIP-1 and 13-344 epitope expression of cells grown one week in presence of VIP-1 and 13-344 and subsequently for two weeks in absence of VIP-1 and 13-344. Growth impaired VIP-1 treated Jurkat and Raji cells contain the same levels of CD71 on the surface compared to the untreated cells. * The fluorescence signal of VIP-1 treated Jurkat and Raji cell was scaled to the same extend of the cell number of untreated cells due to the lack of an adequate number of cells.

4.3 Proteome analysis of VIP-1 treated Jurkat cells

For 84 hours, 1×10^5 Jurkat cells were cultured in absence or presence of 10 $\mu\text{g/mL}$ VIP-1 and a significant inhibition of proliferation was already observed (fig. 26). Subsequently, aliquots were sent for proteome analysis. 3330 proteins in untreated and 2922 proteins in VIP-1 treated

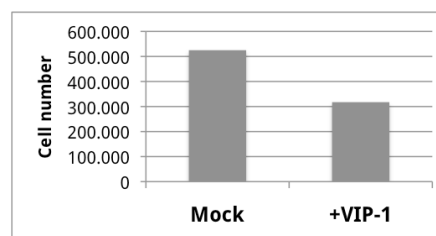


Figure 26 Proliferation of Jurkat cells after 84 hours.

Jurkat cells were identified with a high confidence scale. Proteins, which were not identified in VIP-1 treated cells but in untreated and vice versa, were qualitatively selected. From this pool, proteins, which were not directly related to cell proliferation and immunological processes and had less than 2 peptides and 5% coverage were excluded. The minimized pools of less than ten candidates are enlisted in table 4 and 5.

RESULTS

UniProt Access Nr	Score	Coverage [%]	# Proteins	# Unique Peptides	# Peptides	#PSMs	#AAs	MW [kDa]	calc. pI
O75122	138.90	6.41	1	5	6	6	1294	141.0	8.47
CLIP-associating protein 2									
Q8WVJ2	171.76	31.21	1	5	5	5	157	17.7	5.07
NudC domain-containing protein 2									
P51148	206.32	36.57	2	3	5	5	216	23.5	8.41
Ras-related protein Rab-5C									
P51151	97.46	13.93	1	2	2	2	201	22.8	5.47
Ras-related protein Rab-9A									
Q15907	253.41	32.11	2	7	7	8	218	24.5	5.94
Ras-related protein Rab-11B									
Q92769	83.05	12.30	1	3	3	3	488	55.3	5.91
Histone deacetylase 2									
Q9UBK9	59.62	15.29	1	2	2	3	157	18.2	7.59
Protein UXT									

Table 4 A selected list of proteins that are absent in VIP-1 treated Jurkat cells.

UniProt Access Nr	Score	Coverage [%]	# Proteins	# Unique Peptides	# Peptides	#PSMs	#AAs	MW [kDa]	calc. pI
Q8N5F7	82.73	3.86	1	2	2	2	415	47.1	10.11
NF-kappa-B-activating protein									
Q9Y316	80.91	6.73	1	2	2	2	297	33.7	7.14
Protein MEMO1									
Q14699	33.31	8.30	1	3	3	3	578	63.1	5.67
Raftlin									
Q96E39	301.50	31.28	2	1	9	12	390	42.1	9.89
RNA binding motif protein, X-linked-like-1									
O95630	26.06	8.73 %	1	2	2	2	424	48.0	6.29
STAM-binding protein									
O15405	68.28	5.90 %	4	2	2	2	576	63.3	7.85
TOX high mobility group box family member 3									

Table 5 A selected list of proteins induced in VIP-1 treated Jurkat cells.

4.4 VIP-1 and 15-221 induce a hypoproliferative state

In order to examine if VIP-1 or 15-221 treatment leads also to a sustained growth inhibitory effect in primary T cells, CD71mAb pretreated T lymphocytes were restimulated via the corresponding receptors CD3, CD3+CD28 and CD3+CD63 of the primary stimulation, but in the absence of soluble CD71 mAbs. T cells, which were activated in presence of VIP-1 and 15-221 during primary stimulation, still had an impaired proliferation response. CD3+CD63 restimulated T cells had a reduced cell count of over 90%. T lymphocytes, which were activated via CD3+CD28, 15-221 pretreated cells were less affected than VIP-1 pretreated cells, which score 32% of measured cpm to control cells. Rescue of proliferation was not achieved by addition of exogenous IL-2 in CD3+CD28 stimulated T cells, and was marginally successful in case of 15-221 pretreated T cells activated via CD3+CD63, which was elevated by 5% (fig. 27).

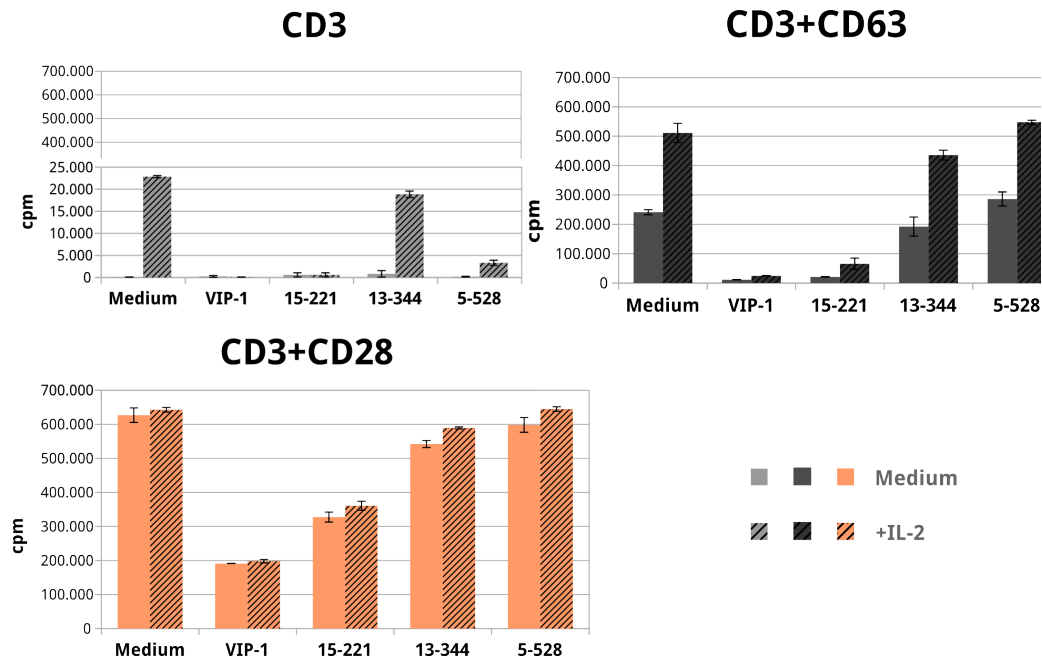


Figure 27 Proliferation of VIP-1 and 15-221 pretreated and restimulated T cells is still impaired. Addition of exogenous IL-2 does not abolish this substandard effect. Human, primary T cells that were previously activated in the presence of soluble CD71 mAbs, are restimulated in the absence of CD71 mAbs either alone or with exogenous IL-2. T cells, treated with VIP-1 or 15-221 in the primary stimulation show diminished proliferation capacities. Bars represent the mean ($\pm$ standard deviation) of triplicates of one representative experiment of three independent experiments.

4.4.1 CD71 is entirely expressed on hypoproliferative T cells

T lymphocytes, which were analyzed in the previous section were additionally immuno-stained for CD71 expression to test whether the cell growth was inhibited due to impaired transferrin uptake or not. Clearly, the results indicate that the TfR of CD71 pretreated cells was fully expressed like the untreated cells upon restimulation (fig. 28).

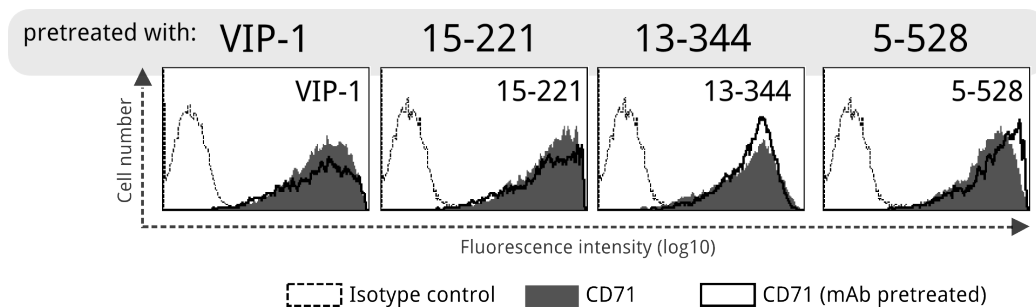


Figure 28 Unchanged CD71 expression detected after three days of restimulation via CD3 and CD28 of CD71 pretreated T lymphocytes. Detection of CD71 via biotinylated mAbs and Strep.-PE.

