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Jennifer Brell, BSc

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1. INTRODUCTION

1.1. Human iron homeostasis: intrinsic factor in biological processes

Elemental iron is an essential micronutrient that is required for the proliferation and survival of virtually all living organism. It plays a crucial role in the formation of heme- and iron-sulfur cluster (ISC)-containing enzymes that are involved in biological processes including energy metabolism (cytochromes, mitochondrial aconitase, Fe-S proteins - electron transport), DNA synthesis (ribonucleotide reductase), cellular respiration and oxygen transport (hemoglobin, myoglobin) [1, 2]. Moreover, iron is a decisive factor for immunosurveillance because it facilitates cell growth, influences cytokine activities and modulates immune cell-proliferating as well as cell-mediated immune effector pathways [3, 4]. To avoid anemia and growth arrest associated with iron deficiency as well as cellular damage associated with iron overload, iron metabolism has to be tightly regulated through the cooperation of functional compartments, including the control of iron uptake, recycling, storage and export [5].

1.1.1. Non-heme iron

Non-heme iron, the inorganic form, is found in several metalloproteins as iron-sulfur clusters (ISCs) such as 2Fe-2S, 3Fe-4S or 4Fe-4S, which are located within mitochondria, the cytosols and the nucleus of eukaryotes. These ubiquitous co-factors are crucial for electron transfer processes (respiratory enzyme complexes within the electron transport chains (ETC)), enzyme catalysis (mitochondrial aconitase within the citric acid cycle), gene expression and repair, homeostasis as well as signal transduction [6]. Mitochondrial aconitase are required for energy production and directly interacts with enzyme substrates through ISCs. Other non-heme proteins such as ribonucleotide reductase for DNA synthesis containing iron-oxo clusters or oxygenase and lipoxygenase, which regulates inflammation containing mononuclear iron centers [1]. The maturation of cellular iron-sulfur proteins occurs in the mitochondrial ISC assembly machinery whereas the ISC export and cytosolic iron-sulfur protein assembly machinery participate the formation of cytosolic and nucleus iron-sulfur proteins [7].

Iron regulatory proteins (IRP1 and IRP2) are regulated by iron-dependent and iron-independent mechanisms and shares homology with mitochondrial aconitase. The

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bifunctional protein IRP1 serves as an IRE binding protein or as a cytosolic aconitase, depending on the cellular iron status. The 4Fe-4S cluster in IRP1 controls the RNA binding activity: Iron deficiency leads to the loss of 4Fe-4S cluster and aconitase activity, however it binds with high affinity to IREs. In case of iron-repletion IRP1 exhibit aconitase activity and binds mRNA with low affinity [8]. Furthermore, IRP1 is an important sensor to reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as a responder to oxygen availability [9].

Iron has the ability to cycle between two oxidation states, the reduced ferrous (Fe(II)) and the oxidized ferric state (Fe(III)). Under aerobic conditions Fe atom exists in its oxidized ferric state, which has the ability to catalyze the generation of deleterious free radicals through Fenton and Haber-Weiss-type reaction [5]. Under conditions of iron overload, ferrous iron reacts with hydrogen peroxides (H_2O_2) and generates hydroxyl radicals ($\text{OH}^\cdot$), a reactive oxygen species [10, 11]. This causes oxidative stress, DNA damage and lipid peroxidation associated with genomic instability and DNA repair defects as well as promotes cell death and tissue injury that accelerate tumor initiation. Furthermore, in the presence of superoxide ($\text{O}_2^{\cdot -}$) the oxidized metal can be reduced back to ferrous iron. These reactive oxygen intermediates are byproducts of respiratory processes as well as are also produced by membrane anchored NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex of macrophages and monocytes. Therefore, iron is essential but also potentially toxic [1].

1.1.2. Heme-derived iron

A vast majority of cellular iron Fe(II) is located in the center of the protoporphyrin ring of heme, an essential iron chelate. Heme serves as a prosthetic group in various proteins involved in oxygen transport, drug metabolism, cellular antioxidant defense, mitochondrial respiration, signal transductions process and steroid biosynthesis [12], but is highly toxic when it appears in the extracellular milieu. The most frequent hemoproteins are hemoglobin in erythroid tissue, and myoglobin in muscle tissue, which are crucial for oxygen transport in vertebrates [13], where the heme moieties enables oxygen binding [1]. Other heme-containing proteins include enzymes such as oxygenases and nitric oxide synthases as well as antioxidant enzymes such as ROS protective catalases and peroxidases. Furthermore, heme also occurs in cytochromes, which are important as electron-transfer molecules of the respiratory chain [12]. Heme is synthesized from the precursor 5-amminolevulinic acid (ALA)

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in all cells, mainly in liver and bone marrow, through eight enzymatic reactions in the mitochondria and in the cytosol during differentiation of erythroid progenitors. The incorporation of Iron into the protoporphyrin is catalyzed by the mitochondrial enzyme ferrochelatase, followed by the translocation and insertion of heme into hemoproteins in the cytosol [1, 6, 14].

1.1.3. Iron distribution in humans

The healthy human body absorbs daily a small amount of dietary iron (1-2 mg), which occurs in intestinal epithelial cells (enterocytes) in the duodenum, to compensate iron losses, whereas an adult body contains approximately 3-5 g of iron. Macrophages provide the basic source of plasma iron (20-25 mg) through degradation of senescent erythrocytes in the spleen, liver and bone marrow [15, 16]. The majority amount of body iron (2.1 g in human, 60-70%) is distributed in hemoglobin of developing erythroid precursors and mature red blood cells. High concentrations of iron are stored in macrophages and hepatocytes within ferritin (20-30%) and the remaining amount is present as myoglobin in muscles (5-15%) [13]. Due to the daily loss of iron, the balance is maintained by the control of dietary iron absorption through duodenal enterocytes [1].

1.1.3.1. Dietary iron absorption and subsequent transport

Two forms of dietary iron exist: Heme iron, which derives from ingested hemoproteins such as myoglobin and hemoglobin and non-heme iron, which is liberated as free iron from carrier proteins such as ferritin.

Inorganic iron uptake is well described since the last years [5]. During the uptake of nutritional iron, Fe(III) is reduced to Fe(II) in the intestinal lumen catalyzed by membrane-associated ferrireductases DcytB (duodenal cytochrome-b) and is subsequently transported across the apical membrane into the enterocytes by the divalent metal ion transporter DMT1 [17].

Another source of dietary iron is heme, arising from the degradation of hemoglobin and myoglobin, which is more bioavailable than inorganic iron. Heme can be also transported into the enterocytes by a distinct, not well characterized mechanism. It is proposed that the proton coupled folate transporter/ heme carrier protein-1 (PCFT/HCP1), a transmembrane transporter that is localized in the apical membrane of enterocyte plasmalemma is responsible

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for heme uptake [18]. However, heme is internalized by receptor-mediated endocytosis or via direct transport [16]. Heme transport is posttranscriptional regulated by iron stores because during iron-depletion HCP1 is localized to the apical membrane of enterocytes, whereas iron-repletion lead to the localization of the receptor in the endosome. In addition, heme iron is enzymatically liberated by the inducible microsomal enzyme heme oxygenase-1 (HO-1), which can be induced by numerous chemical and physical stimuli [12].

The liberated iron from heme or directly internalized iron into enterocytes by DMT1 that is illustrated in Figure 1 enters the labile iron pool (LIP), which is comprised of chaperone proteins or small organic chelates such as citrate, peptides, ATP and AMP that bind and transport iron. The cytosolic LIP contains only a low amount of total cellular iron, but representing the cellular iron status, which is controlled by intracellular sensors. It is utilized for direct iron incorporation into proteins or for iron transport to mitochondria [15, 16]. In erythrocytes released iron can be used for cellular processes (for iron-sulfur cluster biogenesis in mitochondria) or can be stored in ferritin, which is strongly expressed in spleen and liver. Furthermore, iron can be also exported from enterocytes into the bloodstream through the transmembrane transporter ferroportin (FPN1), followed by re-oxidized to Fe(III) by the membrane-bound ferroxidase hephaestin (Hp). In blood plasma one or two atoms of oxidized iron are subsequent loaded to apo-transferrin (apo-Tfn), a soluble protein in the blood, which transport and distribute iron to tissues [10].

Moreover, heme can be also exported as an intact molecule across the basolateral membrane of hematopoietic cells such as erythrocytes and most non-hematopoietic cells through the putative heme exporter FLVCR1 (feline leukemia virus subgroup C cellular receptor 1) to ensure sufficient amount of heme in the cytosol [19]. The ATP-binding cassette transporter G2 (ABCG2) is recognized as a second heme exporter, which is expressed on tissues including hematopoietic stem cells and erythroid progenitors, interacting directly with heme [20]. Heme in the blood stream binds to hemopexin (Hpx), a glycoprotein of blood plasma, which protects cells against its cytotoxic effect and cell death [15]. Moreover, the Hpx complex can be taken up by macrophages and hepatocytes, which is recognized by hemopexin receptors on the cell membrane (CD91) and internalized by endocytosis. Heme is subsequently metabolized by HO-1 or HO-2 in endosomes and iron is exported into the cytosol [13, 17].

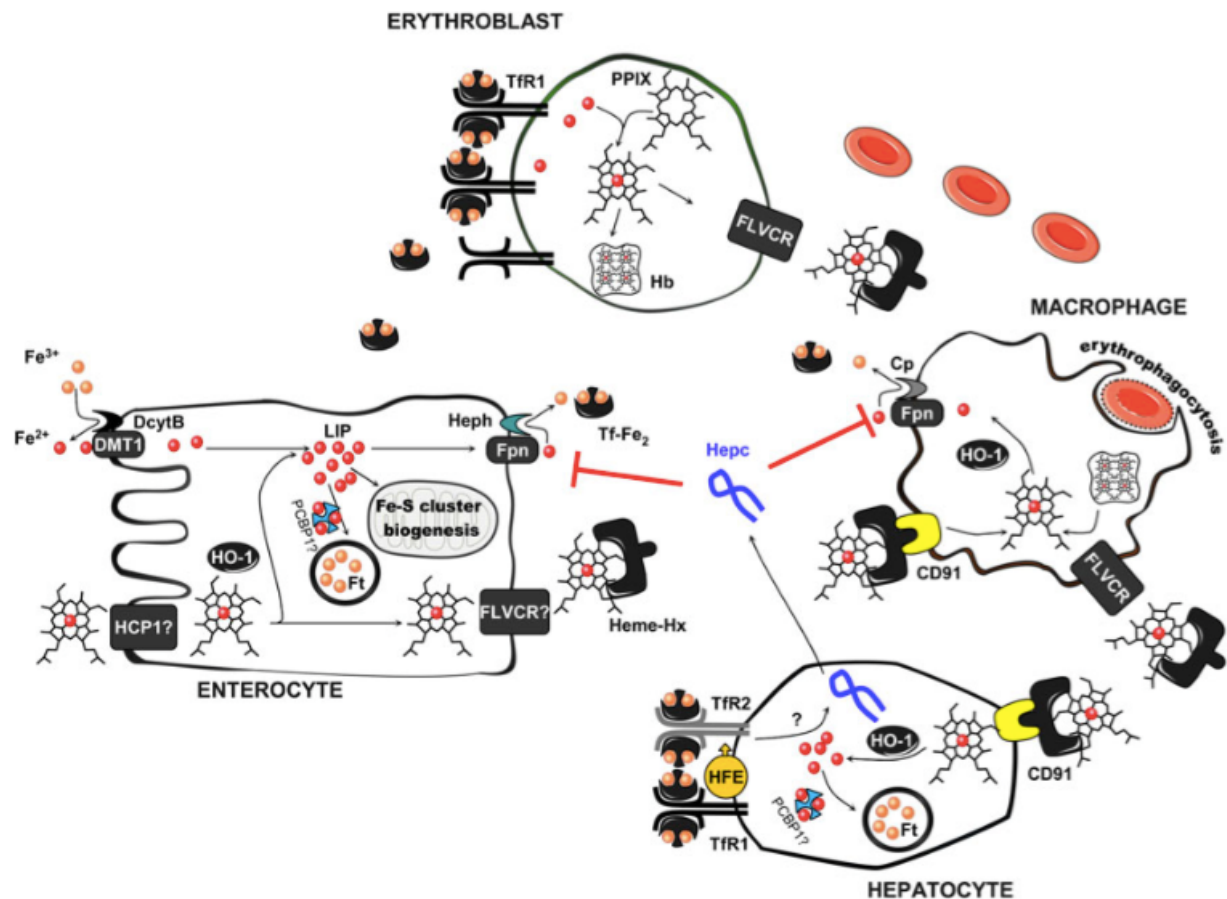


Figure 1 Overview of iron absorption by enterocytes, erythroblasts, macrophages and hepatocytes. In the duodenum non-heme iron is taken up by DMT-1 in enterocytes with the reduction of ferric to ferrous iron, which is mediated by DcytB. Heme is transported through enterocytes via PCFT/HCP1 and further catalyzed by HO-1. Iron in cells is needed for cellular processes or is exported via Fpn into the bloodstream after oxidation via hephaestin to ferric iron. Ferric iron binds to Tfn and is taken up via receptor-mediated endocytosis, whereas TfR1 is expressed in all cell types and TfR2 only in hepatocytes. Heme export is mediated by FLVCR1 to avoid heme toxicity in cells and subsequently binds to Hp in the circulation. Heme-Hp is taken up by hepatocytes and macrophages via CD91 receptor. In addition, hepcidin is responsible for the regulation of iron homeostasis, which is primarily expressed in hepatocytes. Adapted from Lipinski *et al* [a].

1.1.4. Regulation of cellular iron traffic/metabolism

1.1.4.1. Hepcidin - central iron regulatory molecule of iron homeostasis

The liver-derived peptide hormone hepcidin is predominantly expressed in hepatocytes and controls iron trafficking and extracellular iron concentration through the regulation of dietary iron absorption and the release of iron in macrophages. Hepcidin negatively regulates ferroportin-1 through phosphorylation, internalization and lysosomal degradation of FPN1 in intestinal cells, causing decreased iron export into the plasma [18]. It is highly expressed in

response to iron as well as during inflammation and infection causing decreased dietary iron adsorption and iron retention in macrophages to deprive iron from rapidly growing pathogens. In contrast, iron deficiency or hypoxia decreases hepcidin secretion, which triggers again increased iron adsorption from duodenum and iron release from macrophages [10]. Increased pathological amounts of hepcidin lead to iron deficiency and triggers the development of anaemia of chronic diseases. Conversely, inherited and acquired disorders that disrupt hepcidin production cause severe iron overload called hereditary hemochromatosis [1, 16].

1.1.4.2. Hereditary hemochromatosis protein (HFE)

The hereditary hemochromatosis protein regulates also the iron homeostasis by interacting with the TfR1. The protein exhibits a homolog structure to the major histocompatibility complex (MHC) class 1 type molecule including β 2-microglobulin (β 2m) [21]. HFE binds to the helical domain of the TfR1 in the endoplasmatic reticulum and translocated to the cell surface, leading to negative regulation of Tfn mediated iron uptake. In endosomes, under acidified conditions, HFE dissociate from the TfR1 [22]. Studies found out that Tfn and HFE compete for the TfR1 binding because both shares the same binding site on the receptor within the helical domain. Due to binding of HFE to TfR1, followed by disruption of the recycling pathway and degradation of the TfR1, the level of cellular iron uptake decreases [21, 22]. Additionally, studies have observed that sTfR also has the ability to interact with HFE [23].

Hereditary hemochromatosis (HH) is an autosomal recessive disease, which is characterized by excessive absorption of dietary iron leading to severe iron overload in various tissues especially in the liver and progressive organ failure [21]. Notably, HH is also associated with multiple T cell abnormalities [24]. Patients with HH have mostly a mutation in the HFE gene, which prevents the correct folding of HFE and their interaction with β 2-microglobulin, leading to the abolishment of correct trafficking [23, 24].

1.1.5. Mitochondrial Iron storage via ferritin complexes

Cells can remove excess intracellular iron by the transport of heme through the FLVCR or the secretion of Fe(II) through FPN1. However, cells have also the ability to detoxify and store excess intracellular iron within ferritin. The mitochondria play a vital role in cellular iron homeostasis because iron is used by these organelles for the synthesis of heme and biogenesis

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of ISCs as well as have a protective role against free iron and radicals [13]. Iron is taken up directly from the cytosol into the organelles via mitochondrial-specific iron importer proteins such as mitoferrin 1 and mitoferrin 2, expressed in erythroid and non-erythroid tissues [19], or by a kiss-and-run mechanism where Tf_n-laden endosomes interact with mitochondrial membrane [6]. In previous studies the ferritin protein, a homopolymer in the mitochondrial matrix, has been described as a conserved iron storage molecule, which consists of 24 subunits of heavy (H) and light (L) chains. The majority amount of ferritin is observed in sex cells of the testis. The common role of ferritin is to sequester iron molecules (up to 4500 Fe atoms), which enter ferritin via an iron chaperone protein, PCBP1 (poly(rC)-binding protein), followed by their oxidation through the ferroxidase activity of the H-chain subunit before incorporation. In some mammalian tissues, mitochondria express a distinct ferritin protein (FtMt), which is homologous to the H-subunit of cytosolic ferritin [10, 13]. During conventional conditions, ferritin is translated at low levels but in case of pathological conditions such as in sideroblastic anaemia it is induced for the detoxification of excessive iron [10, 15]. FtMt is expressed in the cytosol, which encodes the precursor and is taken up by mitochondria, cleaving the N-terminal target sequence. Under iron deficiency, cytosolic ferritin is degraded and recycled in proteasomes or lysosomes, allowing the release of iron. The disruption of the mitochondrial iron metabolism, including proteins such as ferritin and mitoferrins, leads to severe diseases such as neurodegenerative diseases.

1.2. Transferrin receptor system

1.2.1. Transferrin

Transferrin (Tf_n), the major plasma iron carrier, is a non-heme iron-binding glycoprotein with a molecular weight of approximately 80 kDa. The polypeptide is primarily synthesized in the liver (hepatocytes), but also a small amount in Sertoli, oligodendroglia and ependymal cell lines, which is secreted into the blood plasma [21, 25]. In vertebrates, Tf_n consists of two structural similar (40 %) but functionally different subunits, the N-terminal and C-terminal iron-binding subunit, separated by a short spacer sequence (peptide), which are further subdivided into two lobes (N1 and N2; C1 and C2) [26]. The two domains of iron-free Tf_n (apo-Tf_n) are widely separated into a “V” shaped conformation and form a deep cleft for an easy access of Fe(III). Apo-Tf_n has the capacity to bind two atoms of Fe(III) with high affinity to

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solubilize it and mediates iron uptake into cells through the interaction with its transferrin receptor (TfR) followed by receptor-mediated endocytosis [27]. Diferric Tfn (holo-Tfn) has a higher affinity to its receptor than the monoferric Tfn and apo-Tfn at physiological pH of 7.4.

Transferrin facilitates iron transport and cellular iron uptake, and maintains Fe(III) in a soluble form as well as in a redox-inert state to avoid free toxic radicals [15]. Serum Tfn is present in blood and other mammalian fluids and only 30% of Tfn is saturated with iron in the blood stream [22]. At the metal binding site carbonate is the relevant synergistic anion, which coordinates Fe(III) to Tfn and is essential for strong binding. Additionally, Fe(III) interaction with apo-Tfn depends on two tyrosine residues, one histidine and one aspartic acid residue [28]. Recent studies investigated that not only iron can bind to the specific iron binding site in Tfn but also many other metal ions [21, 22].

Other members of the transferrin family have been characterized, including ovotransferrin distributed in egg white, avian and reptilian oviduct secretions and lactoferrin located in milk, saliva, tears and sweat. Both proteins exhibit antimicrobial activities and bind circulating Fe(III), preventing cells against its toxic effect. Additionally, melano-transferrin, an integral membrane protein, is found in human malignant melanoma cells and in some fetal tissues [22].

1.2.2. Transferrin receptor 1 – CD71

The transferrin receptor TfR1 (CD71, here referred as TfR) is a transmembrane bound glycoprotein with a molecular weight of 180 kDa that constitutively cycles between the plasma membrane and endosomal compartments. The surface receptor is expressed on all virtually cells with the exception of mature red blood cells whereas the expression correlates with cellular proliferation as well as its iron status. The majority amount of TfR is located on rapidly dividing cells such as erythrocytes, the minority on the surface of resting cells and confined differentiated cells. Due to defects in erythropoiesis and neurological development, TfR knockout mice die before embryonic day 12.5 [21, 28].