RESULTS

4.5 CD71 mAbs alter serum IgA interaction

4.5.1 Binding of the alternative CD71 ligand IgA detected in B and T cell lines

Only a limited fraction of lymphoid cell lines like Daudi, Jurkat or U937 were used in studies regarding CD71 as a new IgA receptor [47]. We further investigated the mIgA1 binding abilities of two other B cell lines like Raji, and OTMA, as well as the myeloid cell line KG-1 and the human TfR transfected murine BW cell line. All tested cell lines and primary, activated lymphocytes were able to bind serum IgA, except KG-1. The major IgA receptor, CD89, was absent on Jurkat cells and marginally expressed on Raji cells.

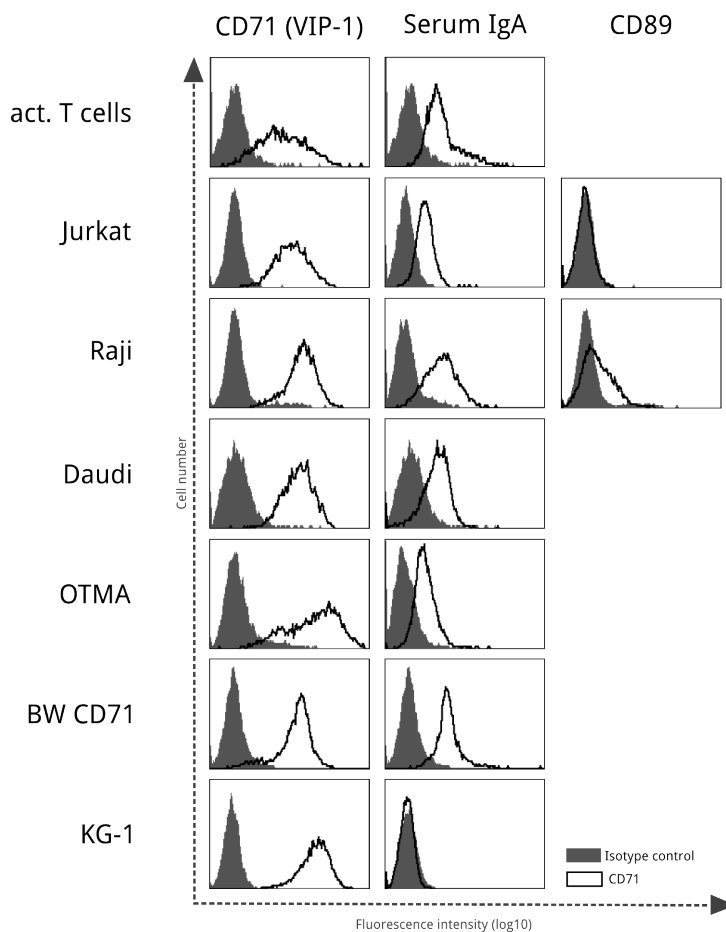


Figure 29 B and T cell lines bind serum IgA1. Surface staining with 1 mg/ml IgA and polyclonal FITC conjugated goat anti-human IgA (α chain) Ab as secondary Ab. Several cell lines were tested for their ability to bind serum IgA1. Except for KG-1, all B and T cell lines, the CD71 transfected cell line as well as activated T cells can bind human, purified mIgA1 on their surface.

4.5.2 VIP-1 and 15-221 deplete serum IgA engagement

A competitive binding assay of VIP-1, 15-221 and serum IgA was performed with alternating preincubation of one of them. Pretreatment of Raji or BWCD71 cells with serum IgA1 did not alter the binding intensity of each antibody. In contrast to the previous results, CD71 mAb preincubated cells, showed a reduced IgA binding of 70% with VIP-1 and a limited reduction of 36% of 15-221 pretreated Raji cells. In BWCD71 cells, 15-221 is the stronger inhibitor with 81% serum IgA binding reduction. VIP-1 was still efficient at blocking IgA (fig. 30).

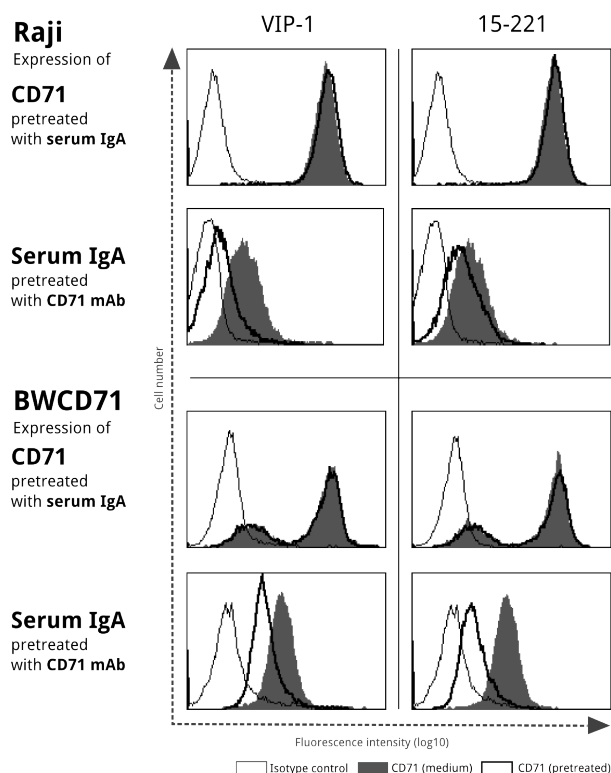


Figure 30 Efficient hindrance of serum IgA binding by CD71 mAbs in Raji and BW-CD71 cell lines. To IgA or CD71mAb pretreated Raji and BW-CD71 cells were CD71mAb or IgA added. VIP-1 or 15-221 were detected with OG and biotinylated IgA with Streptavidin as developing reagent. Preincubation with IgA does not alter the binding intensity of VIP-1 and 15-221 in Raji and BW-CD71 cell line. In the human B cell line Raji, VIP-1 efficiently reduces IgA binding by 70%, 15-221 has a weaker inhibition ability (~36%). In BW-CD71, the outcome is the opposite with 15-221, which exhibits the stronger inhibitory effect with 81% IgA binding reduction. VIP-1 reduces the IgA fluorescence by 60%.

4.5.3 Serum IgA fails to down-modulate CD71, transferrin uptake remains unaffected

The potential ability of IgA as natural ligand to down-modulate the TfR was analyzed under the same conditions described in section 4.2.2. Clearly, the results showed that IgA failed to down-modulate CD71 although minor alterations of the epitope expressions of 13-344 and 5-528 could be observed as shown in figure 32. Tf uptake of long-term IgA treated cells was unaltered (fig.31).

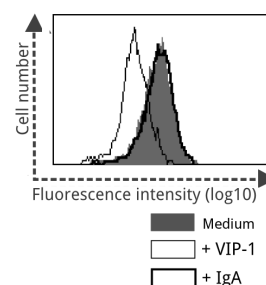


Figure 31 Long-term IgA treatment has no effect on transferrin uptake. Transferrin uptake was measured with Jurkat cells, which were treated with IgA and VIP-1 for 18 hours.

RESULTS

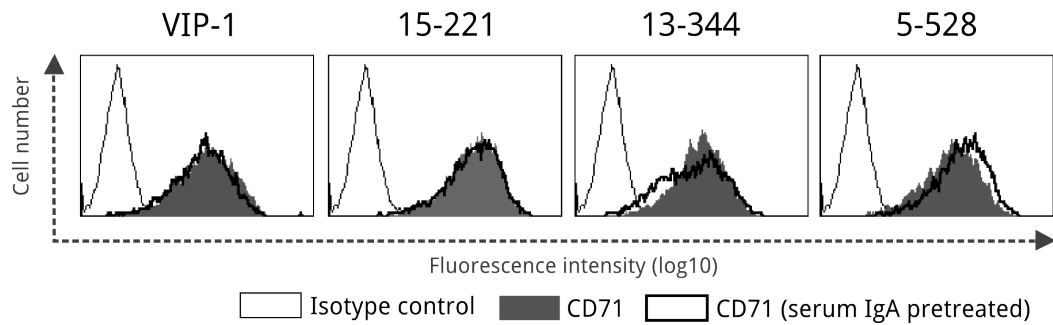


Figure 32 Serum IgA does not down-modulate CD71. Treatment of Jurkat cells with 1 mg/mL IgA for 18 hours at 37°C has no impact on CD71 expression. Marginal downmodulation of 13-344 and upmodulation of 5-528 can be observed.

The previous experiment showed that the Tf uptake after 30 minutes remains steady after serum IgA application of 18 hours (fig. 32). In order to cover different time points of analysis, Jurkat cells were pretreated with IgA and Tf internalization was measured after different periods of time. Only after the shortest time span of 5 minutes, a minor inhibition in presence of IgA was detected. Starting at the time point of 10 minutes, no variation was observed (fig. 33).

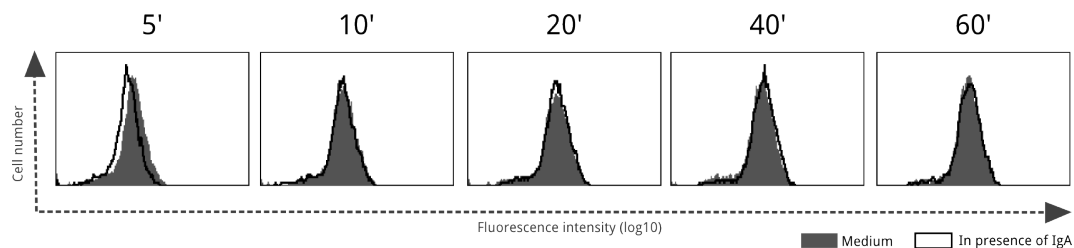


Figure 33 Tf levels remain constant over time upon IgA treatment in Jurkat cells. Jurkat cells were pretreated for one hour at 4°C with 1 mg/mL IgA, 50 µg/mL FITC conjugated Tf was added and were incubated for 5, 10, 20, 40 or 60 minutes at 37°C. After 5 minutes, Tf uptake is marginally reduced by 27% of median fluorescence. This difference is already absent after additional 5 minutes and does not occur at the other time points.

4.5.4 Proliferation of B and T cell lines are not influenced by serum IgA

By culturing Raji and Jurkat cells in presence of different soluble serum IgA concentrations for three days, the effect of IgA on the cell growth of malignant lymphoid cells was examined. Figure 34 clearly illustrates that none of the cell lines was significantly compromised in its proliferation, which is represented by cpm values (fig. 34).

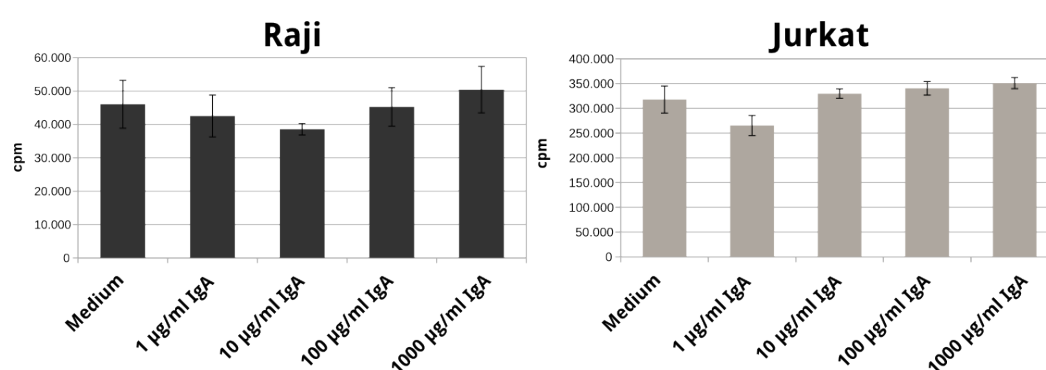


Figure 34 Unaffected proliferation of Raji and Jurkat cell lines in presence of different IgA concentrations. 5×10^4 cells / well were seeded and treated with 1, 10, 100 or 1000 µg/mL IgA for 3 days. The time span of ^3H T incorporation was 18 hours. Data from one representative experiment out of three is shown. Bars represent the mean ($\pm$ standard deviation) of triplicates.

4.5.5 Addition of serum IgA during T cell activation has no proliferative impact

To determine whether soluble IgA may influence T cell activation via immobilized CD3 or CD3 + CD28 in a dose-dependent manner, T cells were cultured in presence or absence of different IgA concentrations for 3 days. The number of recorded cell counts was nearly equal with increasing concentrations of IgA in CD3 and CD3+CD28 activated T cells (fig. 35).

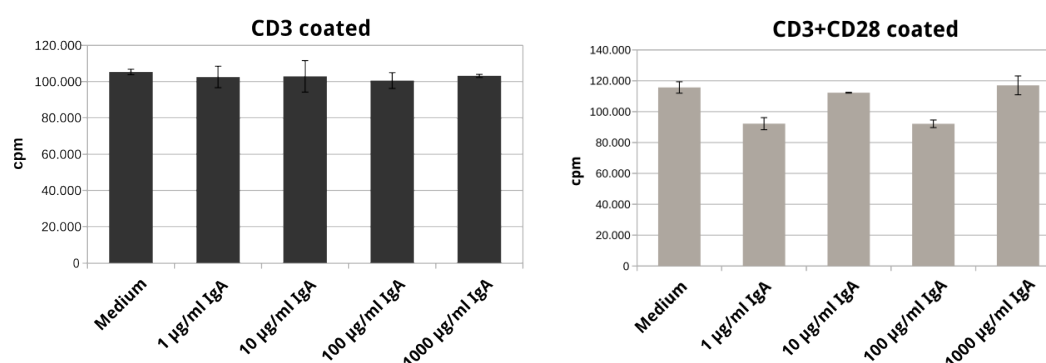


Figure 35 T cell activation via plate-bound CD3 or CD3+ CD28 is not modulated by serum IgA. Isolated human T lymphocytes were activated for three days via immobilized CD3 or CD3+CD28 in presence of 1, 10, 100 or 1000 µg/mL IgA. Thymidine incorporation was measured on day three after 17 hours. Bars represent the mean ($\pm$ standard deviation) of triplicates.

RESULTS

4.5.6 Cytokine secretion is moderately modulated by serum IgA in CD3 activated T cells

Cytokine secretion of activated T cells treated with increasing concentrations of serum IgA from the previous section 4.5.5 was analyzed. In CD3 stimulated T lymphocytes, production of all measured cytokines decreased with increasing concentrations of serum IgA, whereas IL-2 concentrations remained constant. T cells, which underwent activation via CD3 and CD28, showed only minor reduction of IL-2 and IL-10, whereas the cytokine levels of IL-22, IFN γ and IL-4 were minimally enhanced. In summary, cytokine production is mildly affected by serum IgA, and does not directly correlate with the enhanced IgA concentrations by tenfold.

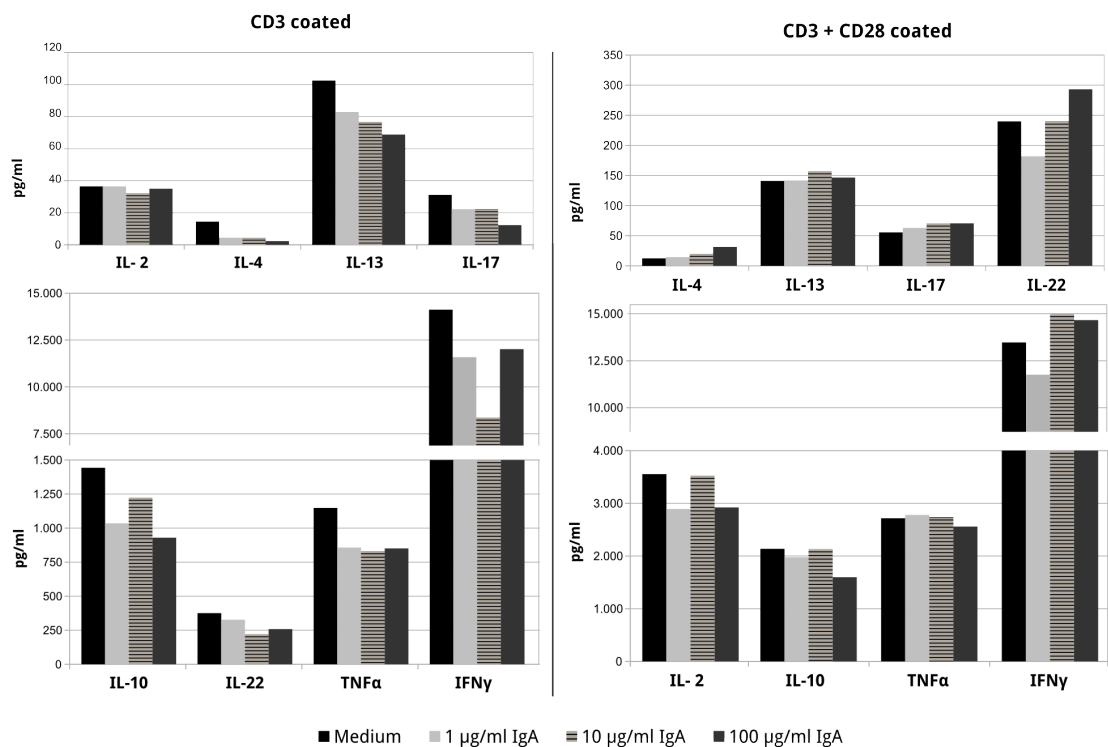


Figure 36 Modest changes of cytokine secretion by serum IgA in T cells. Supernatants of human T cells activated for three days in presence of 1, 10 or 100 µg/mL serum IgA with plate-bound CD3 or CD+CD28 were measured for cytokine secretion. CD3 activated T cells show decreasing cytokine levels, except for IL2, while the results in CD3+CD28 activated are heterogenous.