1.2.2.1. Structural properties

TfR has been identified as a transmembrane type II glycoprotein through monoclonal antibody (mAb) studies [29], which is primarily found as a cell surface homodimer. TfR is composed of two identical glycosylated subunits linked by two disulfide bridges to form a dimer, which facilitates cellular iron uptake via internalization of iron-loaded Tfn, including recycling of the iron binding serum protein transferrin, as well as in the regulation of cell growth. The disulfide bridges are located in the extracellular part and consist of cysteine residues 89 and 98 [22]. Each monomer consists of a large extracellular C-terminal domain (ectodomain) containing the Tfn binding site, a hydrophobic transmembrane domain and a short cytoplasmic N-terminal tail [21]. The ectodomain is subdivided into the protease-like domain, the apical domain and the helical domain. Studies have shown through site-directed mutagenesis that the helical domain is critical for Tfn binding [22, 28].

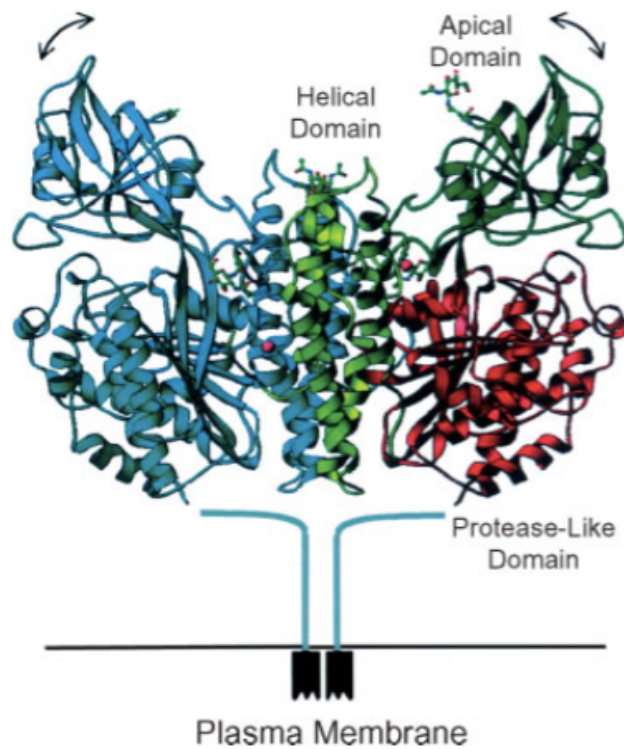


Figure 2 Schematic representation of the transmembrane bound transferrin receptor 1, CD71 in its crystal structure. The TfR1 is a type 2 glycoprotein, which is primarily found as a homodimer on the cell surface. TfR consist of two monomers linked by disulfide bridges, whereas each monomer consists of three domains, an intracellular domain, a transmembrane domain and extracellular domain. The Figure represents the extracellular domain, which is subdivided into the protease-like domain (red), the apical domain (dark green) and the helical domain (light green) in one monomer. The transmembrane segment is colored in black and the stalk in gray. Figure is adapted from Lawrence *et al* 1999 [b].

The ectodomain is subdivided into the protease-like domain, the apical domain and the helical domain. Studies have shown through site-directed mutagenesis that the helical domain is critical for Tfn binding [22, 28].

The TfR is present on the surface of most cell types, increasing the expression on high proliferating cells that need a high iron concentration such as cells of the basal epidermis and intestinal epithelium [21, 30]. Additionally, during fetal development the TfR is also highly expressed on those cells that require a large amount of iron from maternal circulating Tfn, including placental trophoplast [15, 29].

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The receptor is synthesized in the endoplasmic reticulum and is modified by phosphorylation and fatty acylation. The expression of TfR is mainly regulated at the post-transcriptional level in response to intracellular iron concentration [22]. The membrane-bound TfR contains three N-linked glycosylations and one O-linked glycosylation site, which are important for the correct functionality of the receptor [21].

The circulating form of TfR1, termed serum transferrin receptor 1 (sTfR1), is a soluble truncated monomer of tissue receptor which is produced by proteolysis (shedding) of the TfR C-terminal trypsin-sensitive site (Arg 100) via membrane-associated serine proteases. The concentration of sTfR is used to investigate the erythropoietic activity as well as is an indicator for functional iron deficiency which leads to an sTfR increase proportional to the severity of iron deficit. It reflects the iron demand of the body as well as the iron availability in the blood stream. In case of inflammation and chronic diseases the concentration of sTfR is not affected [31]. Dassler *et. al* has investigated that ferric Tfn has an inhibitory effect on TfR shedding [23]. A study has shown that the cleavage of the remaining N-terminal fragment (NTF) of the TfR1 is regulated by the intramembrane proteolysis, including the peptides peptidase-like 2a and 2b [32].

1.2.3. Intracellular traffic of iron – cellular iron uptake

Iron can be transported into cells by two major routes: transferrin-bound iron uptake and non-transferrin-bound iron uptake (NTBI).

1.2.3.1. Transferrin-independent iron uptake

Cells can also obtain heme indirectly. Senescent erythrocytes are phagocytosed by macrophages in the spleen, liver and bone marrow followed by intravascular hemolysis to acquire high amounts of iron. Moreover, macrophages can also receive heme iron through receptor-mediated internalization of Hpx associated with free heme (CD91) or hemoglobin which binds to haptoglobin (CD163), a liver-derived plasma protein. During endocytosis, macrophages metabolize hemoglobin, and heme degradation is catalyzed by microsomal heme oxygenase (HO-1 or HO-2 in endosomes) in the spleen to liberate inorganic ferrous iron which is reutilized. Subsequently, carbon monoxide (CO) and biliverdin is generated in which the latter is enzymatically further processed to the antioxidant bilirubin in the liver and provides cellular defense mechanism during oxidative stress [33]. Macrophage recycling

provides an approximately 20-fold higher iron amount than iron is absorbed through intestine. Analog to intestinal enterocytes, macrophages transport reduced iron across the plasma membranes into the cytosol via the transmembrane transporter FPN1, coupled by re-oxidation by the ferroxidase ceruloplasmin. Iron can also be stored in ferritin, which is delivered by the chaperon PCBP or is incorporated into iron-containing proteins [13, 15].

1.2.3.2. Transferrin-mediated mechanism: Tfn-Tfn receptor cycle

In comparison to transferrin-independent iron uptake, rapidly dividing cells and erythroid progenitor cells acquire metabolic iron by receptor-mediated endocytosis of dimeric Tfn through TfR1. Dietary iron adsorption is mediated through DMT-1, followed by the export of iron via FPN1 on the basolateral surface. Before iron is subsequently loaded to apo-Tfn, Fe(II) has to be re-oxidized to Fe(III). Dimeric Tfn, which is loaded with two atoms of iron, has a 30 to 500 fold higher binding affinity to TfR than monoferric Tf or apo-Tfn [15].

Two iron-bearing Tfn molecules can bind the TfR1 on the cell surface at the extracellular pH of 7.4, leading to the internalization of the complex in clathrin-coated vesicles, which are budded from the plasma membrane. However, at this conditions apo-Tfn is not able to interact with TfR1. Due to subsequent acidification to pH 5.5 of early endosomes by the proton pump ATPase, a conformational change of Tfn is induced allowing the dissociation of iron [22]. At this acidic pH, apo-Tfn remains bound to TfR1, until the receptor is recycled back to the cell surface where the molecule dissociates from the receptor due to pH increase [26]. Afterwards Fe(III) is reduced to Fe(II) by the ferric reductase STEAP3 (six-transmembrane epithelial antigen of the prostate 3) in immature erythroid cells and is translocated through the endosomal membrane via the trafficking protein Sec15l1 and the divalent metal transporter DMT1 to the cytosol (Figure 3) [15, 16].

Moreover, an alternative low-affinity mechanism of Tfn-bound iron uptake has also been described which acts in a TfR1-independent manner, but the mechanism is still unclear. It is disclaimed that TfR2 which is tissue-limited expressed is involved in the alternative mechanism because TfR2 mutations did not lead to defects in iron distribution. However, the involvement of other receptor molecules such as cubilin on kidney cells are not well characterized [15].

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Both mechanisms for cellular iron acquisition depend on the concentration of Tfn and the cell type. TfR1-mediated uptake predominates during low Tfn level, while the TfR1-independent uptake occurs at higher Tfn concentration. Hepatocytes as well as intestinal crypt cells receive holo-Tfn by the use of both pathways. However, immature erythroid cells only acquire holo-Tfn via the TfR1-mediated mechanism, which is turned out by the disruption of TfR1 followed by embryonic lethality of mice, leading to hematopoietic and neuronal defects [15].

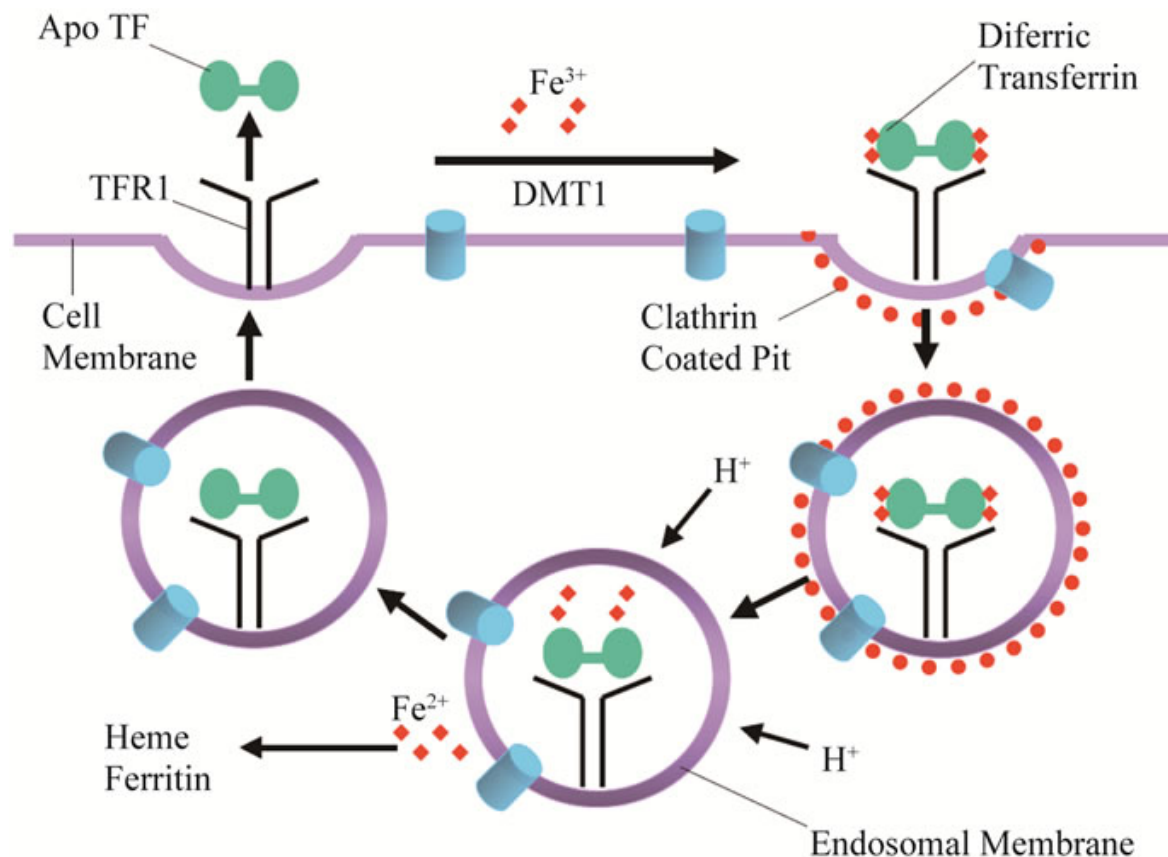


Figure 3 Image of the cellular iron uptake mechanism by the Tfn/TfR cycle via receptor-mediated endocytosis. Iron-bearing transferrin (holo-Tfn) binds to the TfR homodimer and the internalization of the Tfn/TfR complex is mediated by clathrin coated vesicles. Tfn/TfR is delivered into the endosomes, where the endosomal pH drops (pH 5.5), stimulating conformational changes in Tfn. The released Iron is subsequently reduced and transported into the cytosol by DMT-1. The apo-Tfn/TfR complex is recycled back to the surface, followed by the dissociation of apo-Tfn. Adapted from Elliott *et al* [c].

1.2.4. Regulation of transferrin receptor expression: IRE-IRP system

The expression of TfR1 is regulated by many factors including proliferation, differentiation and cellular iron levels [34]. In comparison to slowly or non-proliferating cells, higher levels of TfR are expressed on rapidly dividing cells, including activated peripheral blood mononuclear cells,

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epidermal cells, intestinal cells or malignant cells. During fetal development the Tfn mRNA increases continuously in the liver, remaining high in adults [15]. The TfR gene is located on the chromosome 3 and its expression is controlled in response to intracellular iron at the post-transcriptional level by mRNA-protein interaction. The expression of the TfR1 and the iron storage protein ferritin are coordinately regulated through the binding of the iron-regulatory protein 1 and 2 (IRP1 and IRP2) to iron-responsive elements (IREs), in their 3' or 5' untranslated regions (UTRs). The IREs of TfR1 mRNA form 5 hairpin stem loop structures, consisting of about 30 nucleotides, whereas the mRNAs encoding H- and L-ferritin contain one IRE [10]. IRP1 and IRP2, two trans-acting proteins which control mRNA stability and the rate of translation, belong to the protein family of iron-sulfur cluster isomerase, including mitochondrial aconitase [1]. IRP1 is a bifunctional sensor and ubiquitously expressed in all cells, containing a 4Fe-4S cluster [21]. Depending on cellular iron status, IRP1 can interact with RNA as well as has enzymatic activities as aconitase, sharing 30% sequence identity with mitochondrial aconitase. In contrast, IRP2 lacks aconitase activity because it does not contain 4Fe-4S cluster, however it is able to bind IRE sequences [22].

During iron starvation, the aconitase activity of IRP1 is lost due to the disassembling of 4Fe-4S cluster, allowing to bind with high affinity to the IREs in the 3' UTR region of TfR which increases the stabilization and translation of TfR1 mRNA, but inhibits ferritin expression when IRP1 binds to the IREs in the 5' UTR of ferritin mRNA. The same applies to IRP2 which also increases TfR expression and blocks ferritin translation. This leads in iron-deficient cells to the increase of iron uptake through Tfn-TfR system and to the inhibition of iron storage via ferritin [21]. Under high concentration of intracellular iron, IRP1 acts as an aconitase and does not bind to the IRE regions of the TfR1 mRNA following degradation. Additionally, ferritin mRNA is efficiently translated because IRP2 is targeted for proteasomal degradation, leading to the inhibition of IRP2 binding to IREs in the 5' UTR of ferritin mRNA [22].

1.2.5. Transferrin receptor 2

A homologous receptor, the transferrin receptor 2, is also a transmembrane type II glycoprotein. Due to alternative splicing two different receptor variants of TfR2 are produced, an alpha (TfR2 α) and a beta form (TfR2 β). TfR2 α contains 66% similarity to the extracellular domain of TfR1. In contrast to TfR1, TfR2 mRNA is predominantly expressed in cells, which are responsible for iron metabolism regulation such as hepatocytes and erythroid precursors

(enterocytes) of the small intestine, but not in mature red blood cells [26, 29, 35]. Another difference is that the expression of TfR2 does not correlate to intracellular iron levels in cells or is regulated by the interaction with HFE, however the expression might be regulated by the cell cycle. Furthermore, the TfR2 mRNA does not contain IRE sequences and has a 30-fold lower binding affinity for transferrin, in comparison to TfR1. A similarity between these two receptors is that both are glycosylated [21, 34].

1.3. Overview of the human immune system

The human immune system is comprised by two different kinds of immune defense systems, the innate immune system that is the natural non-specific immunity and evolutionary older, and the adaptive immune system that acts specifically. The first line of defense, including outer barriers such as the skin and mucous membranes, different cell types of the mononuclear phagocyte lineage, proinflammatory cytokine and complement system, protects the human body rapidly against common infectious pathogens [36, 37]. In particular, neutrophils, macrophages and natural killer cells (NK cells) are essential for the regulation of bacterial infections through phagocytosis as well as for the initiation and subsequent direction of the long-lasting adaptive immunity [38].

1.3.1. B and T cell immunity

In case of inefficient removal of infectious microorganism, the adaptive immune system is activated after the development of lymphocytes, which are subdivided into two major groups: B lymphocytes, which are responsible for antibody response and T lymphocytes. T cells are further subdivided into effector T lymphocytes for cell-mediated immune response and regulatory T lymphocytes (Treg) for the regulation of autoimmunity and maintenance of self-tolerance [39]. B cells are responsible for the humoral immunity because they recognize specific molecular structures through membrane immunoglobulins, IgM and IgD. Mature B lymphocytes produce and release specific antibodies against extracellular microbial antigens and toxins, leading to the elimination of the foreign particle and infection [40].

In contrary, T cells are involved in cell-mediated immunity and proliferate, grow and differentiate to different subsets in response to intracellular invaders. T cells recognize intracellular processed pathogens or peptide fragments, followed by the induction of killing affected cells, which cannot be detected by antibodies [36]. Immature dendritic cells (imDCs),

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which belong to the crucial group of antigen-presenting cells (APCs) of the innate immune system of mammalian are the linkage between the innate and adaptive immunity and have a important role in the initiation of T cell-mediated response [38]. APCs such as DCs, phagocytic cells and B lymphocytes have the function to recognize, phagocytose and degrade pathogens, presenting the processed antigen by the major histocompatibility complex (MHC) class 1 or class 2 molecule to antigen-specific naïve T lymphocytes in secondary lymphoid organs such as lymph nodes, interacting with the T cell receptor (TCR) on T cells [40, 41]. The TCR associated CD3 complex is phosphorylated by the kinase Lck on ITAMS (intracellular immunoreceptor tyrosine-based activation motif) which induces the recruitment of adaptor proteins such as LAT (Linker for Activated T cells) [42]. Moreover, between the interface of T cell and APCs the immunological synapse is formed which is important for the delivery of intracellular signals in T lymphocytes. In addition, proinflammatory cytokines as well as many different cell-surface molecules influence the immune response and trigger lymphocyte homing [37, 43, 44].

Mature T lymphocytes are comprised by two main types which are based on different co-receptor profiles. Fragments of antigens, which are bound to MHC class 2 stimulate T-helper cells (CD4+). CD4+ cells are important for the activation of different immune cells such as B lymphocytes, macrophages as well as CD8+ T-cells and stimulate the recruitment of other immune cells for the elimination of infectious pathogens. In particular, the activation of T-helper (Th) 0 cells, leads to the further division into three effector subtypes, including T helper cells type-1 (Th1), type-2 (Th2), type-17 (Th17) and regulatory T cells, depending on specific costimulatory molecules and cytokine expression [39]. In general, Th1 cells produce interferon (IFN)- γ , Interleukin (IL)-2 and tumor necrosis factor (TFN)- α and - β as well as mediate immunity against intracellular pathogens such as bacteria and protozoa [3]. In addition, Th1 cells are responsible for the activation of CD8+ cells and are associated with several inflammatory and autoimmune diseases [38, 39, 45]. For the initiation of T helper cell differentiation, different amounts of cellular iron levels are necessary, including the requirement of higher iron concentration for Th1 cell development than for Th2 cells. The effector function of Th1 cells is more sensitive due to the treatment of TfR with anti-TfR antibodies than it was observed in Th2 cells, leading to the inhibition of DNA synthesis [3]. In contrary, Th2 response is associated with allergic diseases and contributes to the B-cell mediated humoral immune response in case of extracellular pathogen invasion, enhancing

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the B cell isotype switch to IgE secreting plasma cells. A new T cell effector subunit has been observed, the Th17 cells, which is crucial for the elimination of extracellular bacteria and fungi through stimulation of neutrophil cells via IL-17 [39] .

In contrary, MHC class 1 bound peptides stimulate cytotoxic T cells (CD8+) that produce IFN γ and TFN- α , followed by the clearance of viral infections and elimination of pathogens [38]. CD8+ cells are responsible for antiviral response as well as recognize tumor cells, which are eliminated by cytotoxicity [40]. After activation, clonal expansion and contraction (apoptosis) of lymphocytes due to antigen exposure during the primary immune response, few antigen-specific T cells that survive programmed cell death differentiate into long-lived memory cells and remain in the host. During a secondary infection, memory cells protect the host against antigen-mediated activation as well as directly kill infected cells. In particular, memory cells become quickly reactivated by cognate antigens, followed by the promotion of the adaptive immune system [41, 46, 47].