4.5.7 Serum IgA does not compensate the inhibitory effect of VIP-1 and 15-221

The potential compensation for the growth-inhibitory effect of VIP-1 and 15-221 with serum IgA was analyzed in T cell proliferation assays with different stimulation signals. Apparently, serum IgA failed to abolish the inhibited proliferation by VIP-1 and 15-221. Increasing concentrations of IgA showed no effect upon VIP-1 and 15-221 treated T lymphocytes. Further, no effect upon proliferation of 13-344 or 5-528 together with serum IgA was observed.

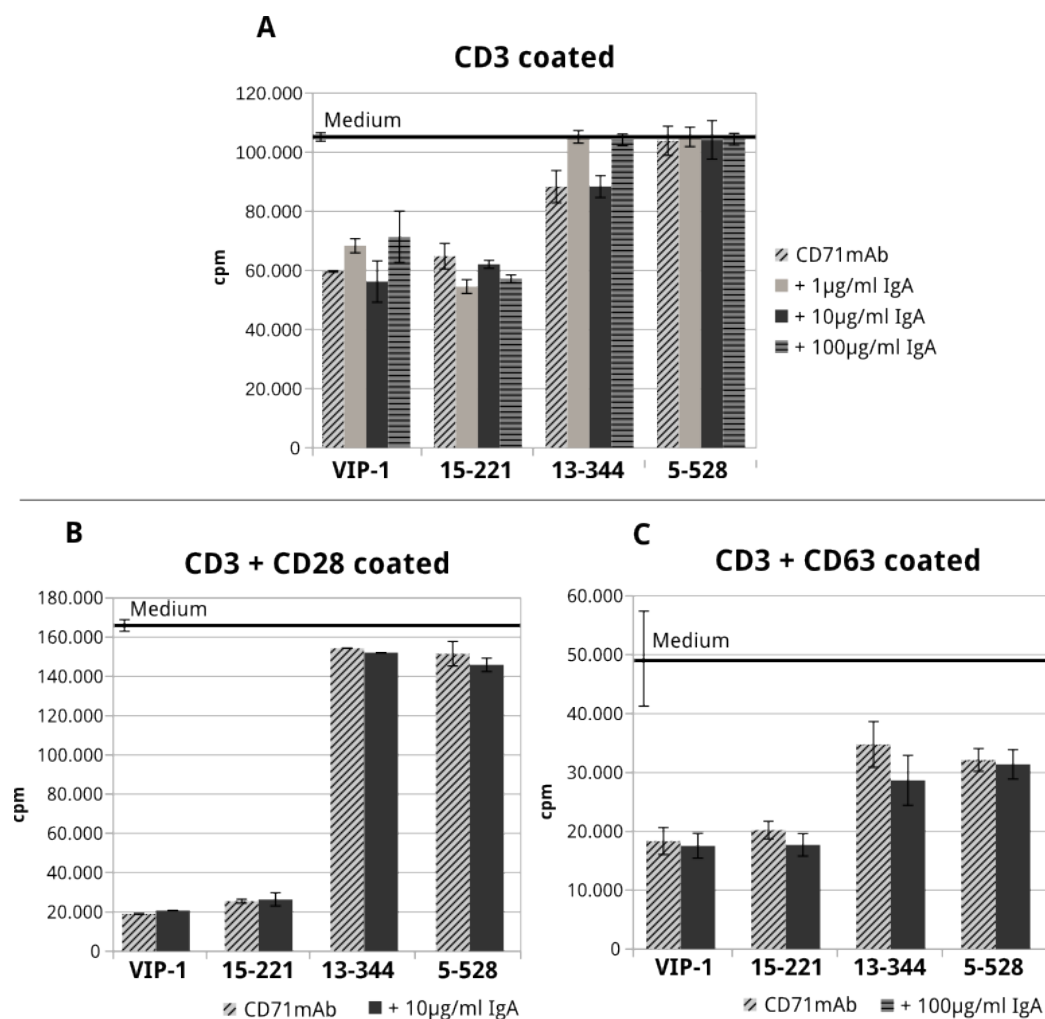


Figure 37 VIP-1 and 15-221 treated human T lymphocytes unaltered reduced proliferation rates in presence of serum IgA. In presence of 10 µg/mL CD71 mAbs, 1×10^5 isolated, human T cells were stimulated for three days via (A) plate-bound anti CD3 mAb and cultured in medium containing 1, 10 or 100 µg/mL serum IgA, (B) via immobilized anti CD3 and CD28 mAb in medium containing 10 µg/mL IgA or (C) via immobilized anti CD3 and CD63 mAb in medium containing 100 µg/mL IgA.

RESULTS

4.5.8 Immobilized serum IgA during T cell activation via CD3+CD63 increases proliferation

All previous experiments were performed with serum IgA in suspension. In order to test if there exists a difference between the application of soluble or plate-bound IgA regarding T cell activation, T cells were cultivated in presence of immobilized or soluble serum IgA. T lymphocytes, which were activated via CD3 with dissolved serum IgA, showed unchanged proliferation rates, independent of the IgA concentrations as expected. In contrast, T cells showed in presence of plate-bound IgA under the same conditions a lessened thymidine incorporation with a maximum of 28% with 100 $\mu\text{g}/\text{mL}$. Co-stimulation via CD3 + CD63 of T cells in presence of soluble IgA led to a reduced growth rate with the mean of -44%, autonomous from the IgA dosage. Compared to these results, plate-bound IgA had a contrary effect and promoted proliferation of CD3 and CD3 stimulated T cells up to 1.7 fold of recorded cpm.

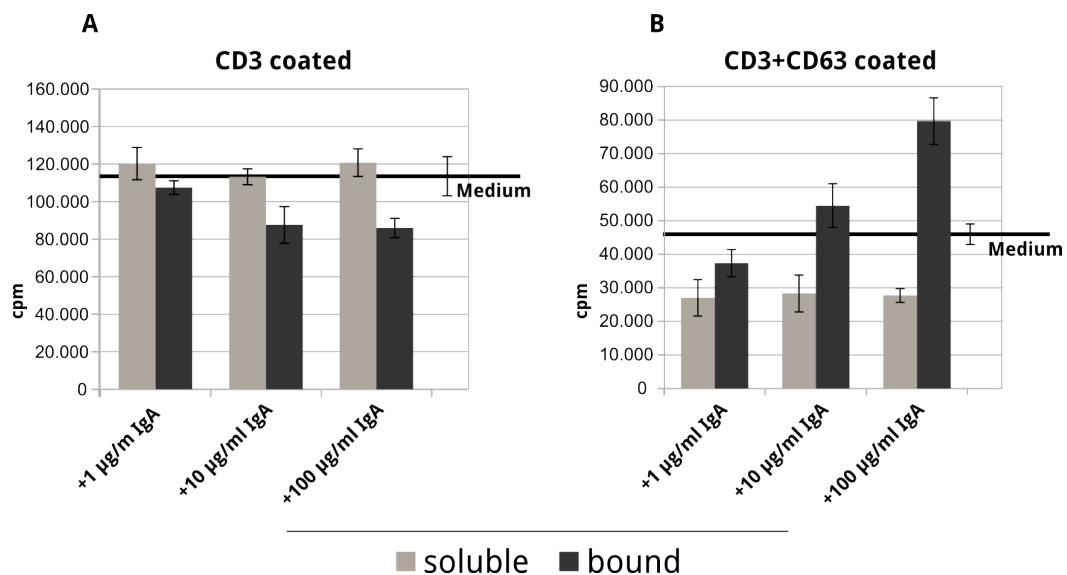
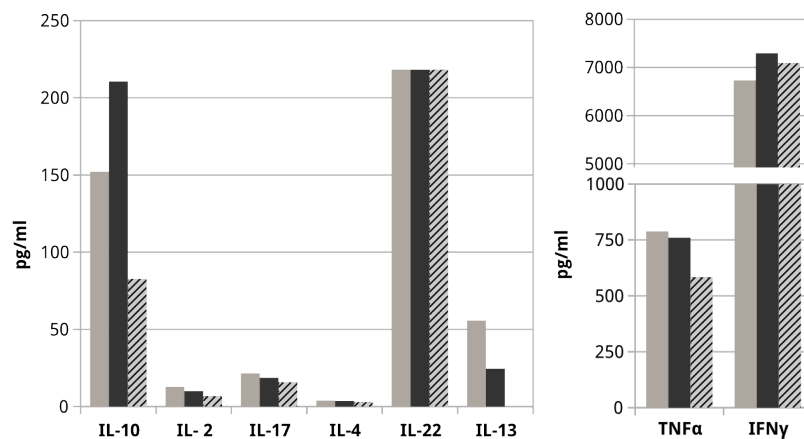


Figure 38 Different proliferation rates of CD3 or CD3+CD63 activated T cells upon increasing plate-bound or soluble serum IgA concentrations. In presence of increasing concentrations of soluble or plate-bound serum IgA, 1×10^5 human T cells were activated with immobilized (A) anti CD3 mAbs or (B) anti CD3 and anti CD63 mAbs. Bars represent the mean ($\pm$ standard deviation) of triplicates.