1.3.2. The role of Iron and TfR in the human immune system

1.3.2.1. Iron metabolism by T cells and their function in the human immune system

It has been observed that a cross-regulatory interaction between iron homeostasis and immune system exists [4]. Iron has the ability to affect immune effector mechanisms such as the proliferation of immune cells, cytokine expression, radical production, causing the modulation of host immune surveillance. Moreover, cellular iron can alter the immune response against invaders in association with cytokines and cell-mediated immune effector cells such as macrophages [3].

During iron-related diseases, the homeostasis of iron is lost. Limited amount of iron is associated with abnormal absorption of metals from the digestive lumen. The impact of iron deficiency on T cell accumulation causes an uncontrolled decrease of the total number of circulating T lymphocytes in blood, which is a result of thymus atrophy and decreased thymocyte proliferation. However, the thymocyte apoptosis is not increased during iron deficiency [24]. Furthermore, the ability to induce NO synthase and to kill intracellular pathogens through IFN- γ -mediated pathways, such as the expression of MHC class 2 and proinflammatory cytokines, is lost when macrophages loaded with iron [3]. The weak immune

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system allowed the invasion of pathogens which are independent of iron, however extracellular bacteria that depend on iron concentration are inhibited in their growth [4].

Reduced proportions of T lymphocytes and circulating CD4⁺ T cells as well as a shift of the ratio between CD4⁺ and CD8⁺ T cells occur during iron overload conditions. Additionally, the adsorption of dietary iron is dramatically increased which is associated with diseases such as thalassemia, hemochromatosis and cirrhosis [3, 24]. Increased iron availability in tissues and plasma contribute to malignant growth and increase the risk for several tumors. Many cancers such as breast cancer, leukemia, lung cancer and others highly upregulate TfR on their surface as well as store excess intracellular iron in the iron storage protein ferritin [11].

The regulation of iron homeostasis is crucial in host-pathogen interaction. Microorganisms take up free heme or heme proteins through specific membrane receptors and proteins like hemophores, which serves as the favorite iron source, leading to infections. Therefore, it is crucial that heme is sequestered by proteins, which counteracts bacteria invasion [48]. Additionally, during pathological conditions, cytokines, hormones and cellular proteins such as hepcidin affect the iron homeostasis to reduce the availability of circulating iron for extracellular microorganism, allowing the abatement of infections. However, the decrease of iron leads to the development of anemia of chronic diseases (ACD) [4].

1.3.2.2. TfR for the precise maturation of T cells

A majority amount of iron is not only required during erythropoiesis for the generation of hemoglobin through TfR, but also for the differentiation and proliferation of other hematopoietic cells such as B and T lymphocytes during early lymphoid development. Little or no detectable amount of TfR are expressed on the surface of resting or mature T and B cells, allowing no adsorption of iron. However, during the activation of normal peripheral blood T and B cells upon antigen or mitogenic stimulation, which has a central role in the immune response, TfR is expressed on their surface in the G0/G1 phase during cell cycle to facilitate cellular iron uptake by receptor-mediated endocytosis [49-51].

T lymphocyte activation requires two signal processes, including antigen-specific signals through the interaction of MHC molecules and TCR, as well as costimulatory signals, which are mediated by co-receptor-ligand interaction. Afterwards, T cell activation is associated with the expression of IL-2 receptor as well as cytokine production, followed by the upregulation of TfR

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for the initiation of proliferation of quiescent T cells, depending on IL-2 detection. In addition, T cell activation is linked to the availability of high amounts of iron during proliferation, leading to the modulation of TfR by cellular iron concentration [52].

A recent study has observed that the TfR is crucial for the normal maturation of T lymphocytes because the absence of TfR leading to completely T cell arrest at a very early stage in their development (CD4⁺8⁺3⁺) [52].

Normally, TfR is not present in lipid rafts at steady state, however during T cell activation TfR is compartmentalized in lipid rafts [21]. Batista *et al.* have observed this partial translocation of TfR from endosomal compartments into lipid rafts of activated T cells via anti-CD3 and anti-CD28 antibodies (Abs) or superantigen followed by redistribution of surface proteins and the induction of intracellular signaling pathways. Through the interaction between MHC class 2 of APCs and T cell receptor (TCR) of T cells, the central supramolecular activation cluster (c-SMAC) is formed, including the TCR, which is surrounded by peripheral SMAC (p-SMAC) where TfR is accumulated [42, 53]. It is suggested that TfR is needed for the formation of the immunological synapse between APCs and T cells because pre-treatment of T cells with anti-TfR mAb (FG2/12) affects lymphocytes to form immunological synapses [53].

However, TfR serves as an iron transporter, but seems to be also important for signal transduction through TCR/CD3- ζ -chain, contributing to T cell activation and antigen presentation. Due to the stimulation of TfR with the mAb F1/6, the formation of the immunological synapse is inhibited [21]. Nevertheless, F1/6 binding to TfR induces a tyrosine phosphorylation of proteins (TCR/CD3 ζ), which depends on TCR expression on the cell surface, leading to the physically association with the ζ -chain of the TCR/CD3 complex that is associated with the T cell specific protein tyrosine kinase 70 kDa (ZAP70). In particular, anti-TfR activates the protein kinase C and releases IL-2. Conversely, the direct stimulation of TCR causes TfR tyrosine phosphorylation and the activation of a protein tyrosine kinase which is associated with TfR [54].

1.3.3. The TfR as therapeutic approach and the inhibition of lymphocyte proliferation by monoclonal antibodies

TfR is highly upregulated in tumor cell lines and malignant tissues compared to healthy compartments, which is associated with the malignancy of tumor progression. During cancer

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immunotherapy mAbs are used against TfR, which represents a potential therapeutic target, leading to the decrease of TfR expression and privation of iron followed by the inhibition of tumor growth [11, 29, 45].

Inhibitory effects have been discovered via the application of other CD71 mAbs such as A24 (IgG isotype) [55], FG2/12 (IgG1 isotype) [49] or 42/6 (IgA isotype) [50, 56]. Guerrero *et al* have shown that the location of mAb FG2/12 on the TfR molecule is related to the binding site of Tfn, leading to the efficiently inhibition of Tfn to its receptor [49]. Furthermore, it was observed that FG2/12 influences the formation of the immunological synapse by T cell-APC interaction [53]. Moura *et al*. have investigated the therapeutic effect of the mAb A24 against the human TfR in adult T-cell leukemia (ATL). The mAb A24 competes with Fe-Tfn which leads to the reduction of Tfn binding on TfR and Fe-Tfn uptake in activated T cells followed by TfR recycling in endosomal compartments as well as the induction of apoptosis of malignant cells [55]. In another way acts the mAb 42/6, which sterically inhibits the binding of Tfn without any competition between the mAb and Tfn. This was suggested because Tfn does not block the binding of mAb to the transferrin receptor. In comparison to mAb 42/6, it was observed that the anti-Tfn IgG mAb B3/25 does not inhibit Tfn binding, Fe-Tfn uptake and lymphocyte growth. However, in the presence of mAb 42/6 and B3/25 the TfR expression is decreased, but only 42/6 has the ability to inhibit Fe-Tfn uptake, resulting in the inhibition of cell growth of normal and malignant human lymphocytes by iron deprivation [56].

1.3.4. The role of TfR in B lymphocyte proliferation

The accumulation of TfR on B cell surface can be detected during the activation of B cells, which is probably regulated by the B cell growth factors (BCGFs), leading to B cell proliferation. In addition, a second signal, which depends on T cell derived mitogen stimulation, but not on TfR stimulation, leads to the secretion of immunoglobulins. However, resting B lymphocytes only express low amounts of TfR [51]. Neckers *et al*. has observed due to mAb 42/6 binding studies that TfR expression is required for B cell proliferation because mAb 42/6 blocks the binding of Tfn to its receptor, mediating inhibition of B cell growth by the prevention of DNA synthesis. Nevertheless, another study has investigated by the usage of the Rag2^{-/-} mouse model that the development of TfR deficient B cells is not as affected as T lymphocyte development. Iron has a crucial role in early steps of erythrocyte and lymphocyte development. Immature B cells, which go through heavy-chain gene rearrangement, develop

functional IgM⁺ antibodies during TfR knockout followed by iron deprivation. Nevertheless, B cells proceed normal differentiation during early steps in the absence of TfR, but only less amount of B cells are permitted to mature to effector B lymphocytes due to a defect in proliferation of precursor B cells, suggesting the importance of iron uptake via TfR during proliferation [52].

1.4. Hemoglobin, heme scavenging and cytotoxic effect of heme

Hemoglobin (Hb), a cooperative hetero-tetrameric heme-protein, binds and transports oxygen (O₂) in the blood stream, depending on the prosthetic group termed heme *b*, which has a central iron atom and a pyrrol ring. Hb consists of four homologous subunits, two α - and two β -domains, which form identical $\alpha\beta$ dimers. When oxygen interacts with Hb structural changes occur, allowing the transition from the T state to the R state and the increase of oxygen affinity [57].

The synthesis of Hb during erythrocyte development is coordinated because free α - and β -subunits are cytotoxic. Additionally, under physiological conditions the concentration of extra-erythrocytic Hb (free hemoglobin) and free heme is low, but can become dangerously high under pathological state [48]. Therefore, an abundant acute phase glycoprotein called haptoglobin (Hp), which is primarily expressed in the liver, interacts with released Hb in the plasma to prevent extra-erythrocytic Hb-induced cell damage and preoxidative modifications of Hb [58]. Afterwards, the Hp-Hb complex interacts with its receptor CD163, located on macrophages and hepatocytes and internalizes through receptor-mediated endocytosis [59].

During the Hb metabolism, heme-protein and free heme molecules are released into extracellular fluids [48]. Free heme has the ability to intercalate into lipoproteins and lipid membranes with its porphyrin ring, consisting of a central iron atom, followed by destabilization and increase of membrane permeability. In particular, heme promotes membrane lipid peroxidation and the generation of reactive oxygen species. Additionally, heme induces the activation of innate immune cells as well as inflammation by non-hematopoietic cells [59]. Due to the consequences of heme toxicity, extracellular and intracellular proteins have crucial functions to regulate free heme availability. Free heme is sequestered by serum albumin, Hpx and high and low density lipoproteins to protect cells against free heme oxidative damage as well as maintain and recycle the iron pool [48]. This

plasma scavenger proteins transport free heme to intracellular compartments, allowed the catabolism of heme through HO enzymes [59].

In case of infections, malaria, trauma and sickle-cell anemia (SCD) the intravascular hemolysis increases rapidly which leads to the saturation of Hb/heme scavenging mechanisms, followed by heme-mediated oxidative damage [59].

1.5. Human serum Albumin (HSA)

Human serum albumin (HSA) is the major soluble protein component of the blood plasma, comprising 60% of the total protein amount [60] and is crucial for homeostasis. It is important in heme transfer from high and low lipoproteins to hemopexin (Hpx) [61].

Under physiological conditions, only 20-30% of SA is synthesized in hepatocytes of the liver as a precursor, termed pre-pro-albumin, which is regulated transcriptional and posttranscriptional [62, 63]. Afterwards the processed protein pro-albumin is secreted into the lumen of the endoplasmatic reticulum, followed by proteolysis in the Golgi complex. The synthesis can be altered by hormonal changes, changes in colloid osmotic pressure or in the osmolality of the extravascular hepatic space [64].

HSA is circulating from the intravascular space to the extravascular space, where it is in the latter case, including blood fluid, predominantly available [62]. The majority of HSA is released into the vascular space and only a small amount of HSA is stored in hepatocytes. The remaining HSA level is in extracellular locations such as muscle and skin [63].

1.5.1. Structure and biochemical properties

Serum albumin (SA) is an abundant non-glycosylated plasma protein with a molecular weight of 66 kDa, which consists of a single chain of 585 amino acid sequences with 17 disulfide bridges, including few aromatic amino acids but many amino acids with functional groups to stabilize the blood pH [65]. In particular, the heard shaped structure of HSA contains 35 cysteine residues, which are all involved in disulfide linkage, with the exception of one cysteine residue (Cys-34), remaining as a free reduced thiol that maintains the redox state of plasma [62, 66]. HSA is composed of three homologous domains (I–III), which are subdivided in two subunits (A and B), seven FA binding sites as well as two major structurally-selective small molecule sites [60]. Each sub-domain is composed of six and four α -helices, connected by

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flexible loops [66]. The primarily binding regions in HSA are located in the subdomain 2A and 3A, which exhibit structural differences in the location of bound ligand [67]. The FA binding sites are the relevant cleft hosting ligands, where heme, FAs, bilirubin as well as drugs interacts [68]. Under physiologically conditions, HSA predominantly exists in its reduced form in extracellular fluids, comprising a free thiol group. The thiol-redox state of HSA is used as an indicator for oxidative stress, liver diseases and diabetes mellitus [63, 65].

HSA represents the depot and transporter of biologically important ligands, modulates fluid distribution between the compartments, regulates the colloid osmotic pressure (COP) as well as act as an antioxidant in cell culture, including the ability to bind metal ions, scavenge free radicals as well as protect bound substances from peroxidative damage [69]. In particular, HSA may modulate inflammatory processes, including neutrophil/endothelial cell interactions and part of cell signaling after shock and resuscitation [63]. Due to its strong negatively charge, the common function of albumin is the transport of a large number of hydrophobic small molecules such as fatty acids, steroids, metal ions, toxic metabolites and a wide variety of drugs across organ-circulatory interfaces such as liver, kidney, brain and intestine [70, 71].

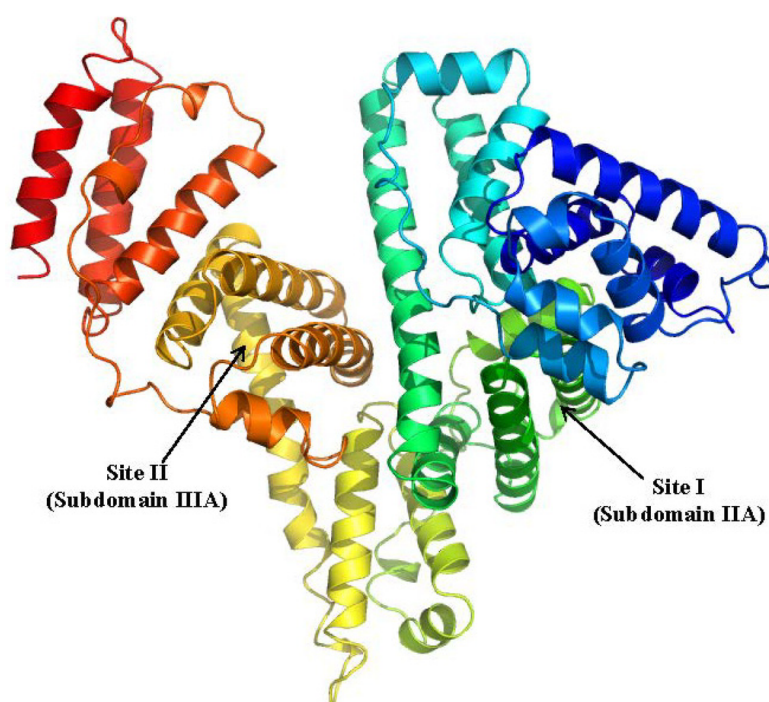


Figure 4 The secondary structure of human serum albumin is shown. The Figure represents the binding domains of HSA. The domain IA is shown in blue; the domain IB is cyan; the domain IIA is green; domain IIB is light green-yellow domain IIIA is orange and the domain IIIB is red. Hydrophobic binding sites, including the Sudlow I (Sub-domain IIIA) and Sudlow II (Sub-domain IIA), are indicated. The Figure is obtained from Chuang *et al* [d].

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SA is commonly used as an essential medium supplement in cell culture experiments; in particular, SA is crucial for the development of cell lines such as monocyte-derived dendritic cells as well as support the cell growth of various cell types. However, bovine serum albumin (BSA) is more widely used for cell culture than HSA because of the supply and costs [65, 66].

Activation of lymphocytes needs an antigen or mitogenic stimulation such as concanavalin (Con A) or bacterial lipopolysaccharide (LPS). However, SA has observed to facilitate the proliferation of activated T and B lymphocytes through an unknown factor, replacing unspecific mitogens such as Con A for growth stimulation. The study investigated that the factor which is responsible for cell growth does not involve fatty acids or hormones. However, the effect of SA contributes to a molecule which is tightly bound to SA or to the SA itself [72].

1.5.2. HSA delivery system

Numerous albumin-binding proteins have been identified in different tissues and cell lines, including kidney, fibroblasts and endothelium: Glycoprotein 60 (gp60), glycoprotein 18 (gp18), glycoprotein 30 (gp30), neonatal Fc receptor (FcRn), heterogeneous nuclear ribonucleoproteins (hnRNPs), calreticulin, cubilin and megalin damage [69].

In kidney cells, SA is taken up by endocytosis through clathrin-dependent mechanism, involving the glycoprotein megalin [66]. The albumin surface receptor gp60, which is expressed on the plasma membrane of continuous endothelium except in the brain, specifically binds the plasma protein, allowing internalization through caveolin-dependent endocytosis and transcytosis. Through additional interaction with long-chain fatty acids (LCFAs) or glycosylation of SA, the rate of albumin transfer increases [73]. HSA is protected against degradation in acidified endosomes via the transport receptor FcRn, which is expressed on APCs, and tissues including kidneys, lung and gut [66, 69].

1.5.3. Fatty acids in plasma and role of albumin in their transport

Fatty acids (FAs) adsorption is the energy source of many tissues such as resting skeletal muscles, renal cortex and liver. The majority of FAs in plasma is represented by LCFAs that are released into the blood stream and transported by serum albumin. Short and medium chain FAs are taken up in the blood followed by dietary intake. In the circulation, HSA is the main carrier for the delivery of unesterified FAs and has at least seven binding sites for medium- as

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well as long-chain FAs such as myristate with different affinity (FA1 to FA7) [66, 74]. Due to low affinity to the subdomain IIA, FAs are slightly replaced by drugs. In contrast, the subdomain IIIA is the strongest binding site of FAs, allowing during interaction the inhibition of drug and steroid binding (e.g. cholesterol) to HSA [75, 76] .

1.5.4. Heme-albumin

HSA has a high affinity to heme-Fe(III) and a crucial role in heme scavenging during physiological and pathological conditions, binding heme from lipoproteins to form methemalbumin and further transporting to the heme-binding protein Hpx. In contrast to hemoglobin and myoglobin, native heme-albumin does not have the ability to bind oxygen because it provides no molecular structures similar to the other hemoproteins [77].

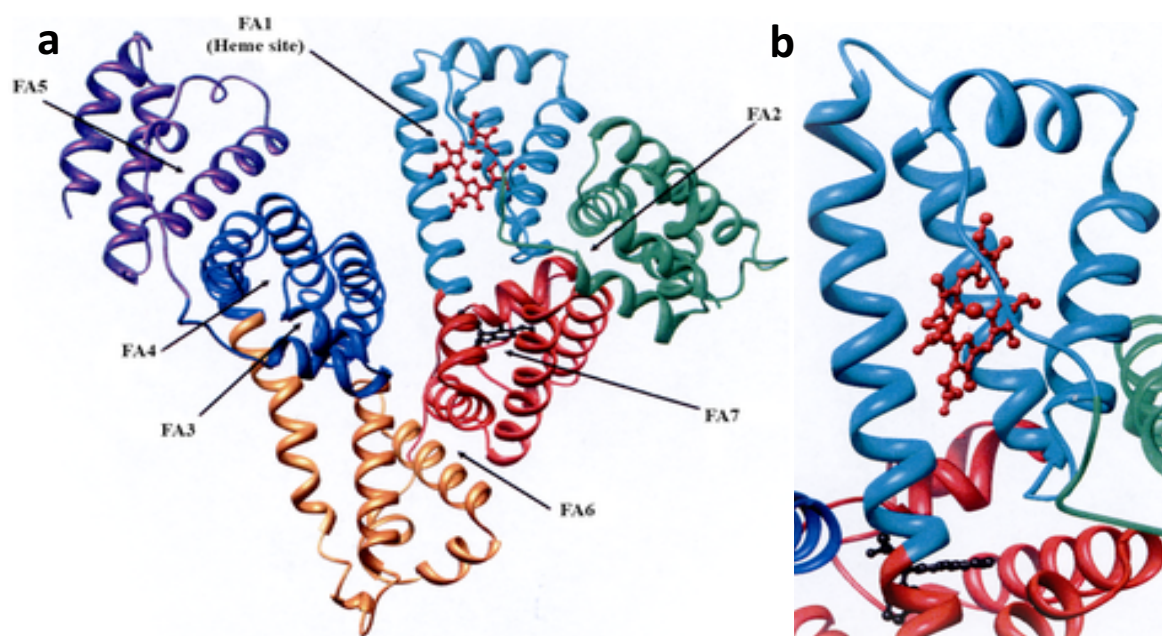


Figure 5 Crystal structure of human serum albumin molecule which is complexed with hemin and fatty acids.

(a) The secondary structure of HSA is shown with three homologous domains (I–III), which are subdivided in two subunits (A and B). IA is green; IB is cyan; IIA is red; IIB is orange; IIIA is blue; IIIB is purple. Each subdomain is composed of six and four α -helices, connected by flexible loops. Binding sites 1–7 correlates to the seven fatty acid binding sites which are identified in previous studies, whereas the fatty acid site 1 is occupied by heme-Fe in this Figure (in red) [66, 74] and FA7 by warfarin (in black). (b) The schematic ribbon diagram represents the binding site of heme in the subdomain IB. Figure is obtained from Bocedi *et al* [e].

Through binding studies, the heme-binding site was discovered in the subdomain IB of HSA molecule, binding to HSA by the IA-IB loop, which corresponds to a binding location for FAs.

Notably, when FAs associate with the heme-binding site as well as bind to the Sudlow's site 1 (subdomain IIA), the interaction of heme to HSA is competitively and allosterically modulated. Moreover, various pharmaceutical agents such as warfarin can modulate allosterically heme-based catalytic properties of HSA through the interaction with the Sudlow's site 1, which influences the coordination state of heme-Fe atoms. Accordingly, heme-binding decreases the drug affinity to the Sudlow's site 1 [48, 61, 77]. In particular, the plasma protein protects the body against free-heme oxidative damage [61]. When Hpx becomes saturated by heme, heme binds after hemolysis to HSA, which provides a reserve binding capacity. Afterwards, heme is transported to the liver for metabolism or excretion [77, 78].

During pathological conditions, microorganisms take up free heme or heme proteins through specific membrane receptors and proteins like hemophores, which serves as the favorite iron source, leading to infections. Therefore, it is crucial that heme is sequestered by proteins, which counteracts bacteria invasion [48].

1.5.5. The ability of HSA for drug delivery

In circulation, drugs can represent two forms, a bound or unbound form to plasma proteins. In most cases, the unbounded drug level in tissue depends mostly on the unbound drug level in the plasma. HSA has also the ability to deliver a wide variety of hydrophobic and hydrophilic drugs throughout the circulation to organs or tissues. Additionally, HSA is not only the relevant factor for drug distribution and their biological effectivity, but also protects bound drugs against oxidation. In particular, HSA can affect the pharmacological effects of drugs and induce chemical modifications [60], whereas drugs that bind simultaneously to HSA, can alter the HSA binding behavior [75]. In case of drug displacement from their binding site through other substances, the distribution, metabolism and excretion of the displaced drug as well as the pharmacological action would be altered [60]. Other drugs, which have a high affinity to albumin in plasma, bind at different sites without any competition such as warfarin and diazepam [62].

Recent studies investigated two specific binding sites on HSA for the most pharmaceuticals, site I and site II which are located in the subdomains IIA and IIIA on the albumin molecule. Site I and II have different ligand-binding affinities and overlap with the binding sites of endogenous ligands [63, 77]. Different drugs such as warfarin, phenytoin, some penicillins and

many others bind at the binding site I (Sudlow's site 1) whereas the binding site 2 of drugs (Sudlow's site 2), represent a recognition cleft of diazepam and anti-inflammatory agents [68, 73].

The binding of drugs is relevant in patient with critically illness. However, many agents can influence this drug-albumin interaction [73]. Due to severe side effects during cancer treatment, the HSA-based delivery system has been utilized to reduce the side effects and improve the targeting of anticancer agents [60].

1.5.6. Abraxane: An albumin-bound nanoparticle

One of the most effective chemotherapeutic drug is named paclitaxel which is an alkaloid compound and is isolated from bark of Pacific yew tree. The drug has a crucial role in treatment of ovarian, breast, non-small-cell lung cancer (NSCLC), Kaposi's sarcoma and other carcinomas [79-81]. Paclitaxel is associated with Cremophor EL (polyoxyethylated castor oil) and dehydrated ethanol due to poor water solubility which leads to severe side effects such as histamine release, anaphylaxis, abnormal lipoprotein patterns and hyperlipidemia[80, 82].

Abraxane (nab-paclitaxel), a white lyophilized powder, is composed of albumin-bound paclitaxel particles without Cremophor for drug delivery and have a particle size of about 130 nm. The chemotherapeutic drug is a solvent-free colloidal suspension, using albumin as a vehicle, which is derived from human blood, to exploit albumin receptor-mediated endothelial transport. SA has the ability to increase drug delivery to tumors by binding to a selective overexpressed glycoprotein (gp60), an albumin receptor on endothelial cells, followed by endocytosis and drug accumulation in tumor cells bound to SPARC (secreted protein, acidic and rich in cysteine) [82, 83].

In general, microtubules are crucial in the process of mitosis and cell division, including the formation of mitotic spindle during cytokinesis, cell motility and attachment, intracellular transport, regulation of transmembrane signals and shape maintenance, which provide an important target for anticancer agents. Paclitaxel is known to promote and stabilize eukaryotic microtubules, including the stimulation of polymerization of tubulin subunits of microtubules and prevention of depolymerization followed by the inhibition of the G2 or M phase of cell cycle [79, 84]. The binding site of paclitaxel is located in the β -subunit which is arranged on the inside of the microtubule surface, binding with high affinity followed by conformational

changes in the tubulin [81]. Thus, paclitaxel leads to DNA fragmentation, inhibition of cellular division and migration of chromosomes as well as morphological features of apoptosis [79, 84]. In contrast to paclitaxel compound, Abraxane causes fewer side effects as well as exhibit improved effectivity and therapeutic strategies to eliminate cancer cells [83].

1.5.7. Critical illness and therapeutic potential of HSA

In patient with critically illness the rate of synthesis, degradation and distribution of albumin between the intra- and extravascular space is modulated, decreasing the concentration of albumin during sustained inflammatory response. Moreover, the COP is decreased in critically ill patients followed by the increase of morbidity and mortality [73]. Due to its great clinical demand as a potent volume expander for fluid replacement and a compensator for blood loss it has to be acquired by fractionation of donated blood plasma. Moreover, HSA is widely used for the treatment of several diseases such as serious burn injuries, traumatic shock as well as plasmaphoresis [63, 85].

To produce a large amount of HSA molecules, various efficient biosystems have to be developed for the expression and purification of recombinant serum albumin. Recombinant human serum albumin (rHSA) is made in various expression systems, including *Saccharomyces cerevisiae*, *Kluyveromyces* sp., *Pichia pastoris* or transgenic plants such as *Oryza sativa* (OSrHSA) but only few amount of rHSA progresses to clinical trials [86]. Recombinant serum albumin is widely used in different laboratory, clinical fields and pharmaceutical purposes to substitute HSA, avoiding infectious diseases such as hepatitis due to various contaminations [65].

2. AIM OF STUDY

The transferrin receptor system is pivotal for cellular growth and survival. TfR is highly expressed on all dividing cells and serves as an important iron uptake mechanism in cells. Moreover, human serum albumin is a crucial nutrient supplier for cells which represents the depot of biologically important endogenous and exogenous ligands as well as peptides and proteins

In this work we wanted to investigate if heme-albumin plays an important role in the induction of cell proliferation of leukemic T cell lines through CD71. It is already known, that four murine monoclonal antibodies which are directed against CD71 block CD71-dependent iron-uptake. Therefore, it has to be further questioned if CD71 mAbs could inhibit iron supply that is received from heme-albumin.

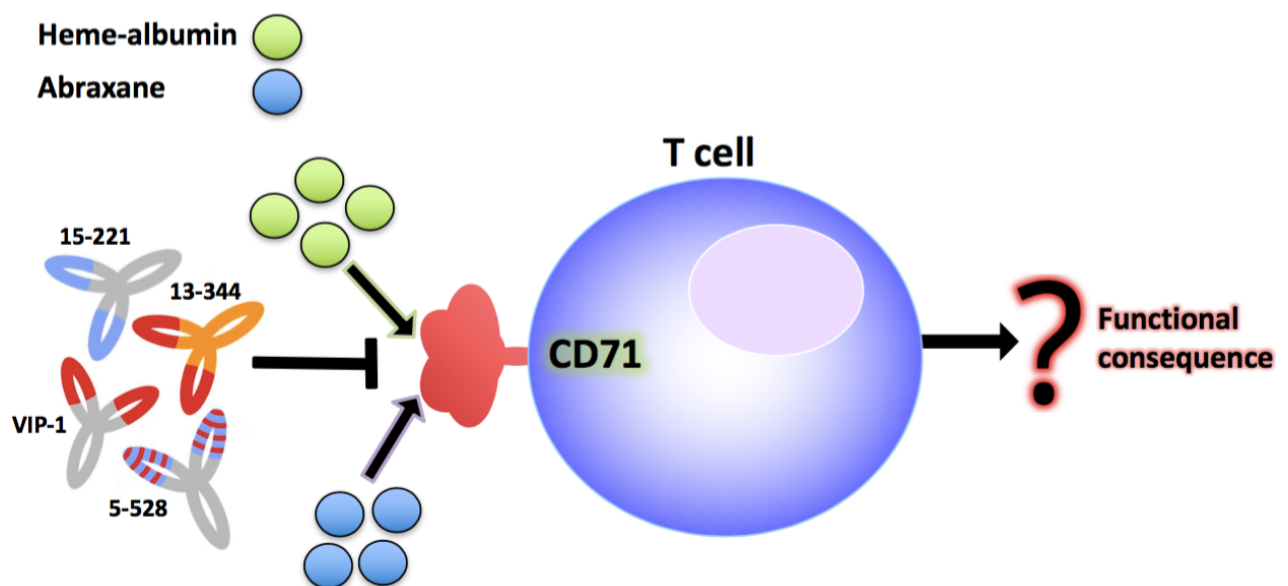


Figure 6 Overview of the aim of my study.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Antibodies

3.1.1.1. Primary antibodies

Specificity	Clone	Isotype	Species	Source
Calf intestine alkaline phosphatase	VIAP-2D5	IgG1	Mouse	Otto Majdic, IFI, Vienna, AT
CD71	5-528	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

Table 1 List of antibodies

3.1.1.2. Secondary antibodies

Specificity	Clone	Isotype	Species	Source
Mouse	Oregon Green 488	IgG	Goat	Molecular Probes, Invitrogen, Eugene, US
Phycoerythrin conjugated Streptavidin (Strep.PE)			Strep.: Sigma-Aldrich, St. Lois, US	
			PE: Cyanotech, Kailua-Kona, US	

Table 2 List of secondary antibodies

3.1.2. Media, Reagents and chemicals

Name	Working Dilution	Source
L-glutamine	2 mM	GIBCO, Invitrogen, Vienna, AT
Fetal calf serum (FCS)	10%	HyClone, Utah, US
Penicillin	100 µg/ml	GIBCO, Invitrogen, Vienna, AT
Streptomycin	100 µg/ml	GIBCO, Invitrogen, Vienna, AT
Phropidium iodid (PI)	50 µg/ml	Sigma Aldrich, St. Loius, US
Bovine serum albumin (BSA)	10 mg/ml	GE Healthcare, Pasching, AT
Albiomin	10 mg/ml	Biotest, Dreieich, DE
Albumin human, recombinant (expressed in <i>S. cerevisiae</i>) A6608	10 mg/ml	Sigma Aldrich, St. Loius, US
Albumin human, recombinant (expressed in rice) A9731	10 mg/ml	Sigma Aldrich, St. Loius, US
HSA, Fatty acid free (dHSA) A3782	10 mg/ml	Sigma Aldrich, St. Loius, US
Holo-Transferrin T0665	10mg/ml	Sigma Aldrich, St. Loius, US
Apo-Transferrin T1147	10mg/ml	Sigma-Aldrich, St. Loius, US
BSA-FITC*	1.4 mg/ml	Sigma Aldrich, St. Loius, US
Albiomin-FITC*	1.2 mg/ml	Biotest, Dreieich, DE
rHSA A6608-FITC*	1.3 mg/ml	Sigma Aldrich, St. Loius, US
rHSA-Hemin A9731-FITC*	1.3 mg/ml	Sigma Aldrich, St. Loius, US
rHSA A9731-FITC*	1.3 mg/ml	Sigma Aldrich, St. Loius, US
dHSA A3782-FITC*	1.8 mg/ml	Sigma Aldrich, St. Loius, US
dHSA-Hemin A3782-FITC*	1.7 mg/ml	Sigma Aldrich, St. Loius, US
Holo-Tfn T0665 -FITC*	1.3 mg/ml	Sigma Aldrich, St. Loius, US
Apo-Tfn T1147-FITC*	1.1 mg/ml	Sigma Aldrich, St. Loius, US

Hemin, from porcine 51280-1G	20 mg/ml	Sigma Aldrich, St. Louis, US
Biliverdin-hydrochlorid 30891-10MG	10 mg/ml	Sigma Aldrich, St. Louis, US
Beriglobin	20 mg/ml (1:8 in 1% BSA-PBS)	Aventis Behring, Vienna, AT
Linoleic acid L1376	10 mg/ml	Sigma Aldrich, St. Louis, US
Oleic acid O1008	10 mg/ml	Sigma Aldrich, St. Louis, US
Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)	2 ng/ml	Fermentas, Burlington, Canada
4-Nitrophenylphosphate Disodium salt 71768	1 mg/ml	Sigma Aldrich, St. Louis, US

Table 3 List of reagents

3.1.2.1. Other reagents

Name	Source
RPMI 1640	GIBCO, Invitrogen, Vienna, AT
PBS (1x), Calcium and Magnesium free	Lonza, Verviers, BE
FACS fluid	Becton Dickinson, New Jersey, US
Ethanol absolute	Merck, Darmstadt, Germany

Table 4 List of reagents

Freezing medium

RPMI 1640 medium was supplemented with 15% FCS and 10% DMSO (Sigma).

Medium for cell culture

RPMI 1640 medium was supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml Streptomycin and 100 U/ml Penicillin.

10 x PBS stock solution

MATERIALS AND METHODS

5.8 g KH_2PO_4 , 16.9 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 72 g NaCl was dissolved in aquabidest (ddH_2O) and filled up to 10 liters (pH 7.2).

PBS/BSA stock solution (20%)

100g BSA and 10g NaN_3 were dissolved in 500 ml PBS buffer, stirred for 1h and filtrated. For the PBS/BSA wash buffer, a 1:20 dilution with PBS buffer was prepared.

Washing buffer 0.05% Tween 20 (for ELISA)

2ml of Tween 20 was diluted with 4L 1x PBS (1:2000)

Coating buffer (for ELISA)

1.59 g Na_2CO_3 and 2.93 g NaHCO_3 was dissolved in aquabidest (ddH_2O) and filled up to 1 L (pH 9.6)

Substrate buffer (for ELISA)

97 ml Diethanolamin ($\text{C}_4\text{H}_{11}\text{NO}_2$) + 100 μl Magnesium chloride hexahydrate, filled up to 1 L with aquabidest (ddH_2O) (pH 9.8)

3.1.3. Cell lines

Cell line	Cell type	Source
Jurkat	Human lymphoblast, T cell	ATTC, Manassas, US
TF1	Human erythroblast, T cell	ATTC, Manassas, US

Table 5 List of cell lines

3.2. Methods

3.2.1. Culturing cells

Jurkat cells were cultured in RPMI 1640 medium which was supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml Penicillin and 100 $\mu\text{g}/\text{ml}$ Streptomycin. In case of TF-1 cells 2 ng/ml GM-CSF was additionally added. Cells were cultured at 37°C in 5% CO_2 humidified atmosphere.

3.2.2. Freezing and thawing cells

3.2.2.1. Freezing of cells

For liquid nitrogen storage human T lymphoblastoid cells were counted via Coulter Counter, centrifuged and the pellet resuspended in freezing medium to obtain a concentration of 5×10^6 cells/ml. Afterwards 1,5 ml aliquots were filled up in cryotubes (Nalgen Nunc International, Roskilde, Denmark) which were placed into a freezing box filled with isopropanol and stored overnight at -80°C . The next day the aliquots were transferred to liquid nitrogen container.

3.2.2.2. Thawing of cells

Cryotubes were thawed as quickly as possible in a water bath at 37°C . Then the cells were slowly resuspended in RPMI 1640 medium supplemented with 10% FCS using a drop-by-drop method. Afterwards the tubes were filled up with medium and the cells were washed twice times. Cells were counted and cultured in 10 ml RPMI 1460 medium supplemented with 10% FCS at 37°C and in 5% CO_2 atmosphere. After 3 days cells were split 1:10 with fresh RPMI 1460 medium supplemented with 10% FCS.

3.2.3. T cell proliferation assay

Jurkat cells were harvested, counted and seeded at a density of 3×10^4 /ml in RPMI 1640 medium in 24-well plate (Costar, Sigma Aldrich). The serum-free medium was supplemented with various proteins at a final concentration of 200 $\mu\text{g}/\text{ml}$ or 10 $\mu\text{g}/\text{ml}$. On the one hand soluble isolated anti-human CD71 monoclonal antibodies (mAbs, 20 $\mu\text{g}/\text{ml}$) were added, on the other hand cells were incubated in combination with linoleic acid and/or oleic acid at a final concentration of 100 mM. In some cases, cells were additionally treated with human transferrin or ferric ammonium citrate (FAC). Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere and the proliferation was monitored with light microscopy. Cells were harvested between the third and seventh day (5 min at 300 g) and the cell viability was measured in triplicates with CASY cell counter (Roche Innovatis AG, Bielefeld, Germany).

Another assay was performed by adding cells at a density of 3×10^4 /ml in RPMI 1640 medium supplemented with FCS in a 24-well plate and incubated in combination with a chemotherapeutic drug (100 ng/ml) and/or soluble isolated CD71 mAbs (20 $\mu\text{g}/\text{ml}$). In one

case iron was additionally added. On the fourth and seventh day cells were counted with CASY counter in triplicates.

3.2.4. T cell titrations assay

Human proteins and recombinant proteins (200 µg/ml) were added into a 24-well plate and diluted to a final concentration of 64 ng/ml. Jurkat T cells were added at a density of 3×10^4 /ml in RPMI 1640 medium and cultured at 37°C in a 5% CO₂ humidified atmosphere.

In case of the chemotherapeutic drug, cells were seeded at a density of 3×10^4 /ml in RPMI 1640 medium supplemented with FCS in a 24-well plate and Abraxane was added (10 ng/ml) and diluted to a final concentration of 0.01 ng/ml. Cells were harvested (5 min at 300 g) and counted in triplicates with CASY cell counter.

3.2.5. Flow cytometry

Flow cytometry can be used to analyze the expression of cell surface and intracellular molecules, to define different cell types, cell size and granularity. The staining mechanism involves making a single-cell suspension from cell culture or tissue samples. The cells are then incubated in tubes or microtiter plates with unlabeled or fluorescent-labeled antibodies and analyzed on the flow cytometer. In a flow cytometry, cell suspension moves as single cell stream through a small nozzle causing cells to pass through a focused laser light beam. Light is hitting the cells and either scattered or absorbed which is then re-emitted (fluorescence). Detectors measure forward (FS) and side scatter (SS) as well as the fluorescence emitted from stained cells or particles. The pattern of light scattering (FS, SS) represent information about the cell size and their granularity, and it can be useful to separate cells into collection tubes relating to size or shape using electrostatic deflection. Moreover, stained cells can be also sorted by fluorescence-based detection which depends on the absorption and re-emission of light at a different wavelength. Flow cytometer is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules such as propidiumiodide binding to DNA. These dyes fluoresce only when the laser excites them, causing the emission of secondary light at a different wavelength. Detection of the fluorescence is used as a measure of the presence of the antigen which is recognized by the antibody on the cell.

3.2.6. Membrane staining with unconjugated or biotinylated mAbs

T cells were counted by Coulter Counter and washed with 1x PBS for 5 min at 300 g. The pellet was resuspended in 1x PBS and pre-treated with various proteins at a final concentration of 200 µg/ml on ice. 50 µl of pre-treated cells (3×10^5 /condition) were transferred into FACS tubes and 20 µl of isolated CD71 mAbs or VIAP as isotype control (Calf intestine alkaline phosphatase specific antibody) at 20 µg/ml were added, mixed and incubated for 30 min on ice at 4°C. Afterwards the cells were mixed and washed twice with 1x PBS, discarding the supernatant. In case of biotinylated mAb, 20µl of 13-344 diluted 1:60 was added and incubated for 30min on ice at 4°C. For unconjugated mAbs 20 µl of Oregon Green (20 µg/ml) and for biotinylated mAbs 20µl of Streptavidin-PE (1:10) were used for secondary labeling. Cells were incubated for 30 min on ice at 4°C and washed two times again. The pellets were resuspended in 50 µl FACS fluid and stored at 4°C until analysis the cell suspensions by flow cytometry. FACScalibur flow cytometer (Becton Dickinson, Palo Alto, CA) and Cell Quest Pro Software were used for data analysis.