4.5.9 Enhanced cytokine secretion in presence of immobilized serum IgA

A potential different influence upon cytokine production regarding soluble and plate-bound serum IgA was analyzed. The results were divergent: While the cytokine levels of T cells stimulated via CD3 were only slightly altered or stayed constant, irrespective from the condition, T cells stimulated via CD3 and CD63 were observed to have enhanced cytokine concentrations in presence of plate-bound serum IgA. Strongest enhancement was observed of IL-10 of which the secreted concentration has doubled in presence of plate-bound serum IgA.

A CD3 coated



B CD3 + CD63 coated

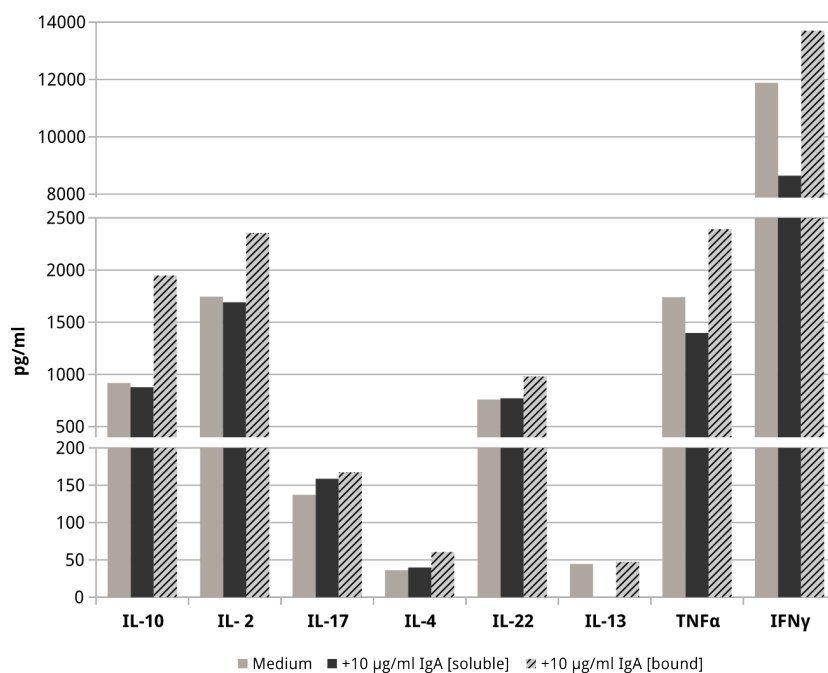


Figure 39 Elevated cytokine levels in presence of plate-bound serum IgA in CD3 and CD63 activated T cells. In presence of 10 µg/mL of soluble or plate-bound serum IgA, 1×10^5 human T cells were activated with immobilized anti CD3 mAbs or anti CD3 and anti CD63 mAbs.

RESULTS

4.6 VIP-1 inhibits the serum IgA promoted T cell lytic activity

In a further approach we characterized the ability of CD71 mAbs VIP-1 and 13-344 as control and serum IgA as well as in combinations to alter the lytic capacity of T lymphocytes against THP-1 target cells at different ratios, which was determined by a 4h ^{51}Cr release assay. Clearly, serum IgA pretreated T cells showed to exhibit an enhanced cytotoxic activity at the used effector/target cell ratio (E/T) 40 and 10, but were at similar levels when the ratio was reduced to 2,5 and 0,8. Serum IgA, in combination with VIP-1 did not alter the effect of VIP-1 alone, which was characterized by slightly increased lysis at the ratio of 40, and an undetectable chromium release at the E/T ratio of 2,5, where the medium control still has a specific lysis of 7%. A different situation was observed with the CD71 mAb 13-344, which showed alone no general effect, but in combination with serum IgA strongly enhanced the cytotoxic activity of T cells by more than two times at highest ratio. This effect faded away with decreasing effector/target cell ratio.

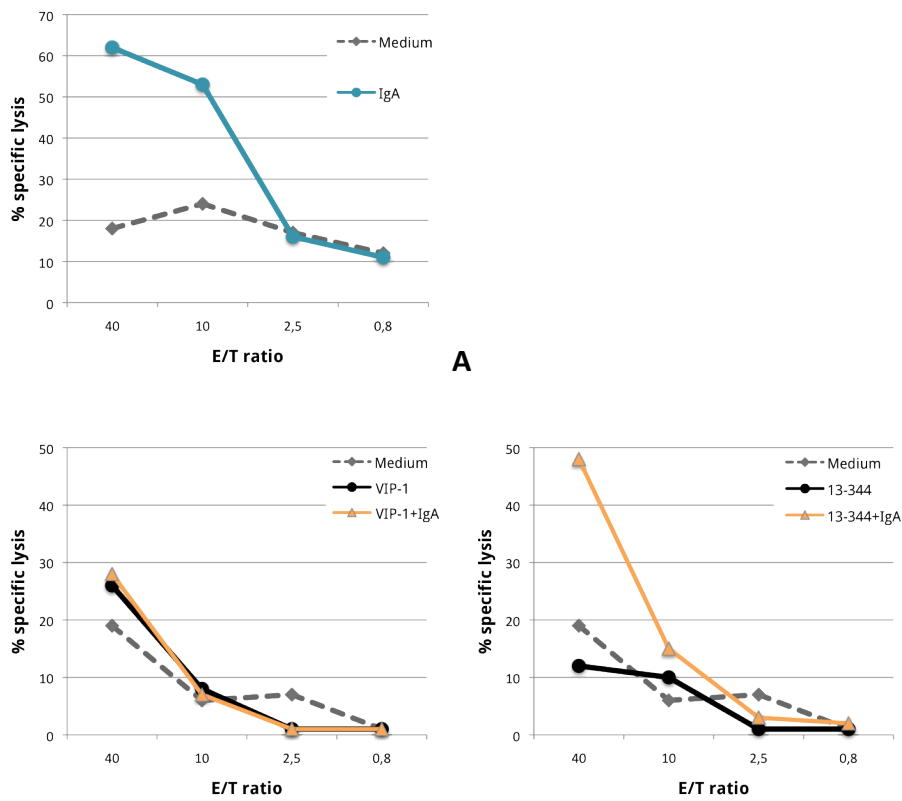


Figure 40 Serum IgA enhances CTL-mediated lysis of THP-1 cells, which is inhibited by VIP-1 but not by 13-344. T cells were co-stimulated for 3 days with CD3 and CD28 mAbs in presence or absence of serum IgA, VIP-1, 13-344 or in combinations of them. These pretreated cells were cultivated for 4 h together with ^{51}Cr labeled THP-1 cells in different cell number ratios and the ^{51}Cr release was measured afterwards that represents the specific lysis.

5 DISCUSSION

The transferrin receptor as crucial iron distribution platform within the strictly regulated iron metabolism is intensively studied regarding cellular mechanisms and pathologies since the 1980s. Its role as immuno-modulatory component became evident over the following years. CD71 mAbs were extensively used to investigate this major iron uptake mechanism. With our in house produced mouse anti human CD71 mAbs VIP-1, 15-211, 13-344 and 5-528 we aimed to analyze immuno-modulatory effects specific for different epitopes in human primary T cells *in vitro*.

Equivalent expression of all epitopes was regularly confirmed in various different cell lines like Jurkat, Raji, KG-1, Daudi and many more. Moreover, CD71 specific binding of all four mAbs was validated by a surface-binding assay in human CD71 transduced murine thymoma BW cell line as reported in previous studies of our group [76]. Reactivity of all four CD71 mAbs showed that all are qualified to reflect the growth rate of each cell line, as slower growing cell lines exhibit lower fluorescence signals than fast growing cell lines. As seen in a former study [76], mAb 13-344 shows cross-reactivity with murine cells, this case was also observed with rabbit cells in this study. The hypothesis that 13-344 covers an epitope of the transferrin receptor that is highly homologous within mammals is hereby supported that *Mus musculus* shares with the human TfR a 77% and with rabbit (*Oryctolagus cuniculus*) 82% sequence identity [117]. To date, no cross-reactivity of mAb 13-344 or other human anti mouse CD71 mAbs with insect cells that have similar transferrin uptake mechanisms was reported [76].