3.2.7. DNA staining with propidiumiodide (PI)

Jurkat cells were harvested, counted and seeded at a density of 1×10^5 /ml in RPMI 1640 medium supplemented with 10% FCS in 96-well plate (Costar, Sigma Aldrich). In triplicates cells were incubated in combination with 100 ng/ml Abraxane and/or 10 µg/ml isolated CD71 mAbs at 37°C in a 5% CO₂ humidified atmosphere. After 24 hr, 48 hr and 72 hr cells were harvested and washed two times with 1x PBS. To analyse the toxic effect, death cells were stained with 50 µl propidiumiodide and stored at 4°C until analysis the cell suspensions by flow cytometry. FACScalibur flow cytometer and Cell Quest Pro Software were used for data analysis.

3.2.8. Membrane staining with conjugated proteins

The cell suspension (for each condition 5×10^5 cells) was centrifuged for 5 min at 300 g, washed with 1x PBS and the pellets were resuspended in 50 µl 1x PBS. In one case 20µl of isolated CD71 mAbs (20 µg/ml) were added and incubated for 30 min on ice at 4°C. Another assay was performed without CD71 mAbs. After the cells were washed twice with 1x PBS (cells were resuspended in 1x PBS and centrifuged 5 min at 300g, discarding the supernatant), 50µl of the respective FITC marked protein (500 µg/ml) was added, mixed and incubated on ice at 4°C for

1hr. Each assay was washed twice with 1x PBS. Afterwards the cells were resuspended in 1x PBS/ 1% FCS medium and the tubes were kept at 4°C until they were analyzed by FACScalibur flow cytometer and Cell Quest Pro Software.

3.2.9. Enzyme Linked Immunosorbent Assay (ELISA)

For ELISA assay a microtiter plate was coated overnight at 4°C with a volume of 50µl 2% HSA and Transferrin (100 µg/ml) in coating buffer. The plate was washed two times with 0.05% PBS-Tween and covered with 2% PBS-BSA (200 µl/well) over night. Upon washing the plate with 0.05% PBS-Tween, 50 µl of isolated mAb 13-344 was added into each well at a concentration of 5 µg/ml, 1 µg/ml, 200 ng/ml and 40 ng/ml and the plate was incubated at RT for 2 hr. Microtiter plate was washed again and 50µl of conjugate α -mouse-AP (1:2000) was added and incubated at RT for 1 hr. After a further washing step 50µl of the substrate 4-nitrophenylphosphatedisodium salt (1 mg/ml) was added into each well and incubated at RT for 30 min in dark. The reaction was stopped by adding 50µl of 1N NaOH. The readings were taken by the use of Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

3.2.10. Statistical analysis

For statistical analysis we used the GraphPad Prism software (La Jolla, CA) and performed compared paired t-test for the comparison of our data. The p-values <0.05 are used to determine the significance. Significant values are represented as one star (*) to indicate p<0.05, two stars (**) to indicate p<0.01 and three stars (***) to indicate p<0.001.

4. RESULTS

4.1. Monoclonal Antibodies 5-528, VIP-1, 15-221 and 13-344 recognize human TfR on Jurkat and TF1 cell line

It is well-known that lymphoma cells require an effective iron delivery mechanism for their high proliferations rate. The transferrin receptor (TfR, CD71) represents the classical iron uptake mechanism which is mediated by endocytosis. In the first part we wanted to analyze the binding affinity of murine anti-human CD71 mAbs 5-528, VIP-1, 15-221 and 13-344 to CD71. Thus, we used human lymphoma as well as human erythroblast cells which highly express the transferrin receptor on their surface. In particular, due to that VIP-1 is well characterized as mAb to recognize defined epitopes of the human transferrin receptor, we used VIP-1 as a positive control in this study. Former studies characterized CD71 mAbs and determined specific epitopes on the transferrin receptor, whereas mAb 5-528 and VIP-1 exhibit overlapping binding sites [87].

It is reported that Jurkat lymphoma cells are isolated from T cell lymphoma patients. Moreover, it is already known that Jurkat T cells express a high number of CD71 on the cell surface [88]. During membrane staining, the fluorescence signal is proportional to the binding affinity of the antibodies, depending on the degree of CD71 expression. Jurkat T cell line was analyzed by flow cytometry for the surface staining of CD71 via CD71 mAbs VIP-1, 5-528, 15-221 and 13-344 (Figure 7). It appeared that VIP-1, 5-528 and 15-221 bound to the surface receptor of Jurkat T cells, whereas 5-528 and 15-221 represented similar fluorescence signals compared to the control antibody VIP-1. Interestingly, there was no observable amount of mAb 13-344 was observed on the surface of Jurkat T cells.

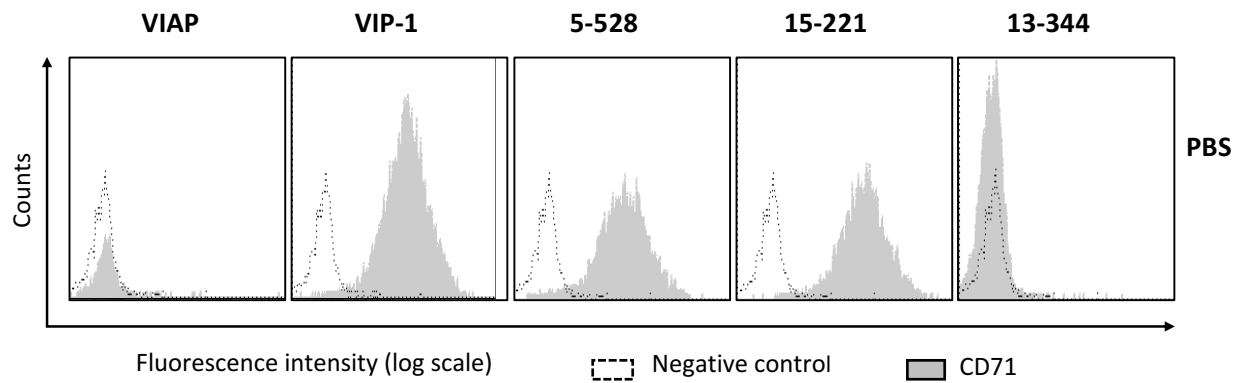


Figure 7 Transferrin receptor (CD71) recognition by CD71 mAbs. Human lymphoma cells were pre-treated with CD71 mAbs (20 µg/ml) for 30' on ice per conditions. The membrane staining was accomplished with Oregon green-labeled (OG) secondary antibodies to determine the binding of CD71 mAbs to CD71 via flow cytometry. VIP-1 was used as positive control and VIAP as isotype control. Data represent one experiment.

4.1.1. The impact of VIP-1 and 15-221 on the proliferation of leukemic cells

According to previous studies, which demonstrated the inhibitory function of CD71 mAbs on the proliferation of T and B cells, including binding studies with the anti-human TfR antibody 42/6 (IgA isotype) [50, 89], FG2/12 (IgG1 isotype) [49] and antibody A24 (IgG Isotype) [55], we wanted to further investigate the effect of CD71 mAbs VIP-1, 5-528, 15-221 and 13-334 on the expansion of leukemic cell lines. We conducted a T cell proliferation assay, whereas cells were harvested and counted via CASY Counter after six days. A very strong decrease of cell proliferation, in case of VIP-1 or 15-221-treated Jurkat T cells, was detected, whereas a growth-inhibitory effect of 5-528 and 13-344 was less pronounced (Figure 8).

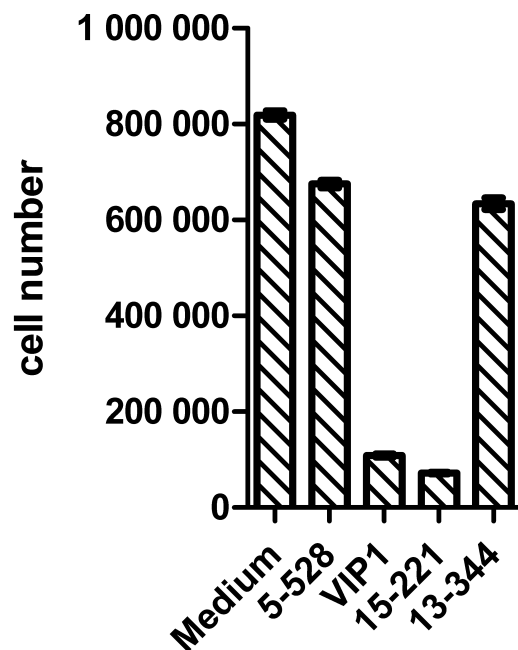


Figure 8 Inhibitory effect of mAbs VIP-1 and 15-221 on Jurkat cell proliferation. Human lymphoma cells were cultivated in RPMI 1640 medium supplemented with 10% FCS. 3×10^4 cells were seeded in 24-well plates and cultivated in the presence or absence of CD71 mAbs (20 µg/ml). On the sixth day, cells were harvested and counted via CASY Counter. Mean values ($\pm$ standard deviation) of one experiment are shown, which was measured in triplicates.

RESULTS

In addition, to further assess growth-inhibitory effects of CD71 mAbs in malignant cells, a second type of human leukemic cell lines was treated with CD71 mAbs to analyze their impact on T cell proliferation.

The TF-1 cell line has been established from an erythroleukemia patient and requires GM-CSF for its cell growth [90]. The experiment with TF-1 cells represented similar results as shown in the previous proliferation assay (Figure 9). All four CD71 monoclonal antibodies are able to reduce TF-1 cell growth, whereas 15-221 was the strongest and 5-528 the weakest inhibitor.

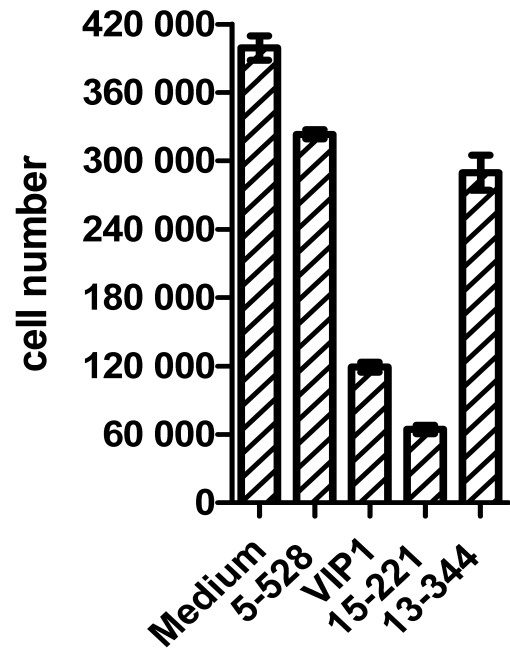


Figure 9 Inhibition of TF-1 cell proliferation by mAbs VIP-1 and 15-221. Human erythroblast cells were cultivated for five days in the same way as mentioned before (Figure 8). Data show mean values ($\pm$ standard deviation) of triplicate determinations.

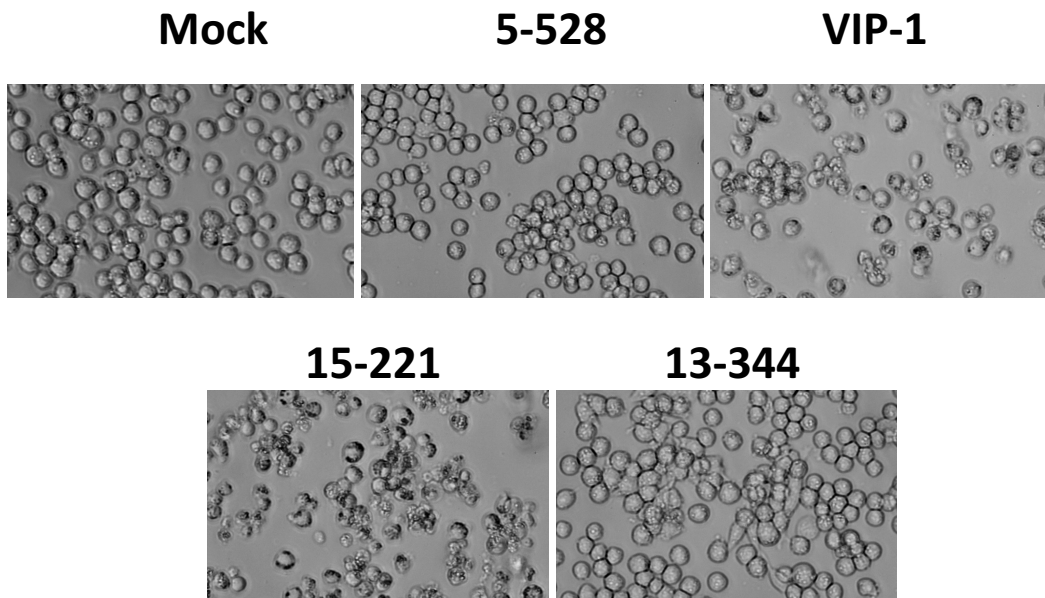


Figure 10 Morphology of CD71 mAb treated Jurkat T cells after seven days. Cells were cultivated in a similar manner as described before. Images were taken from an inverted Nikon Diaphot tissue culture light microscope at 40x magnification. In case of VIP-1 and 15-221 treatment, pictures represent death and granulated T cells.

RESULTS

In addition, Jurkat T cell proliferation and cell death was clearly illustrated via light microscopy after seven days of CD71 mAb treatment. Light microscope images in Figure 10 showed limited cell proliferation and aberrant cell morphology of Jurkat cells in presence of VIP-1 or 15-221 which is may be characterized by apoptosis or necrosis. These observations reflect the cell quantity we have observed in the earlier described experiment, measured via CASY Counter (Figure 8).

4.2. HSA stimulated proliferation is iron-dependent

Elemental iron is an essential micronutrient that is required for the proliferation and survival of virtually all living organism. The high proliferations rate of lymphocyte cells depends on the availability of iron. Some HSA molecules in the human bloodstream have bound hemin in the FA1 region which is secured by the IA–IB loop of HSA. Hemin molecules are metabolized by HO-1 followed by iron liberation and iron uptake into cells [68]. HSA is primarily responsible for the regulation of the colloid osmotic pressure of blood, whereas recombinant HSA (rHSA) has been produced using model organisms such as *S. cerevisiae* which is functional and structural similar to plasma-derived HSA [91].

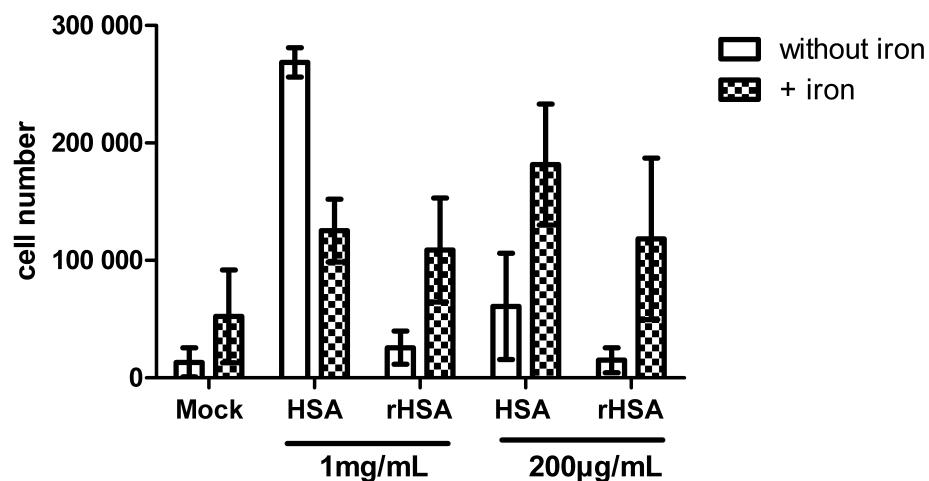


Figure 11 Human serum albumin stimulates proliferation of Jurkat T cells, in which FAC enhances cell proliferation. For growth analysis, human lymphoma cells were cultivated for five days in RPMI 1640 medium, which was supplemented with HSA or rHSA at a concentration of 1 mg/ml or 200 µg/ml, in the presence or absence of 25 µg/ml FAC. HSA strongly induces T cell proliferation, but in case of rHSA no stimulatory effect is obtained. In combination with FAC the growth rate of T cells increases. Mean values ($\pm$ SD) of triple analysis are illustrated.

RESULTS

Based on the fact that T lymphocytes need iron for T cell activation and proliferation, we next examined the stimulatory effect of albumin by performing a T cell proliferation assay. T cells were incubated with HSA from blood plasma or rHSA derived from *S. cerevisiae*. Higher proliferation rate was observed due to HSA treatment with a concentration of 1 mg/ml, whereas rHSA showed no stimulatory effect (Figure 11). As shown in Figure 11, additionally incubation with FAC the T cell proliferation was potently induced in each condition, with the exception of cells, which were treated with HSA at a concentration of 1 mg/ml. Even at the concentration of 200 µg/ml HSA, FAC strongly increased the T cell proliferation.

4.2.1. Iron compensates the inhibitory effect of VIP-1 and 15-221 in TF1 cell line

As already mentioned in previous experiments, mAbs VIP-1 and 15-221 diminish T cell proliferation, whereas 5-528 and 13-344 only showed weak growth-inhibitory effects (Figure 8, 9). Since VIP-1 and 15-221 inhibited cell growth, this finding raised the question if FAC can compensate the strong growth-inhibitory effect of VIP-1 and 15-221. Thus, a proliferation assay was performed with CD71 mAb treated TF-1 cells in the presence or absence of FAC for five days. When VIP-1 or 15-221 was added to cell culture, the proliferation response was suppressed as already mentioned. As expected, treatment of TF-1 cells with FAC abolish the growth-inhibitory effect of VIP-1 and 15-221. However, Figure 12 clearly illustrates

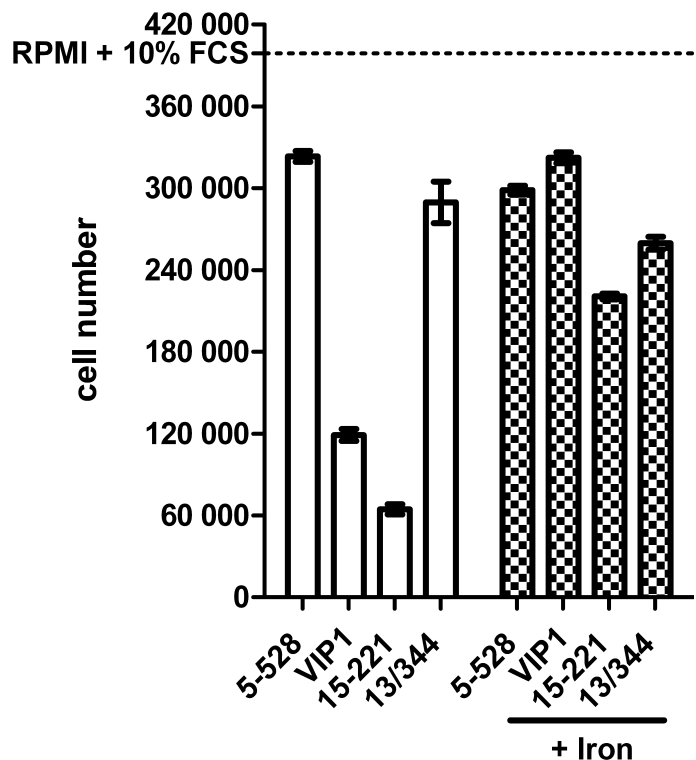


Figure 12 Inhibitory effect of VIP-1 and 15-221 is abolished by FAC treatment. 3×10^4 TF-1 cells were seeded in a 24-well plate, which were incubated with 20 µg/ml CD71 mAbs and 25 µg/ml FAC. Data demonstrate mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

that FAC treatment had only little to no effect on Jurkat T cells, which were cultivated in the presence of 5-528 or 13-344, as there was neither a decrease nor an increase of cell growth.

4.2.2. Human lymphoma cell line proliferation is highly increased by rHSA-hemin and dHSA-hemin supplementation

In the next step, we analyzed the ability of various common and altered albumin proteins to stimulate T cell proliferation in serum-free medium. Recombinant HSA, which is expressed in various expression systems, is widely used in different laboratory, clinical fields and pharmaceutical purposes to substitute plasma HSA [65].