As previous work aimed to analyze effects of CD71 mAbs on DCs [76], this study hereby focused on another immune cell subset. Human primary T cells were activated via CD3, CD3+CD28 or CD3+CD63 in presence of CD71 mAbs *in vitro*. After three days, T cell proliferation was significantly inhibited by VIP-1 and 15-221 independent from co-stimulatory signals. Binding of 13-344 and 5-528 mAbs to the TfR represents epitopes, which are not important to T cell proliferation. According to the suppressed T cell expansion, an inhibited

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production of proinflammatory and proliferative cytokines was expected. Measurement of the cytokine secretion revealed an inconsistent outcome of CD3+CD28 and CD3+CD63 stimulated T cells. IL-2 levels in CD3+CD63 activated T cells were reduced, while CD3+CD28 activated T cells exhibited significant enhanced concentrations of IL-2. This opposed effect of VIP-1 and 15-221 was detected at similar or minor levels of other cytokines like IL-22, IL-13, IFN- γ or IL-10. T cell activation that is accomplished via cross-linkage of TCR (CD3) and CD28 mAbs *in vitro* reflects the standard stimulation pathway at physiological measurements. The co-stimulatory receptor CD63, which belongs to the tetraspanin family, represents an alternative second signal to TCR. Engagement of CD63 is known to induce efficient T cell activation and consistent IL-2 secretion when plate-bound and co-ligated with CD3 [118]. Still, less is known about its interaction partners or the detailed activation cascade. The contradictory to the decreased growth rate of VIP-1 and 15-221 treated T cells detected increase of IL-2 levels of CD28 co-stimulated T cells could be a consequence of a complete activation signal, resulting in IL-2 secretion, which cannot be consumed by the growth deprived cells. Noticeably, this observation indicates that the cells receive stimulation signals and are viable. It would be relevant to measure the expression of activation-related T cell receptors and transcription factors in order to investigate to which measure the T cell integrity is maintained. Further, the differences of the affected cellular mechanisms of CD63 co-stimulated T cells have to be questioned, as they are similarly growth deprived, but have opposed cytokine levels compared to CD28 co-stimulated T lymphocytes. From this situation one can assume that the activation process and cytokine secretion via CD63 stimulation are more sensitive to VIP-1 and 15-221 treatment.

In order to determine if the antiproliferative effect is a result from reduced TfR on the surface, TfR surface expression was measured from overnight treated T cells. Antibody-mediated receptor-downmodulation is a well-known phenomenon when surface receptors are permanently engaged with high affinity ligands. All four CD71 mAbs significantly reduced number of receptor quantity, but only VIP-1 and 15-221 treated cells showed an altered Tf uptake.

Iron depletion seems to be responsible for a reduced proliferative capacity of T cells as they are known to be quite sensitive to it. In addition, we demonstrated that the inhibitory CD71 mAb, namely VIP-1, was found to be rapidly endocytized within the physiological time of Tf uptake [102]. Past studies by St.Pierre *et al* investigated the intracellular fate of TfR/CD71 mAb complexes, which were shown to enter the degradative endosomal pathway and therefore reduces the pool of recycling vesicles containing TfR [37]. Still under discussion, this group in contrast to Lesley *et al* [119] strongly supports the hypothesis that the antibody valence is responsible for redirection of the internalization pathway rather than the Fc region after they did experiments with F(ab')₂-ARG1/rabies G complexes. As they have tested only one type of CD71 mAb (IgG2 A κ) and no Fab'-IgG parts in contrast to Lesley *et al*, the possibility with Fc cannot be ruled out. While 13-344 and 5-528 seem not to cause redirection of the endosomal recycling TfR pathway as Tf uptake is unaltered, but VIP-1 and 15-221 do, it can be argued that both potentially exhibit a higher fluorescence signal compared to 13-344 and 5-528 and therefore possess a higher valency, which would support the hypothesis of St.Pierre *et al*. According to these observations our findings suggest that VIP-1 and 15-221 effectively alter the internalization pathway after 17 hours so that transferrin uptake is affected at this time point. Fluorescence microscopy supports the process, as VIP-1 is internalized along with the TfR at physiological conditions. Also, the possibility of different cross-linking intensities of the TfR-mAbs should be included.

CD71 is highly expressed on malignant cells and we therefore examined the effect of our four CD71 mAbs in different human cancer cell lines. Compared to non-lymphoid cell lines, VIP-1 and 15-221 had a profound inhibitory effect in Raji and Jurkat cells. This observation provided us with the knowledge that B and T cells are especially sensitive to TfR engagement, may it be due to the absence of secondary iron uptake mechanisms or a more sensitive TfR processing. These observations are in context with the examined effects of VIP-1 and 15-221 on Tf binding and uptake after down-modulation. Consequentially, we addressed the question if cells treated with the inhibitory mAb VIP-1 are capable to recover and proliferate again. Surprisingly, Jurkat and

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Raji cells were still strongly growth impaired after one and two weeks of cultivation in absence of VIP-1. These results cannot be explained by the absence of TfR with followed iron depletion as observed within 24h, because we have shown that the expression of CD71 was entirely restored after three weeks. We hypothesize that it has to be a distinct target that is sustainable altered, and which is not exclusively used by the iron metabolism.

In order to gain insight of T cell signaling, reporter cell lines – once stable established – allow a simple and quick analysis of gene induction. The lab of Steinberger *et al* provided us with a multi-channel reporter Jurkat cell line that expresses fluorophore tagged NF- κ B, NFAT and AP-1 sequences. The transcription factors NF- κ B, NFAT and AP-1 are key regulators to orchestrate inflammatory responses of T cells, controlling cellular activation, proliferation and cytokine secretion [50]. The detectable signals of NF- κ B, NFAT and AP-1 during CD3 and CD28

costimulation changed dramatically, when Jurkat cells were pretreated for seven days with VIP-1 and 15-221. Surprisingly, T cell signaling was intact and also significant higher fluorescence signals were detected compared to the untreated cells. Further, these results potentially support the elevated IL-2 levels of CD3 and CD28 co-stimulated primary T cells. Together, we can hypothesize that long-term targeting of CD71 via certain epitopes may

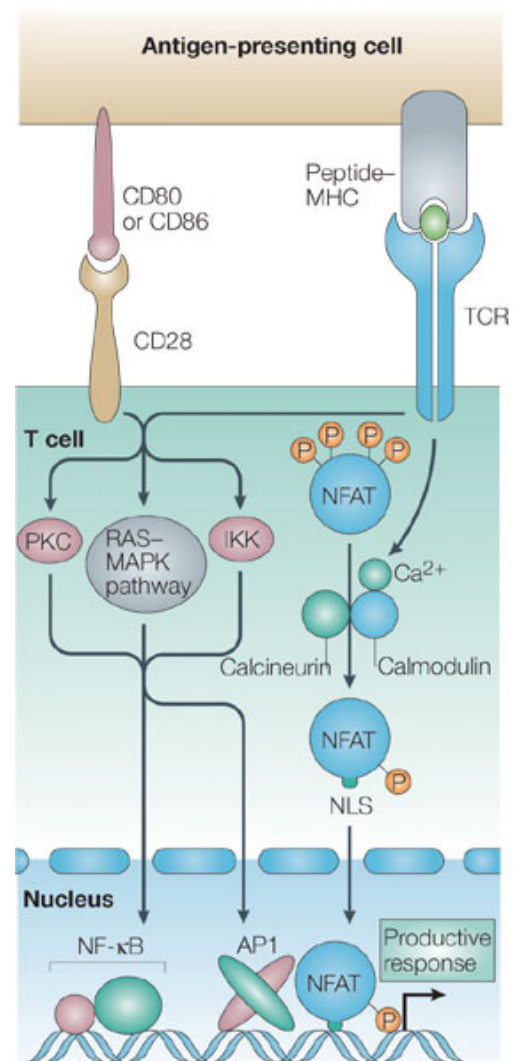


Figure 41 Interaction of a T cell and an APC induces a complex signaling network that involves activation of the transcription factors NFAT, AP-1 and NF- κ B. They are considered to be essential for a complete T cell response that involves cytokine secretion, e.g. IL-2. The image is adapted from Macian F, 2005 [i].

dysregulate T cell responses not exclusively via altered iron metabolism, but also has an influence upon activational signal transduction pathways. Keeping in mind that this read-out system provides us only with indirect information about the strength of activation of those TFs within a T cell line, evaluation of the expression of downstream target genes such as *il2*, *ifng*, *cd40l*, *cdk4*, *fasl* would give us further information. In addition, analysis of expression of T cell activation markers like CD69 and CD25 would confine the cellular effects of VIP-1 and 15-221.