In order to investigate whether different albumin variants represent similar stimulatory effects on T cell growth as serum albumin, we loaded albumin molecules with hemin or biliverdin and conducted proliferation assays in serum-free medium.

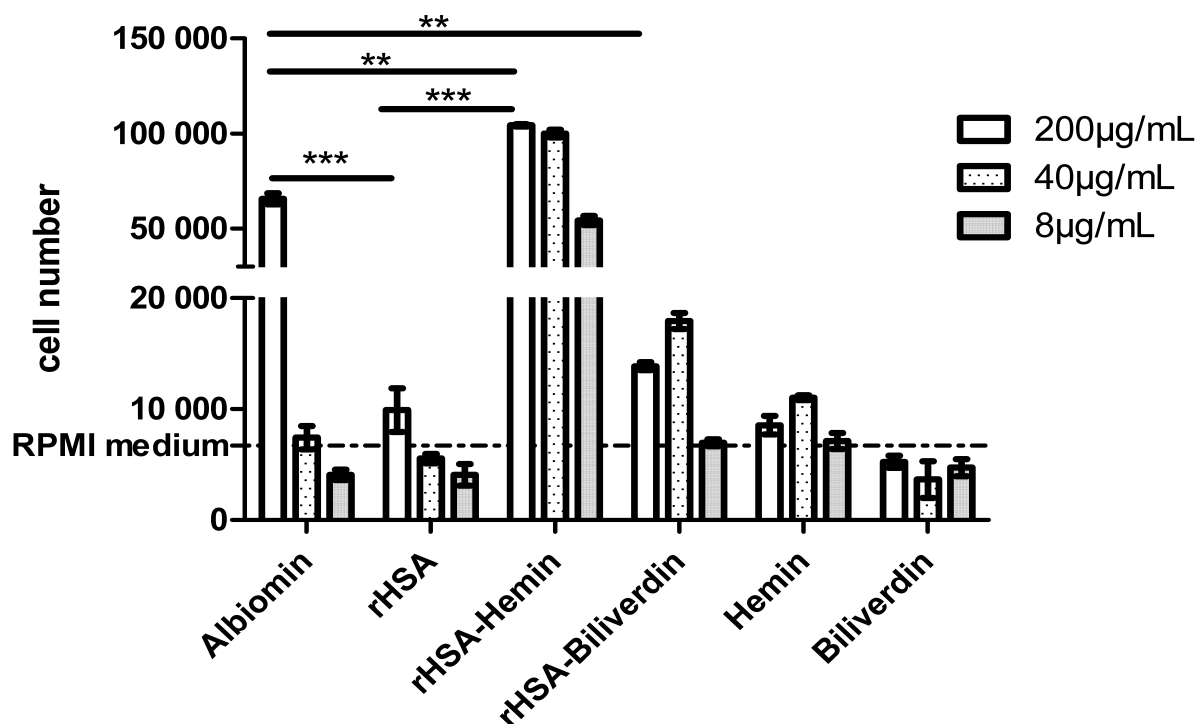


Figure 13 Comparison of growth behavior of Jurkat T cells by different medium supplementations. For cell growth analysis, 3×10^4 cells / well were seeded and incubated for four days with different proteins or components of hemoglobin such as hemin or biliverdin. HSA stimulates T cell proliferation at a concentration of 200 µg/ml, whereas rHSA-hemin enhances cell growth of Jurkat cells in each condition. Figure illustrates unaffected proliferation in presence of rHSA, hemin or biliverdin. Bars show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

RESULTS

Results, shown in Figure 13, demonstrate a potentially increase of T cell growth at different concentrations in cells, which were cultured in medium that was supplemented with rHSA-hemin. In contrast to rHSA-hemin, human serum albumin only stimulated cell proliferation at a concentration of 200 $\mu\text{g}/\text{ml}$. Moreover, no significant growth effect was visible during cell cultivation in presence of rHSA-biliverdin. In addition, to determine the direct effect of hemin and biliverdin on cell expansion, Jurkat T cells were incubated with these molecules for four days. Nonetheless, it is obvious that unbound hemin and biliverdin suppress cell proliferation of leukemic T cells.

A modified variant of serum albumin is the fatty acid-free HSA (dHSA) molecule, which has been identified as a new delivery vehicle for drugs [92]. Since T cells, which were cultivated with rHSA-hemin, are still alive and increased the cell proliferation, the question was if the same effect could be obtained with dHSA-hemin. Thus, Jurkat T cells were cultivated in presence of dHSA or dHSA-hemin at different concentrations. Treatment of cells with dHSA-hemin enhanced T cell growth, whereas dHSA without hemin did not induce cell expansion (Figure 14).

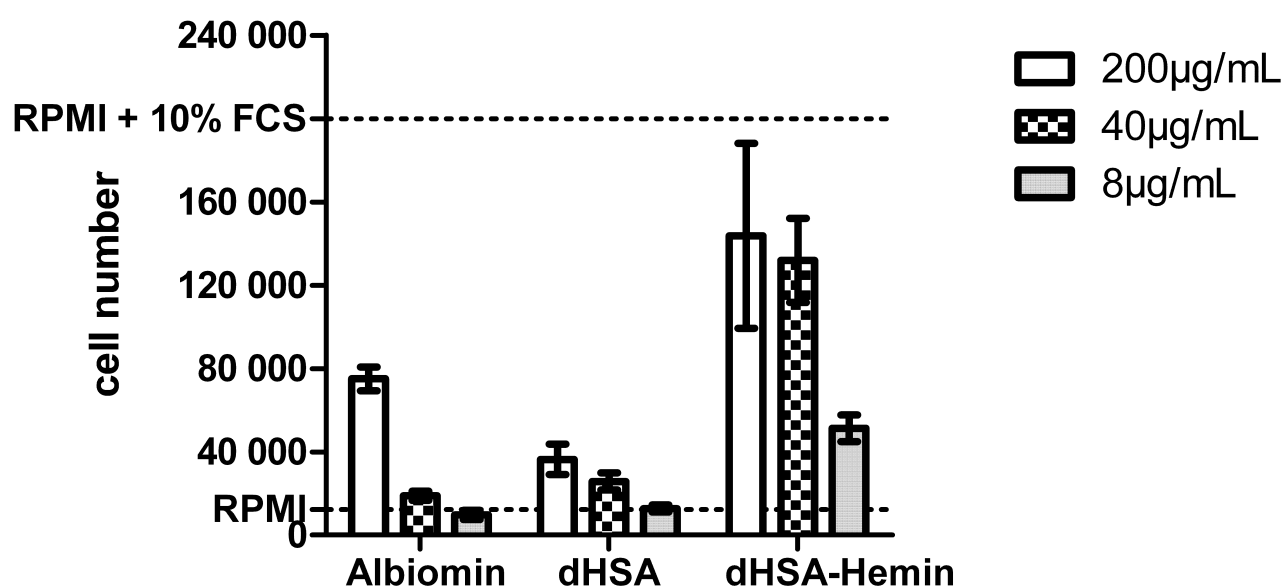


Figure 14 Defatted HSA-bound hemin also has the ability to stimulate cell growth of Jurkat T cells. Human lymphoma cells were cultivated as described before in combination with HSA, dHSA or dHSA-hemin at different concentrations and measured the amount of viable cells on the fourth day via CASY Counter. Cell growth is increased during dHSA-hemin incubation, but is not as high as through FCS cultivation. Bars represent mean values ($\pm$ standard deviation) of two independent experiments.

4.3. HSA stimulated growth is not fatty acid-dependent

Various studies have reported that treatment of B and T lymphocytes with fatty acids causes toxic effects, followed by cell death [93, 94].

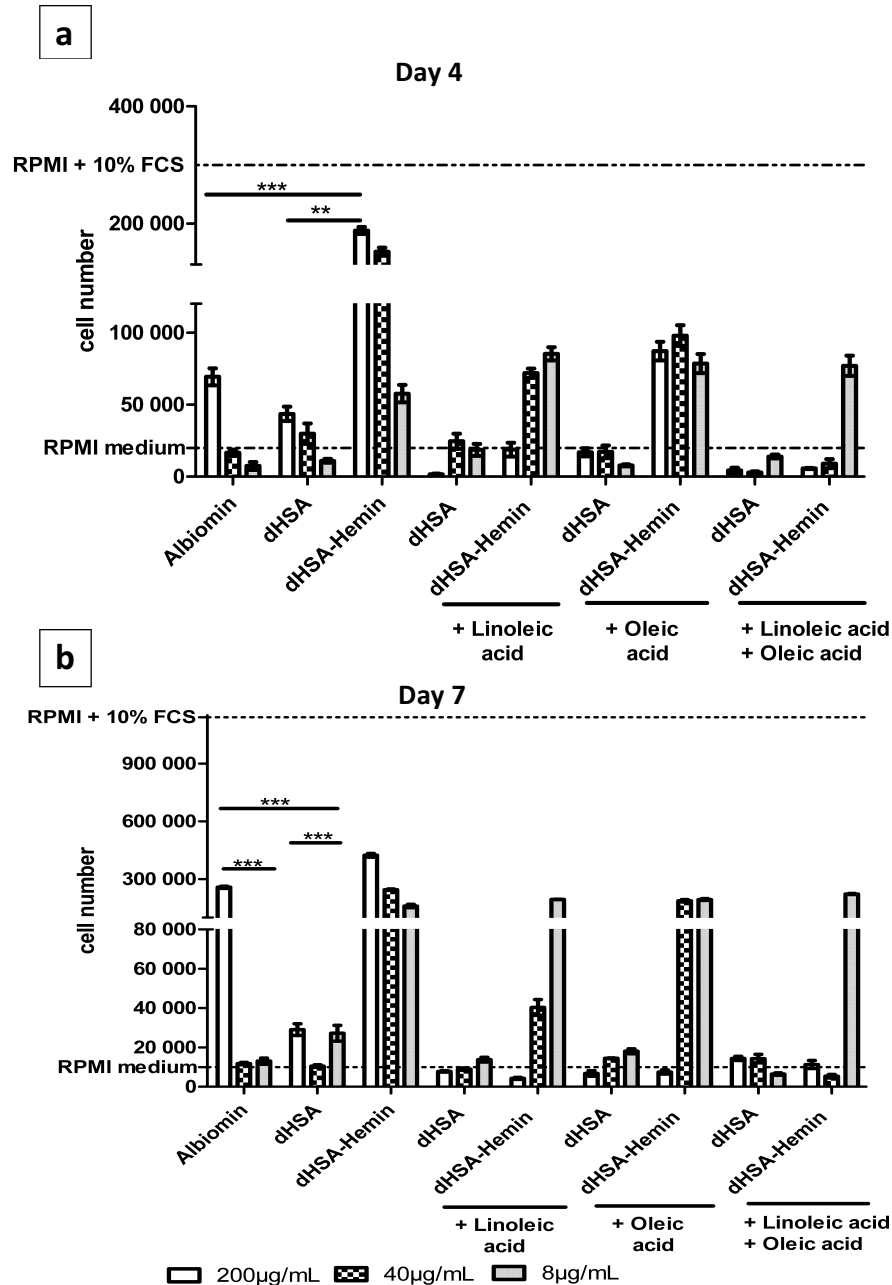


Figure 15 No stimulatory effect of fatty acids on the proliferation of Jurkat cells. (a,b) Human lymphoma cells were cultivated with HSA, dHSA or dHSA-hemin in combination with or without FAs, including linoleic acid and/or oleic acid. Cells were analyzed on the day four and seven. A sustained effect of HSA and dHSA-hemin in cell growth is observed. FAs induce no cell proliferation, followed by the suppression of dHSA-hemin induced cell proliferation. Representative results show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

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It was reported that monounsaturated fatty acids such as oleic acid were less toxic to lymphocytes in comparison to polyunsaturated fatty acids, including the essential FA linoleic acid. Cury-Boaventura *et al.* observed that both FAs reduce the number of lymphocytes due to apoptosis and necrosis causing DNA fragmentation, mitochondrial depolarization, lipid accumulation as well as increase of granularity and size [93].

To confirm that FAs are not the responsible binding factors of HSA for cell growth stimulation, dHSA and dHSA-hemin treated Jurkat T cells were incubated in presence or absence of linoleic acid and/or oleic acid. Before a proliferation assay was performed, 1 mg/ml of each albumin molecule was loaded with 28 µg/ml FAs. Growth-inhibitory effects were detected in linoleic acid and in combination with oleic acid in dHSA-hemin treated cells on the fourth day, which were reflected by cell death. As illustrated in Figure 15, the same toxic effect applied in dHSA treated cells, which are additionally incubated with FAs. Nevertheless, the results indicate that the inhibitory effect of oleic acid was less pronounced than it was observed with linoleic acid. Moreover, linoleic acid in combination with oleic acid led to higher toxicity than FAs alone (Figure 15, a). On the seventh day it was observed that the inhibitory effect increased with time, but within the dilution steps the toxicity declined. T cell proliferation seems to be unaffected in dHSA-hemin-treated cells at a concentration of 8 µg/ml, which were cultivated in presence of FAs. Clearly, results illustrate that rHSA-hemin had still a high stimulatory effect on Jurkat T cells at a concentration of 8 µg/ml, which is similar to the proliferation effect of HSA at a concentration of 200 µg/ml (Figure 15, b).

4.4. HSA stimulated proliferation is mediated via CD71

HSA is the major soluble protein component of the blood plasma [60] and is important in hemin binding as well as transfer [61]. It was identified that HSA is an interaction partner of the C-terminal fragment of the soluble TfR1 [32].

4.4.1. HSA induces T cell proliferation, which is blocked by VIP-1 and 15-221

After observing that VIP-1 and 15-221 strongly reduce cell growth of leukemic cell lines in presence of fetal-bovine serum (Figure 8, 9), we wanted to gain further insights into the function of both mAbs in presence of albumin variants.

RESULTS

Figure 16 clearly illustrates that mAbs VIP-1 and 15-221 efficiently reduce cell growth by 81 % and 77 %, while 13-344 inhibited proliferation by 55 %. 5-528 was found to have a less inhibitory effect (30 %) than the other CD71 mAbs. Clearly, all four CD71 monoclonal antibodies significantly suppress the growth-stimulatory effect of human serum albumin (Figure 16), which was in line with the results described earlier (Figure 8).

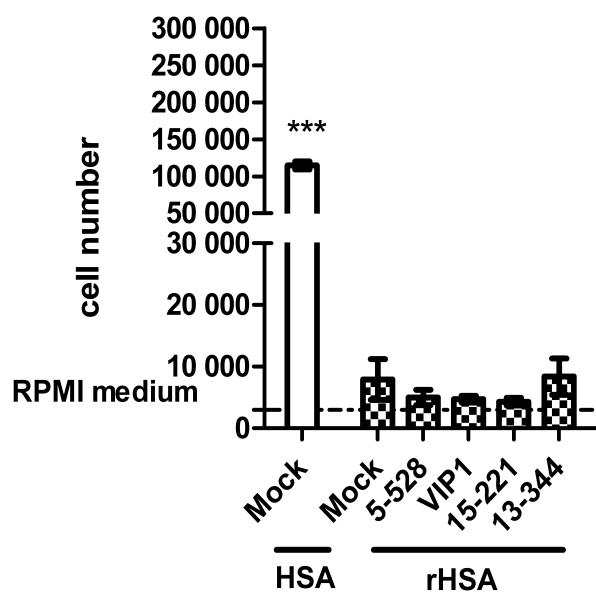


Figure 17 No stimulation or significant inhibition of T cell proliferation via CD71 mAbs during rHSA treatment. Jurkat cells were incubated with 200 µg/ml HSA or 10 µg/ml rHSA for six days as described before. No cell proliferation is obtained during rHSA incubation. CD71 mAbs 5-528, VIP-1 and 15-221 show only weak growth-inhibitory effects. Results show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

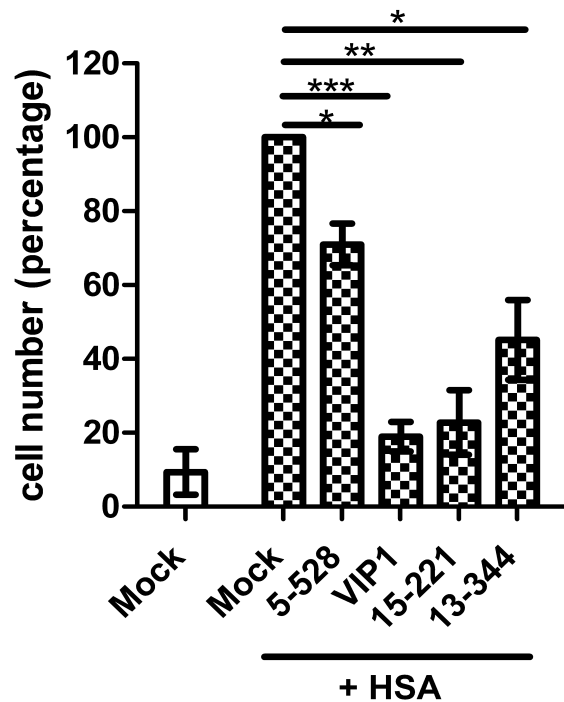


Figure 16 Inhibitory effect of CD71 mAbs on HSA treated Jurkat cells. Jurkat cells were cultivated in 200 µg/ml HSA in presence or absence of CD71 mAbs (20 µg/ml). VIP-1 and 15-221 strongly reduce cell growth. 5-528 and 13-344 have a weaker inhibitory effect. Data represents the cell number in percentage, including four independent experiments, measured in triplicates between the fifth and seventh day.

In addition, the effects of CD71 mAbs were further investigated in rHSA treated cells by proliferation assay. The cell number, which was determined on the sixth day, represents that the proliferation of Jurkat T cells was not stimulated neither with rHSA nor with CD71 mAbs. In particular, a slight reduction in the T cell amount in presence of CD71 mAbs 5-528, VIP-1 or 15-221 was observed (Figure 17).

4.4.2. rHSA-hemin induced T cell proliferation is inhibited by CD71 mAbs

By culturing Jurkat T cells in presence of rHSA-hemin for different days, the effect of rHSA-hemin and additionally the inhibitory effect of CD71 mAbs on cell growth were investigated. Figure 18 indicates that T cell proliferation was stimulated by rHSA-hemin, which was weakly effected by 5-528 and 13-344 showing a slight reduction of 32 % and 23 %. Remarkably, it was shown that in rHSA-hemin treated cells VIP-1 and 15-221 were not as efficient in growth inhibition as shown in previous experiments cultured cells in human serum albumin (Figure 16). Figure 18 illustrates that VIP-1 and 15-221 cause a reduction in cell growth by 48 % and 41 %. Thus, different from the stimulation of cell proliferation via human serum albumin, rHSA-hemin induced strongly the proliferation of leukemic cells.

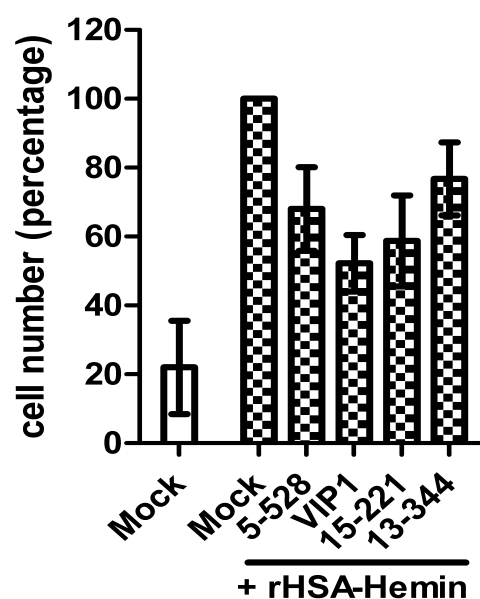


Figure 18 Induced T cell growth during rHSA-hemin incubation is inhibited by VIP-1 and 15-221. Jurkat cells were cultured in RPMI 1640, which was supplemented with 10 $\mu\text{g/ml}$ rHSA-hemin, in presence or absence of 20 $\mu\text{g/ml}$ CD71 mAbs. The results show the stimulation of cell growth via rHSA-hemin, which can be subsequently suppressed by CD71 mAbs. Data demonstrate the cell number in percentage, including four independent experiments.