To identify potential targets of VIP-1, we performed qualitative proteome analysis of untreated and VIP-1 treated Jurkat cells. Three members of the RAS GTPase super family, namely the isoforms Rab-5c, Rab-9a and Rab-11b, which are involved in intra-cellular trafficking pathways, were untraceable in VIP-1 treated Jurkat cells. Ras-related protein Rab-5c down-regulation was shown to be linked to growth deprivation [120] and Rab-5 in general to be directly involved in regulation of TCR downmodulation that is important for proper T cell function after activation. Rab-5 deficient mice show reduced or delayed TCR downmodulation [121]. Only one isoform of

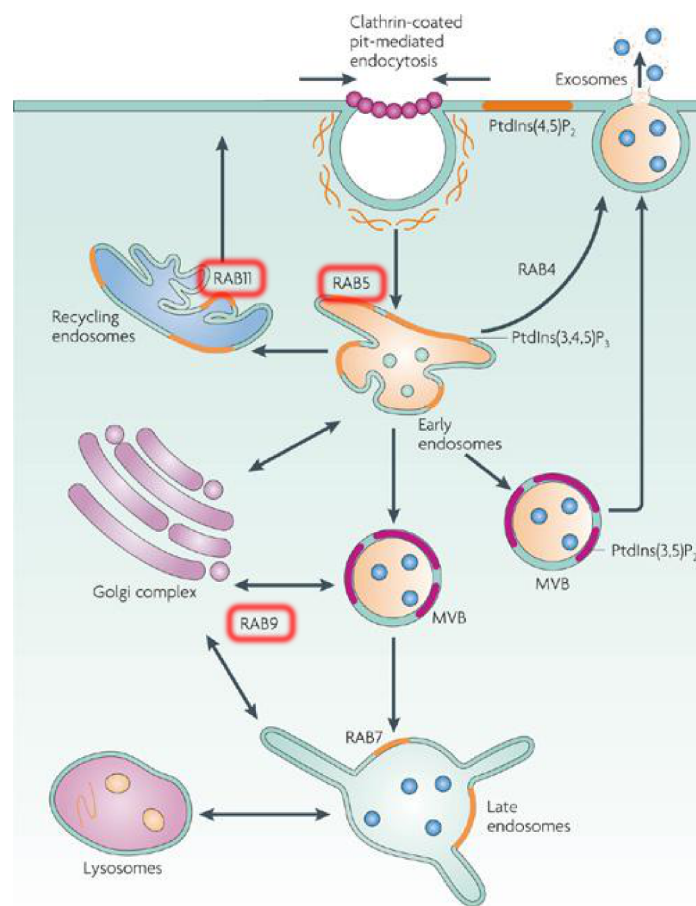


Figure 42 Rab proteins are required for site-directed trafficking between cellular compartments. Several Rab proteins are known of which each is characteristic for a different location. Rab-5 is needed in early endocytic pathways and is therefore found in early endosomes. Rab-9 is necessary for the transport between multivesicular bodies or late endosomes and the trans-golgi network. Rab-11 is involved in the function of recycling endosomes. The image is adapted from Rajendran *et al.*, 2010 [k].

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Rab-9 is present in untreated Jurkat cells, and non in VIP-1 treated cells. Rab-9 contributes to the endo-somal transport to and from the trans Golgi network. Additionally, Rab-11b is missing in the VIP-1 mAb treated cells, but instead posses Rab-11a, which cannot be found in mock-treated cells. The two isoforms might be closely related, but exhibit different characteristics. Rab-11b is shown to colocalize with late stage recycling endosomes and Rab-11a more with trans-golgi-network trafficking. The protein Lck is important for T cell activation and is translocated via Rab-11a endosomes to the IS ^[122]. In contradiction, one report found that Rab-11a together with UNC119a is needed to complete cytokinesis in HeLa cells ^[123], but studies with Rab-11b alone are not provided. In this context, possible aberrant crosstalk between the network of Rab proteins should not be neglected, if certain members are missing. Three other proteins, which are involved in cell cycle progression were absent in VIP-1 treated Jurkat cells: Histon deacetylase 2, CLIP-associating protein 2 and NudC domain-containing protein 2. Whereas the last two proteins that act upon the cytoskeleton are less investigated regarding mitosis, studies revealed that histon deacetylase 2 inactivation can cause a G1/S-phase arrest of cells ^[124]. Furthermore, a recently identified regulatory factor called UXT of Treg cells that is associated with Foxp3 and NF- κ B regulation ^[125] was found to be not expressed in VIP-1 treated cells.

The list of proteins that are identified only in VIP-1 treated cells contains the NF- κ B -activating protein (NKAP), Raftlin, STAM-binding protein, RNA binding motif protein, X-linked-like-1, MEMO1 and TOX high mobility group box family member 3. The investigation of raftlin for example points to modulation of TCR signaling, but its role is still poorly understood, as well as NKAP in T cell maturation and differentiation.

Optionally, a longer incubation time with CD71 mAbs, as evidence give rise to the assumption that it takes days for the inhibitory effect of VIP-1 or 15-221 to be fully established, could yield a more distinct outcome. In addition, a quantitative proteome analysis would provide us with further information. Moreover, a more significant effect could be expected from cells, which were cultivated for a longer time. Results, especially including the Ras GTPase family

members and histon deacetylase 2, point to impairment of cell cycle progression

Following the experiments of long-term culture of Jurkat cells with CD71 mAbs, a similar experiment with primary human T cells was carried out. Not only had T lymphocytes, which were activated with VIP-1 and 15-221 in the primary stimulation, still reduced growth rates, also the attempt with exogenous IL-2 to rescue the impaired proliferation phenotype of VIP-1 and 15-221 treated T cells was ineffective. In regard of this observed impact, VIP-1 has the most dramatic effect on T cell compared to 15-221, which at least showed a lessened phenotyp in restimulation experiments. These unresponsive T cells give us further evidence that targeting CD71 has a long-term effect in regard of cell cycle progression that also has a long-term effect on T cell behavior. Again, the cells expressed all CD71 epitopes and indicate that the TfR is completely expressed on the surface. An evaluation of the cytokine profile after restimulation is needed in order to determine if the cytokine secretion is still intact or not .

In a second part we addressed the question if the novel TfR ligand IgA is able to modulate T cell responses and if IgA can potentially alter the effect of VIP-1 or 15-221. Binding of serum IgA was confirmed in the lymphoid cell lines Jurkat, Raji, Daudi and OTMA as well as in activated primary T cells in contrast to the myeloid cell line KG-1. Cross-inhibition studies of VIP-1 , 15-221 with serum IgA, as well as binding studies with the murine BW CD71 transduced cells supports the CD71 specificity. More cell types have to be tested for an serum IgA binding ability in respect to possible other IgA receptors. If further results show that only distinct cell types can bind serum IgA via CD71, this would point to a pivotal, uninvestigated role of this mechanism. Serum IgA is a natural ligand to CD71 and does not trigger downmodulation of its receptor in Jurkat cells. Serum IgA also does not perturb the Tf uptake cycle. Further experiments showed that serum IgA does not influence the proliferative response of cell lines. While serum IgA administration during T cell activation via CD3 alone or together with CD28 had no impact on the T cell expansion, minor alterations were observed in CD3 activated T cells, when we took a look at the cytokine secretion profile. Serum IgA was also found to be unable to influence the antiproliferative

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effect of VIP-1 and 15-221, but this was expected as we have earlier shown that IgA fails to block binding of VIP-1 and 15-221. As serum IgA was only used in soluble form in all previous experiments, we wondered if immobilized IgA has a different effect to activated T lymphocytes. While the presence of plate-bound serum IgA had no significant impact upon T cell activation via CD3, a dose-dependent effect was detected when T cells were co-stimulated via CD3 and CD63. This pro-proliferative effect was also reflected by an enhanced cytokine production of these cells.

With the knowledge that CD71 mAbs, which block Tf uptake, are capable to inhibit the lytic function of CTLs [126], another aspect of serum IgA including CD71 mAbs was investigated. However, in contrary to the previous study, VIP-1 pretreated T cells showed no significant inhibition of CTL activity. Notably, serum IgA achieved an enhanced induction of lytic activity. This novel discovered effect of serum IgA was completely abrogated in co-pretreatment of T cells with VIP-1, while presence of 13-344 has no influence on the outcome of serum IgA. This data is consistent with results showing VIP-1 to inhibit IgA binding to some extent. The described interference of VIP-1 with serum IgA provides further evidence of CD71 association within CTL function. An improved experimental setup should allow to gain more solid data in future concerning the role of TfR and serum IgA in CTL activity.

In summary, administration of VIP-1 and 15-221 mAbs antagonizes T cell proliferation *in vitro*, but maintains T cell responses to CD3 and CD28 engagement in regard to cytokine secretion and T cell signaling. Although temporarily, the TfR surface expression is affected, it becomes permanently restored – a circumstance, which excludes iron deprivation as the principal reason for the sustained hypoproliferative effect of VIP-1 and 15-221 mAbs. The growth impaired phenotype remains for weeks after removal of CD71 mAbs in B and T cell lines and also cannot be rescued by addition of exogenous IL-2 to primary T cells during restimulation. Altered endosomal traffic may be a potential result and reason for the inability to divide. Further, it is of interest to analyze at which step of the cell cycle the cells are trapped or not.

Further, as our current evidence point to a potential functional role of serum

IgA within T cell function, more studies have to be done to examine especially the effect of serum IgA when cross-linked on T cells and the role of the serum IgA binding site of CD71 during CTL function.