The following experiment was performed with RPMI 1640 medium, which was supplemented with rHSA from yeast bound to hemin. To further investigate if transferrin is the responsible component for the stimulation of cell growth as well as has an impact on the inhibitory effect of CD71 mAbs, a proliferation assay was performed with CD71 mAb treated Jurkat T cells, which are cultured in rHSA-hemin supplemented medium, in the presence or absence of transferrin. The number of observed cell counts was nearly equal in transferrin treated and untreated Jurkat T cells without CD71 mAbs treatment. Finally, the application of transferrin in the presence of CD71 mAbs resulted in the abolishment of the inhibitory-growth effect of VIP-1 and 15-221 (Figure 19).

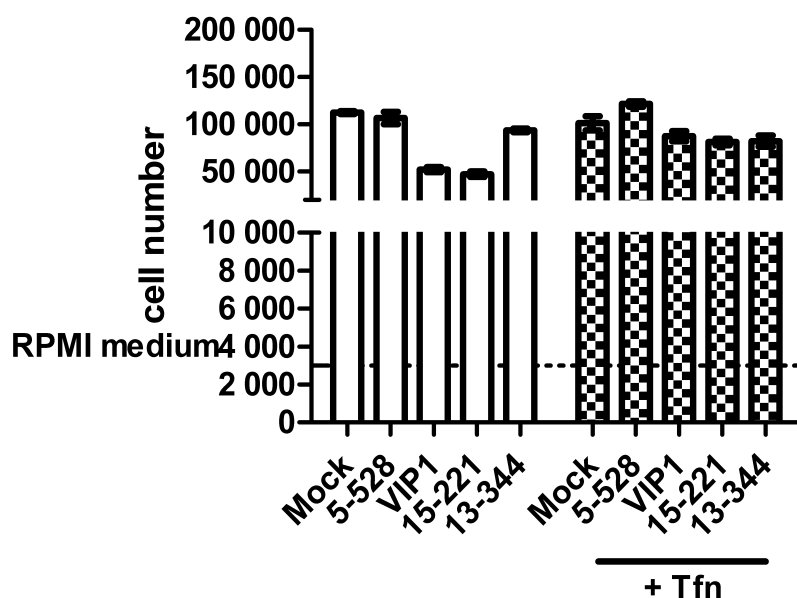


Figure 19 Comparison of different growth conditions for Jurkat cells and the impact of CD71 mAbs. Jurkat cells were incubated for six days with 10 $\mu\text{g/ml}$ rHSA-hemin with or without transferrin supplementation. In both cases increased cell proliferation was obtained. CD71 mAbs VIP-1 and 15-221 (20 $\mu\text{g/ml}$) reduce cell growth, which was induced via rHSA-hemin supplementation. Due to the additional incubation with Tfn, the inhibitory effect of VIP-1 and 15-221 is abolished. Results show mean values ($\pm$ standard deviation) of triple determinations.

4.4.3. Binding of HSA influences the binding affinity of CD71 mAbs to CD71

It is known that the transferrin cell surface receptor is recognized by different CD71 mAbs. In order to define the influence of HSA on the interaction of CD71 mAbs and CD71, we performed staining approaches for leukemic cell lines such as Jurkat T cells and TF-1 cells. T cells were pre-incubated with 1% HSA and subsequently treated with CD71 mAbs. At 0°C, increased binding affinity of 5-528, 15-221 and 13-344 was detectable in HSA treated T cells, of which 13-344 represented the highest enhancement of binding by HSA molecules, increasing the mean of fluorescence intensity (MFI) from 13 to 116. However, the binding affinity of VIP-1 was in fact weaker in HSA pre-treated cells compared to untreated cells. In contrast to Jurkat cells, the fluorescence intensity of CD71 mAbs was much higher on TF-1 treated cells (Figure 20).

RESULTS

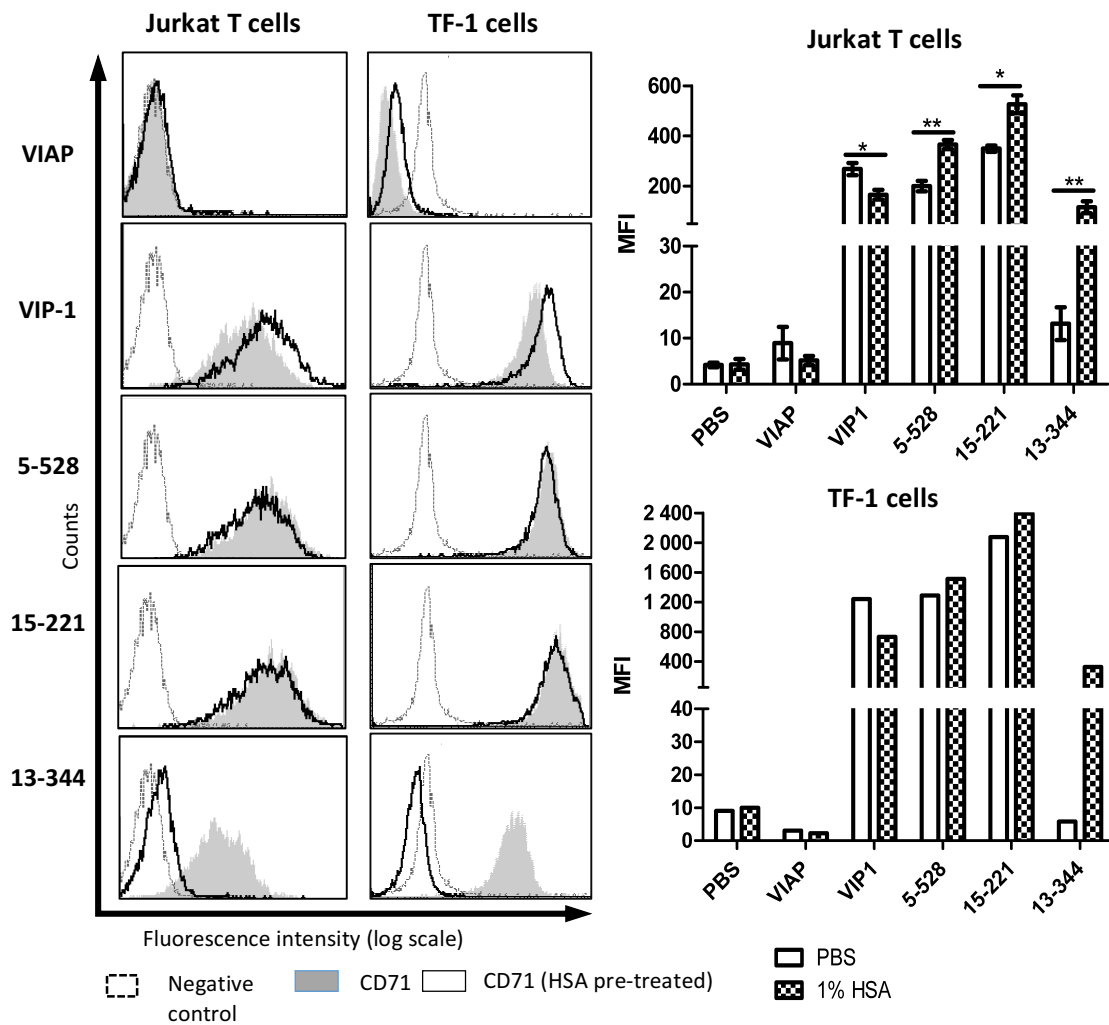


Figure 20 Human lymphoma and erythroblast cell lines were used to determine the binding affinity of CD71 mAbs to CD71 on the cell surface after pre-treatment with HSA. Cells were pre-incubated either with or without 1% HSA, followed by CD71 mAb (20 μ g/ml) incubation for 30' on ice. Negative control represents pre-treated cells with 1% HSA without CD71 mAbs. Membrane staining was performed as described earlier for CD71 binding on Jurkat T cells. All CD71 mAbs have a better binding affinity to CD71 in the presence of HSA, except of VIP-1, which exhibit a weaker affinity. VIAP was used in this study as isotype control. Data represent one of five independent experiments in case of Jurkat cells, but only one with TF1 cell line.

Furthermore, the presence of other albumin proteins had only little or no effect on the binding of CD71 mAbs to the transferrin receptor (Figure 21). In particular, in case of rHSA pre-treatment we observed only a slight increase of VIP-1 (MFI= 113 to 118), whereas dHSA pre-incubated T cells exhibit a low decrease (MFI= 322 to 316). Moreover, the binding affinity of VIP-1 was less blocked via rHSA-hemin (MFI= 452 to 417), but slightly increased in terms of rHSA-biliverdin (MFI= 452 to 511). Remarkably, the binding affinity of 13-344 was unaffected resulted in no detectable amount of 13-344 on the cell surface in untreated and pre-treated

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cells. However, FACS analysis showed in case of HSA pre-treated cells a strong shift to the right which is indicative for the induction of 13-344 binding (Figure 20). Thus, modified and non-modified albumin molecules did not significantly alter the binding affinity of VIP-1 and 13-344 (Figure 21), but it seems like that human serum albumin has the ability to influence significantly binding patterns of CD71 mAbs on leukemic T cells (Figure 20).

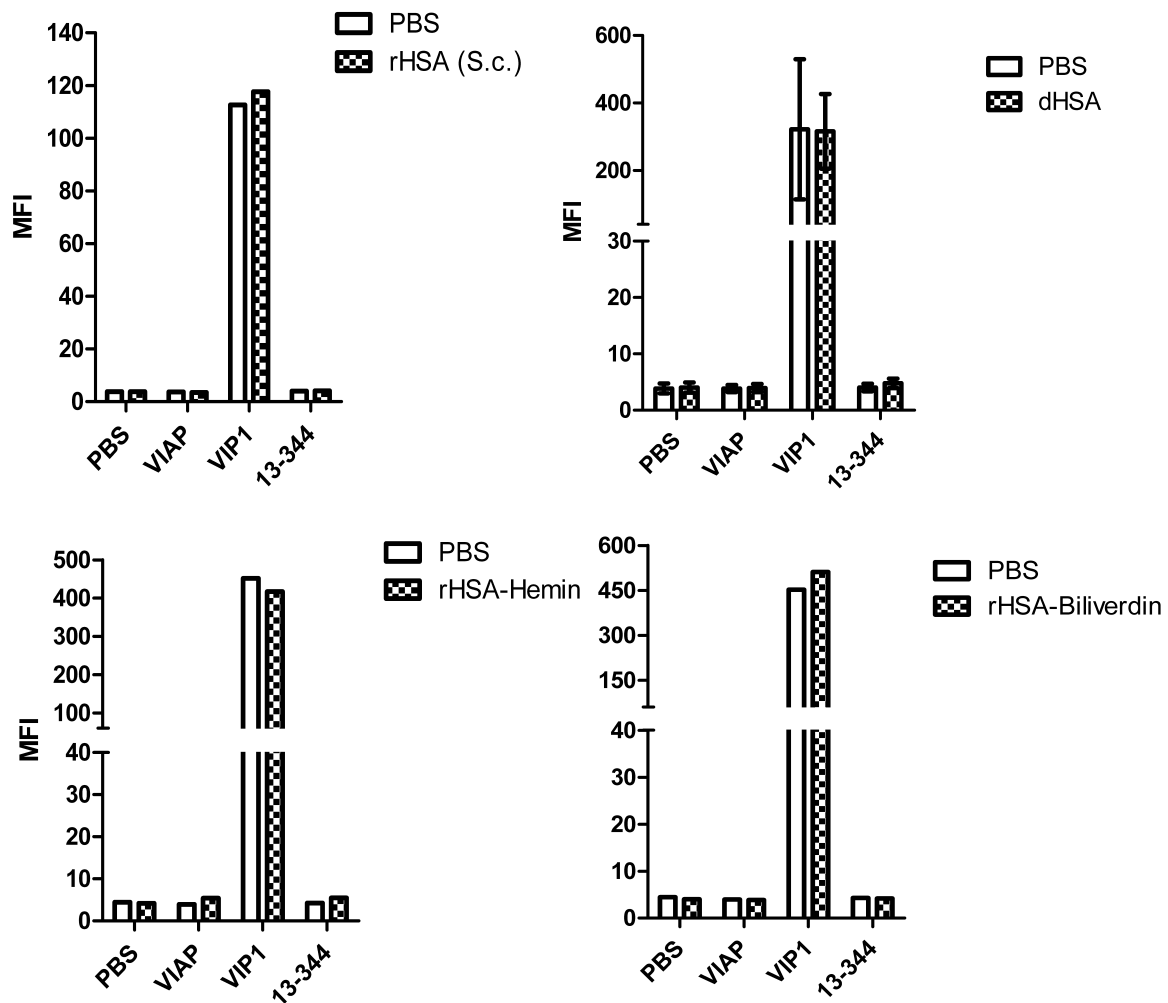


Figure 21 No significant impact of rHSA, dHSA or rHSA-bound hemin/biliverdin to the binding affinity of CD71 mAbs. Jurkat T cells were pre-treated with 200 µg/ml rHSA, rHSA-hemin, rHSA-biliverdin, which was derived from *S. cerevisiae*, or dHSA and subsequently incubated with 20 µg/ml CD71 mAbs. Membrane staining was performed via OG. No clear inhibitory effect of utilized proteins on the binding affinity of CD71 mAb was observed. VIAP was used in this study as isotype control. Results represent one experiment, with the exception of dHSA pre-treated cells showing mean values ($\pm$ standard deviation) of two approaches.

4.4.4. Inhibition of HSA binding to CD71 due to human transferrin, VIP-1 and 5-528

We used staining approaches to study the binding of HSA and BSA proteins to CD71. In particular, we wanted to examine the impact of CD71 mAbs and human transferrin on the interaction between albumin proteins and CD71. To assess this statement, Jurkat T cells were pre-incubated with CD71 mAbs or transferrin and subsequently incubated with FITC-labeled HSA or BSA molecules. Pre-treatment of T cells with CD71 mAbs or transferrin did not alter the binding intensity of BSA. In contrast, pre-incubated cells with CD71 mAbs VIP-1, 5-528 or transferrin showed a reduced capability of HSA binding by 55 % with VIP-1. Moreover, the results represent an inhibition of 45 % with transferrin and a reduction of 37 % by 5-528 (Figure 22).

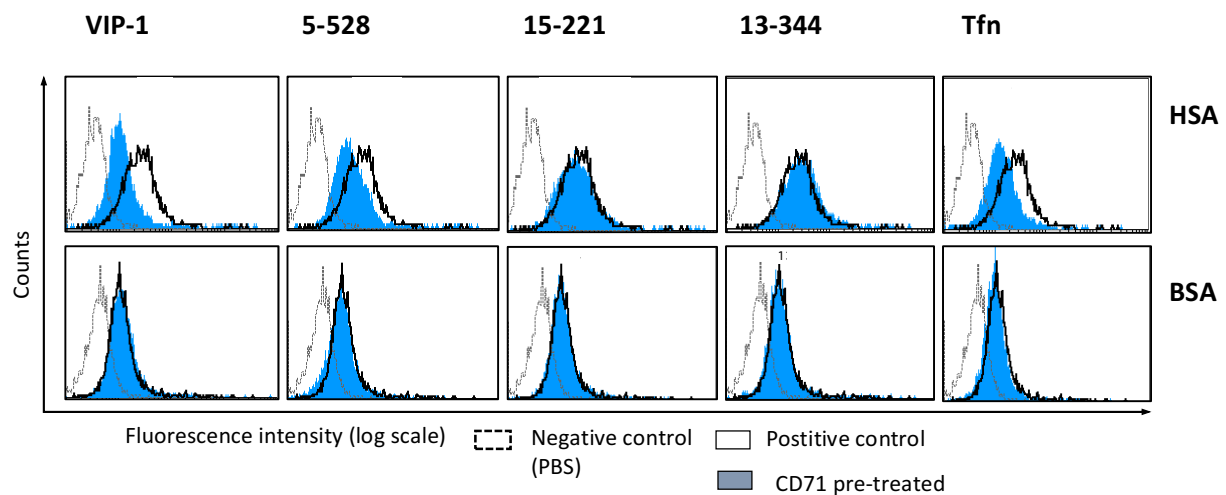


Figure 22 Impact of VIP-1, 5-528 and Tfn on HSA binding to CD71. Jurkat T cells were incubated with 20 $\mu\text{g/ml}$ CD71 mAbs or 1 mg/ml Tfn for 30' on ice. Protein surface staining occurred by direct conjugated (FITC) proteins, HSA or BSA (500 $\mu\text{g/ml}$). One representative data from two independent experiments is shown.

Furthermore, to analyze whether the binding of different albumin molecules is affected by CD71 mAbs or not, a lymphoma cell line was tested. The binding affinity of conjugated proteins was determined via flow cytometry (Figure 23).

RESULTS

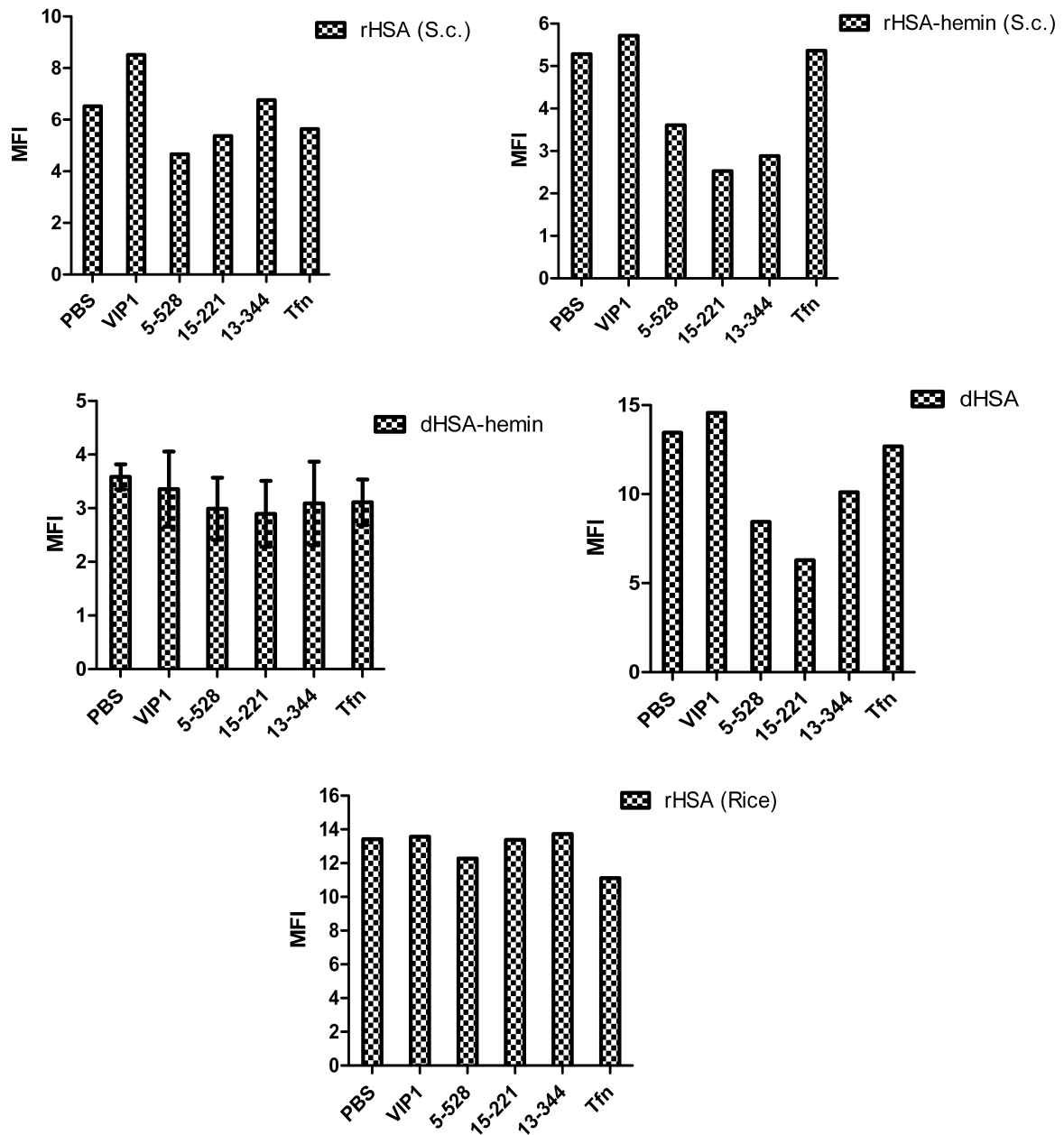


Figure 23 Binding affinity of conjugated albumin proteins is influenced by CD71 mAbs and transferrin. In each case Jurkat T cells were pre-incubated with CD71 mAbs (20 μ g/ml) or 1 mg/ml transferrin and subsequently incubated with FITC labeled albumin proteins. The same staining process has been followed as shown in Figure 22 above. In terms of dHSA-hemin, data show mean values ($\pm$ standard deviation) of three independent experiments, whereas only one experiment was performed for the remaining albumin proteins (rHSA derived from *S. cerevisiae* or rice, rHSA-hemin and dHSA).