6 ABBREVIATIONS

3'UTR	3' untranslated region
Ab	antibody
AP-1	activator protein 1
APC	antigen-presenting cell
ASGP-R	asialoglycoprotein receptor
BBB	blood-brain-barrier
BSA	bovine serum albumin
CD	cluster of differentiation
CFP	cyan fluorescent protein
CSA	Cowan 1 <i>Staphylococcus aureus</i>
CTL	cytotoxic T lymphocyte
DC	dendritic cell
Dcytb	duodenal cytochrome-b
DMT1	divalent metal transporter-1
ETC	electron transport chain
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FP-1	ferroportin-1
Ftmt	mitochondrial ferritin
GAP	GTPase-activating protein
HCP1	haem carrier protein 1
HCV	hepatitis C virus
HFE	high Fe (iron)
HP	hormone hepcidin
HSA	human serum albumin
Ig	immunoglobulin
IKK	inhibitor of NF- κ B kinase
IL	interleukin
INF-γ	interferon γ
IRP	iron-regulatory protein-1
IRE	iron-responsive element
IS	immunological synapses
ISC	iron-sulfur clusters
ITAMi	inhibitory immunoreceptor tyrosine-based activation motif
LAT	linker for activation of T cells
LIP	labile iron pool
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MDR	multi-drug resistance
MFI	mean fluorescence intensity

MHC	histocompatibility complex
MMTV	mouse mammary tumor virus
mo-DC	monocyte derived dendritic cell
MRI	magnetic resonance imaging
MtF	mitochondrial ferritin
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κ B
NFAT	nuclear factor of activated T cells
NK cells	natural killer cells
OG	Oregon Green
PBMNCs	peripheral blood mononuclear cells
PCFT	proton-coupled folate transporter
PE	phycoerythrin
PMA	phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinase
ROIS	reactive oxygen intermediates
ROS	reactive oxygen species
RNS	reactive nitrogen species
SIgA	secretory IgA
siRNA	short-interfering RNA
SMAC	supramolecular activation cluster
STAT-1	signal transducers and activators of transcription
STEAP3	epithelial antigen of the prostate 3
sTfR	soluble transferrin receptor
TCR	T cell receptor
TF	transcription factor
Tf	transferrin
TfR	transferrin receptor
TGF-β	transforming growth factor β
TNFα	tumor necrosis factor α
TLR-4	toll-like receptor-4
Treg cells	T regulatory cells
ZAP-70	ζ chain associated protein kinase 70

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A. ABSTRACT

The transferrin receptor (TfR, CD71) is an abundant surface receptor expressed on all proliferating cells and is fundamental for the human iron metabolism. Engagement of iron-loaded transferrin (Tf) and TfR leads to clathrin-mediated endocytosis of the receptor-ligand complex and release of iron within the cell. Regardless of its central position in cell biology, little is known about the function of CD71 in primary human T cells. In this work, four murine anti-human monoclonal antibodies (mAbs) VIP-1, 15-221, 13-344 and 5-528 that recognize three different epitopes of CD71, were added soluble to human, peripheral blood T cells activated via plate-bound CD3/CD28 mAbs. Among these four mAbs, two (VIP-1, 15-221) reduce Tf binding as well as internalization due to Ab-mediated downmodulation and, moreover, inhibit T cell proliferation. Regardless of the observed negative impact on proliferation, T cells treated with CD71 mAbs maintained their capacity to secrete cytokines like IL-2, IFN- γ or IL-10. Further, despite the restored CD71 expression, induction of an unresponsive state in T cells is detectable upon restimulation in absence of VIP-1 and 15-221 and was not reversible by adding exogenous IL-2. Similarly, Jurkat T cells also showed a sustained growth deprivation though equivalent CD71 expression. Moreover, crucial NFAT/AP-1/NF- κ B signaling needed for T cell activation was not suppressed by VIP-1 and 15-221 in a multi-channel reporter Jurkat T cell line. Concerning proliferation deficiency, proteome analysis of VIP-1 treated Jurkat cells points to an aberrant cell cycle progression due to the absence of necessary trafficking proteins such as Rab-5c, Rab-9a and Rab-11b. In addition to the mAbs, the function of serum IgA, which is a less known natural ligand of CD71, was investigated. Plate-bound serum IgA promoted a dose-dependent proliferative effect that occurred with elevated cytokine levels in CD63 co-stimulated primary T cells. Furthermore, pretreatment with soluble serum IgA increased cytotoxicity of T cells against THP-1 cells, an effect that was neutralized with VIP-1. In summary, targeting of two distinct human TfR epitopes *in vitro* that reduces Tf uptake, but does not alter cytokine secretion, induces sustainable inhibition of T cell proliferation.

B. ZUSAMMENFASSUNG

Der Transferrin Rezeptor (TfR, CD71) ist ein omnipräsender Oberflächenrezeptor jeder proliferierenden Zelle, der essentiell für den menschlichen Eisenmetabolismus ist. Bindung von eisengesättigtem Transferrin (Tf) an dem TfR führt zur Internalisierung des Tf/TfR Komplexes mittels Clathrin – abhängiger Endozytose und zur Freisetzung von Eisen innerhalb der Zelle. Obwohl die zellbiologische Rolle von CD71 im Eisenstoffwechsel hinreichend untersucht ist, ist wenig über dessen immunmodulatorischen Funktion in primären, humanen T-Zellen bekannt. In dieser Arbeit wurden vier monoklonale Maus anti-human CD71 Antikörper (mAk) VIP-1, 15-221, 13-344 und 5-528, die drei unterschiedliche Epitope erkennen, eingesetzt. Diese mAks wurden in löslicher Form zu der Stimulierung von primären und peripheren T-Zellen via Platten gebundenen anti CD3/CD28 mAks beigefügt. Von diesen vier mAks reduzierten zwei (VIP-1, 15-221) die Tf Bindung und Internalisierung durch Ak-vermittelter Rezeptorverminderung, und inhibierten darüber hinaus das Wachstum der T-Zellen, aber nicht deren Zytokinsekretion. Wurden diese T-Zellen in Abwesenheit von VIP-1 und 15-221 restimuliert, waren sie immer noch deutlich in ihrer Proliferation supprimiert – ein Zustand, der auch mit Zugabe von exogenem IL-2 nicht aufgehoben werden konnte, obwohl CD71 an der Zelloberfläche quantitativ wieder vollständig hergestellt war. Eine nachhaltige Wachstumshemmung trotz präsentem CD71 konnte ebenso in der Jurkat T-Zelllinie nachgewiesen werden. Weiters war die für die T-Zellaktivierung wichtige NFAT/AP-1/ NF- κ B Induzierung in der Zelllinie durch VIP-1 und 15-221 nicht unterdrückt, was auf intakte T-Zell Signalwege hindeutet. Eine Proteomanalyse von VIP-1 behandelten Jurkat Zellen zeigte als mögliche Ursache der Wachstumsinhibierung das Fehlen von für den Zellzyklus wichtigen Transportproteinen wie Rab-5c, Rab-9a und Rab-11b auf. Des Weiteren wurde die Funktion von Serum IgA, einem zuletzt neu entdeckten Liganden von TfR, untersucht. Platten gebundenes Serum IgA verursachte eine dosisabhängige Wachstumssteigerung und erhöhte die Zytokinausschüttung in CD63 kostimulierten primären T-Zellen. Weiters steigerte die Vorbehandlung mit

löslichem Serum IgA die Zytotoxizität von T-Zellen gegenüber THP-1 Zellen. Dieser Effekt konnte mit VIP-1 neutralisiert werden. Zusammenfassend kann berichtet werden, dass die *in vitro* eingesetzten mAbs VIP-1 und 15-221, die gegen zwei unterschiedliche humane TfR Epitope gerichtet sind, die Tf Aufnahme reduzieren und zu der nachhaltigen Hemmung des T-Zell Wachstums führen, bei der die Zytokinausschüttung jedoch nicht unterbunden ist.

C. CURRICULUM VITAE

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Since 10/2005	Diploma study of Molecular Biology at the University of Vienna
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1997 – 2005	Secondary school WISKU-BRG Geringergasse, Vienna
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2008 – 2013 Laboratory technician for residue analysis at LVA GmbH, Klosterneuburg

11/2011 Internship at the Children's Cancer Research Institute (CCRI), Vienna, research group Kovar, "Optimizing electroporation of pre-B lymphocytes"

09/2011 Internship at the Institute of Pathophysiology & Allergy Research Vienna, Department for Moleculare Immunopathology, research group Valenta "Investigation of IgE-mediated versus non-IgE mediated mechanisms of birch-allergic patients with atopic dermatitis"

08/2011 Internship at the Institute of Immunology, Vienna, Department for Immunregulation, research group Stöckl "Analysis of ct-CD45 production due to different stimuli in monocytes and granulocytes"

2005 – 2008 Sales assistant at Hofzuckerbäckerei Heiner, Vienna

08/2005 Internship via GENAU-Summerschool at the Institute of Pharmacology, Vienna

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