As shown in Figure 23, the interaction of rHSA to CD71 was slightly reduced via 5-528, 15-221 and Tfn, but increased through VIP-1. Further, 5-528, 15-221 and 13-344 weakly blocked the binding of rHSA-hemin. The same effect was visible in the binding assay of dHSA, which was characterized as lower in the presence of 5-528, 15-221 and 13-344, whereas the binding of

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dHSA-hemin as well as rHSA (Rice) was rather unaffected in presence of CD71 mAbs. In each binding assay, transferrin negatively influences the binding of the hemin-bound and unbound albumin variants. However, a higher binding affinity of each albumin protein to CD71 was visible by VIP-1, with the exception of dHSA-hemin and rHSA, which was derived from rice.

4.5. HSA interaction with TfR is not mediated via Tfn

The main function of transferrin is to bind and transport cellular iron to the cell surface receptor TfR that is highly expressed on proliferating cells as well as malignant cells and further to facilitate iron uptake into cells through receptor-mediated endocytosis [15, 22].

To investigate if transferrin is necessary for the interaction of HSA to the transferrin receptor, binding studies with FITC-labeled HSA and other albumin proteins were performed. Human transferrin displaced a low inhibitory effect on the binding affinity of albumin proteins to CD71 on the surface of Jurkat T cells. The binding of rHSA, produced in yeast or rice, as well as BSA was slightly blocked through human transferrin pre-treatment. Noticeably, the highest inhibitory effect could be observed by the investigation of HSA binding, showing a reduced fluorescence signal from a mean of 33 to 18 (Tfn 2 µg/ml) or rather 17 in transferrin pre-treated cells at a concentration of 200 µg/ml. Thus, we suggest that transferrin as well as albumin molecules comprise similar binding sites to CD71 (Figure 24).

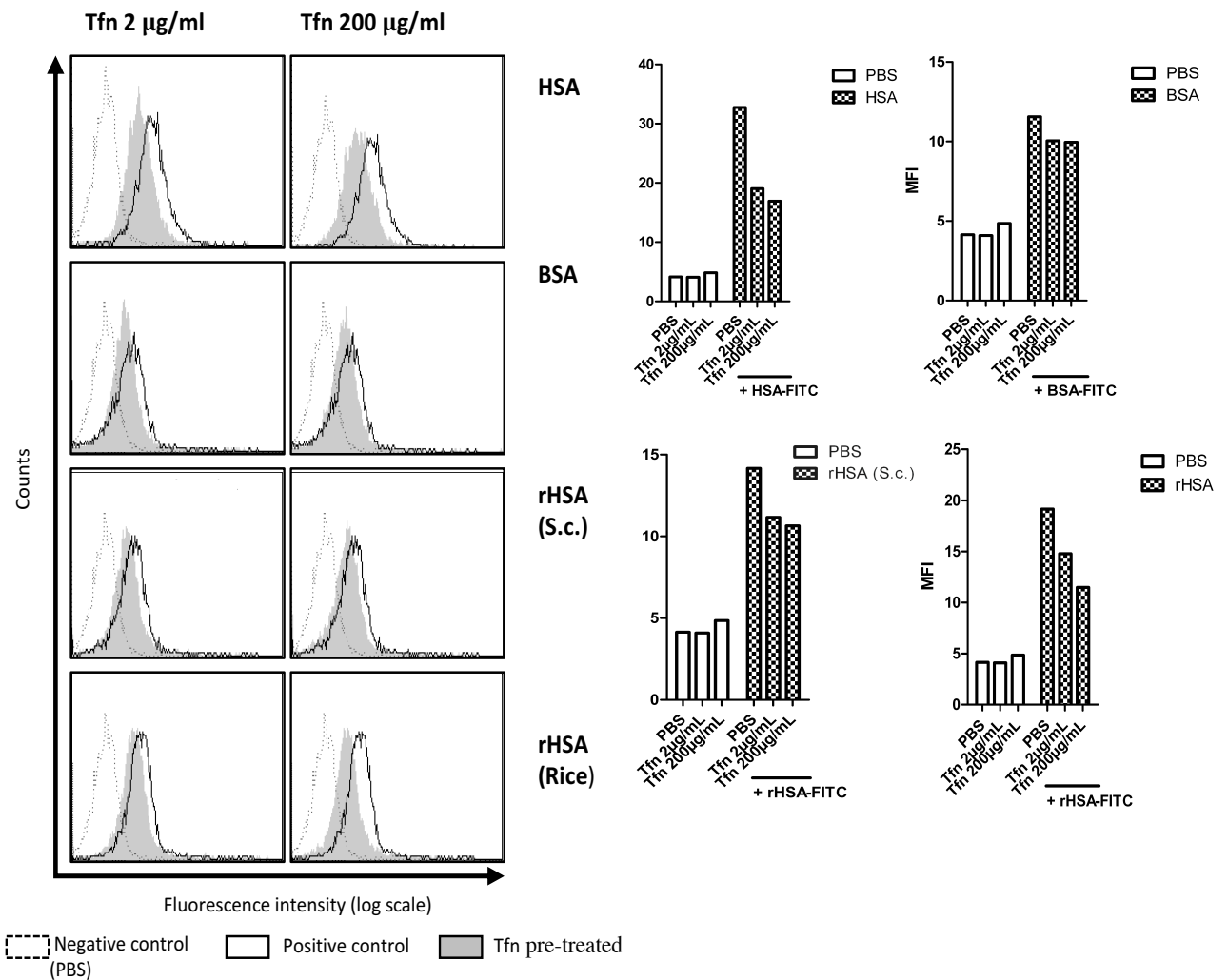


Figure 24 Transferrin is not responsible for HSA binding. Inhibition of protein binding due to transferrin was determined through membrane binding analysis. Human lymphoma cells were used to determine the influence of human transferrin to the binding affinity of different (recombinant) proteins. Cells were pre-treated with two different Tfn concentrations and membrane staining was performed with FITC labeled albumin proteins. Tfn strongly blocks the binding of HSA molecules as well as little bit of BSA and recombinant HSA molecules derived from *S. cerevisiae* and rice.

4.5.1. No inhibitory effect of HSA to transferrin binding

We observed in a previous described experiment that human transferrin negatively influenced the binding of HSA to CD71 (Figure 22), but only slightly reduced the binding of rHSA variants and BSA (Figure 24).

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Moreover, to determine the impact of HSA in the reverse order, particularly to make sure if HSA influences the interaction of transferrin to its receptor, Jurkat T cells were pre-treated with HSA and further incubated with conjugated human transferrin proteins. Figure 25 represents that the fluorescence intensity of human transferrin in HSA pre-treated cells is similar to untreated cells. Thus, it is suggested that human serum albumin has not the ability to block transferrin binding to CD71 (Figure 25).

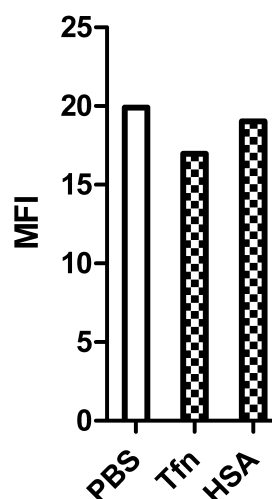


Figure 25 No inhibition of transferrin binding due to HSA. Human lymphoma cells were pre-incubated with 1 mg/ml Tfn or 1 mg/ml HSA for 30'. At 0°C, binding of FITC labeled transferrin (500 µg/ml) was reduced in Tfn incubated cells. HSA has no impact on Tfn binding to CD71. Results demonstrate one experiment.

4.6. CD71 mAb 13-344 interacts with human TfR via plasma proteins

In order to test the binding affinity of 13-344 to CD71, Jurkat T cells were pre-incubated in the presence of modified and non-modified albumin molecules. It was observed that the binding of 13-344 was remarkably sensitive to CD71 in HSA pre-treated cells compared to fatty acid-free and recombinant albumin proteins. In contrast, the pre-treatment of Jurkat T cells with various albumin proteins did not alter the binding intensity of the isotype control VIAP (Figure 26).

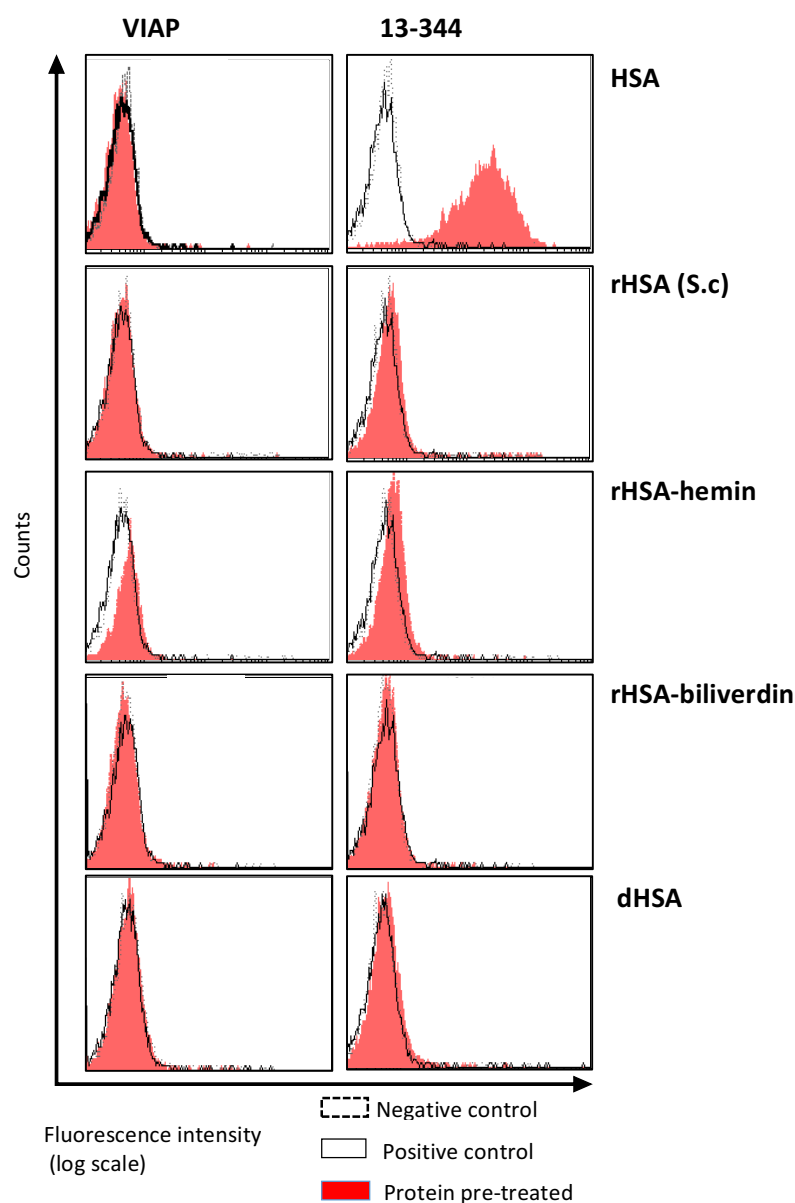


Figure 26 Induction of mAb 13-344 binding in HSA pre-treated Jurkat cells. Cells were pre-incubated with different proteins for 30' on ice and subsequently incubated with CD71 mAb 13-344 and VIAP as isotype control. Membrane staining was performed via OG. Histograms show one representative experiment including three independent experiments for HSA pre-treatment, two experiments for dHSA pre-treatment and one for the remaining conditions.

4.6.1. No Interaction of mAb 13-344 with other CD71 mAbs on Jurkat T cells

In order to characterize the interaction of mAb 13-344 with other CD71 mAbs, a membrane binding assay was performed with a biotinylated antibody. Jurkat T cells were pre-incubated with CD71 mAbs VIP-1, 5-528, 15-221 as well as the isotype control and subsequently immunostained for 13-344, whether to test if 13-344 can bind to CD71 in the presence of

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other CD71 mAbs or not. Clearly, the data indicate that the binding of 13-344 cannot be induced by CD71 mAbs and therefore no interaction was available between 13-344 and CD71 mAbs (Figure 27).

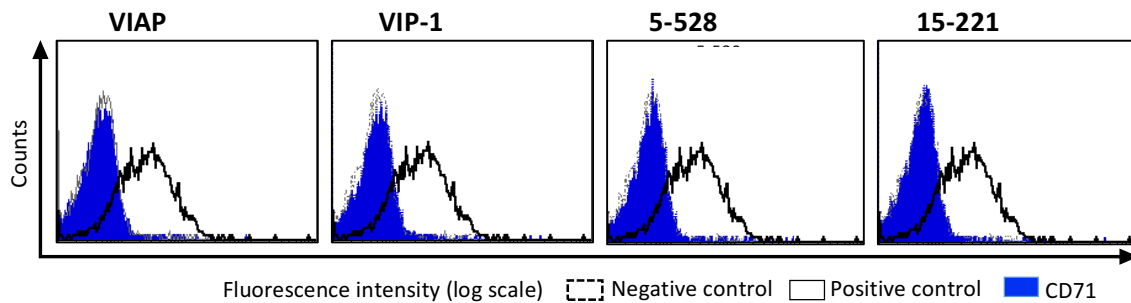


Figure 27 Identification of the Interaction of 13-344 with CD71 mAbs via membrane staining. Jurkat cells were either pre-incubated with CD71 mAbs, PBS or Beriglobin for 30' on ice, using the latter as positive control. After further incubation with biotinylated 13-344 mAb, the membrane staining was accomplished with Streptavidin-PE. No interaction of 13-344 was detected with other CD71 mAbs on the cell surface. One representative histogram for each condition of two independent experiments is illustrated.

4.6.2. 13-344 recognize iron-loaded as well as iron-free human transferrin

As mentioned before, 13-344 can only bind to the cell surface receptor CD71 in the presence of plasma proteins such as human serum albumin or Beriglobin. In order to examine if HSA or human transferrin are the interaction partners for 13-344, an indirect enzyme-linked immunosorbent assay (ELISA) was performed. However, the results represent that iron-loaded as well as iron-free transferrin indicate a binding partner for 13-344. Remarkably, no interaction between mAb 13-344 and HSA was identified (Figure 28).

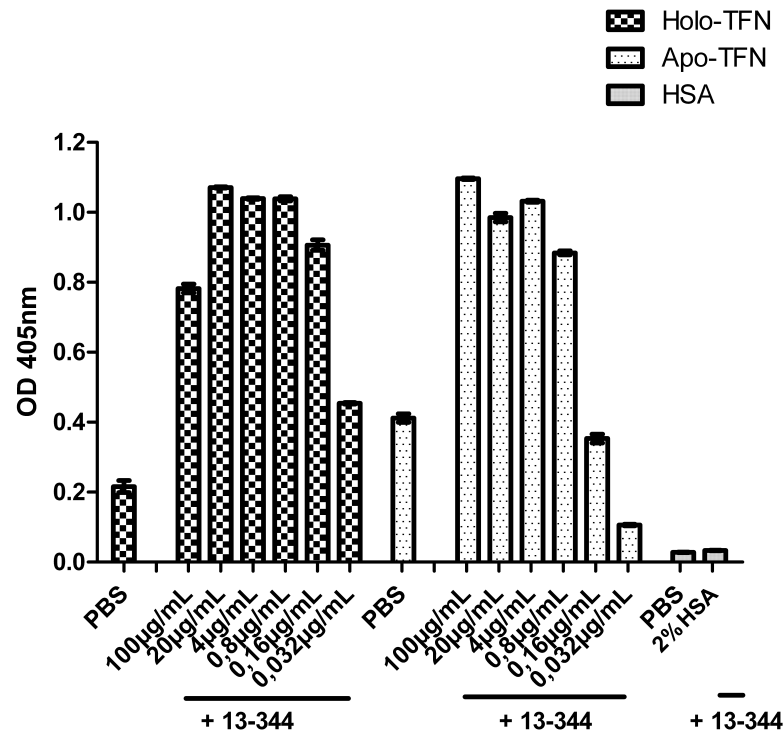


Figure 28 Apo-Tfn as well as holo-Tfn were recognized by mAb 13-344. Elisa plate was coated with either apo-Tfn, holo-Tfn, PBS or HSA as negative control at different concentrations overnight. Afterwards 13-344 binding was detected through fluorescence signals. 13-344 interact with iron loaded and iron-free Tfn, but not with human serum albumin. Results show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

4.6.3. Bovine transferrin is not responsible for 13-344 binding on CD71

Fetal-bovine serum is a common used supplementation in tissue-culture medium. Previous studies have investigated the interaction of isolated bovine transferrin from fetal-bovine serum with human cells in comparison to human isolated transferrin. A past study has already observed that bovine transferrin exhibit a much lower binding affinity to human CD71 than of human transferrin [95]. In order to analyze the impact of bovine transferrin on CD71 mAb binding, Jurkat T cells were incubated with PBS or RPMI 1640 medium supplemented with 10% FCS. Then the cells were subsequently treated with CD71 mAbs and the fluorescence of the antibodies was measured via flow cytometry. The binding capacity of CD71 mAbs on the surface of Jurkat T cells incubated in 10% FCS medium is compared to untreated T cells. Figure 29 demonstrate that in comparison to untreated cells, the binding affinity of CD71 mAbs remained unaltered in bovine transferrin pre-treated cells. In particular, data represent consistent results in case of the binding affinity of 13-344 to CD71 (Figure 29).

RESULTS

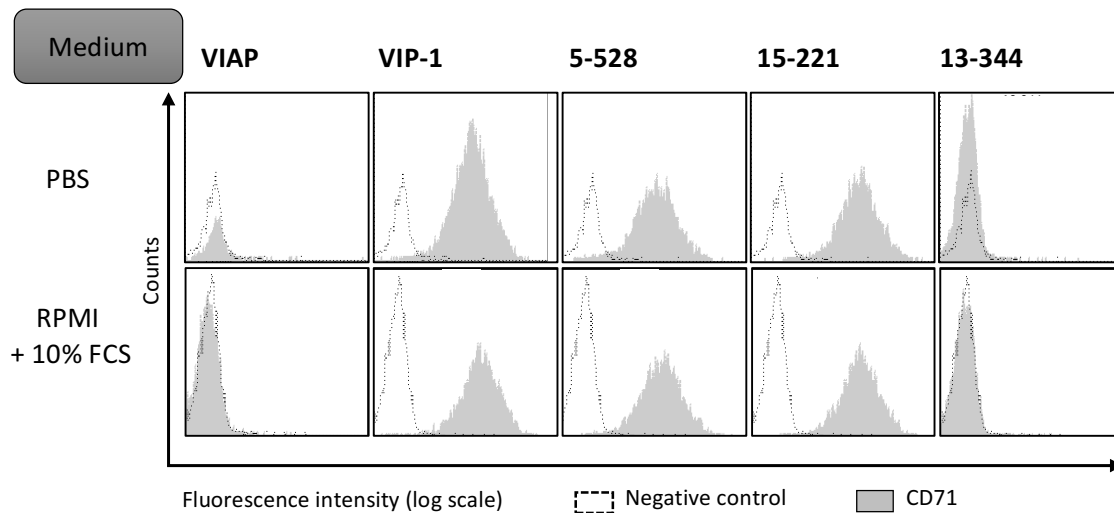


Figure 29 Human lymphoma cells were used to determine the influence of bovine transferrin to the binding affinity of CD71 mAbs. No 13-344 mAbs are detected on the cellular surface either when cells were cultivated in PBS nor in RPMI 1640 medium supplemented with 10% FCS. Bovine transferrin induces no interaction of 13-344 to CD71. VIAP was used in this study as isotype control. Data represent one performed experiment.

4.6.4. Transferrin is responsible for 13-344 interaction to specific epitopes on CD71

Based on the data of the protein binding assay (Figure 28), we examined the binding affinity of 13-344 as well as VIP-1 as positive control to CD71 on Jurkat T cells in the presence of human transferrin. Since we observed binding capability between human transferrin (apo- and holo-Tfn) and 13-344, we were interested in the impact of transferrin to 13-344 binding. Thus, we performed a membrane staining assay, whereas Jurkat T cells were pre-incubated with human holo-transferrin, followed by CD71 mAb treatment and surface staining. Transferrin mediated the binding of 13-344 with a decreasing concentration, whereas very low amount of transferrin at a concentration of 0.2 $\mu\text{g/ml}$ can no longer induce 13-344 binding. However, VIP-1 was inhibited by transferrin, in which the binding affinity was highly affected when the concentration of transferrin decreased (Figure 30).

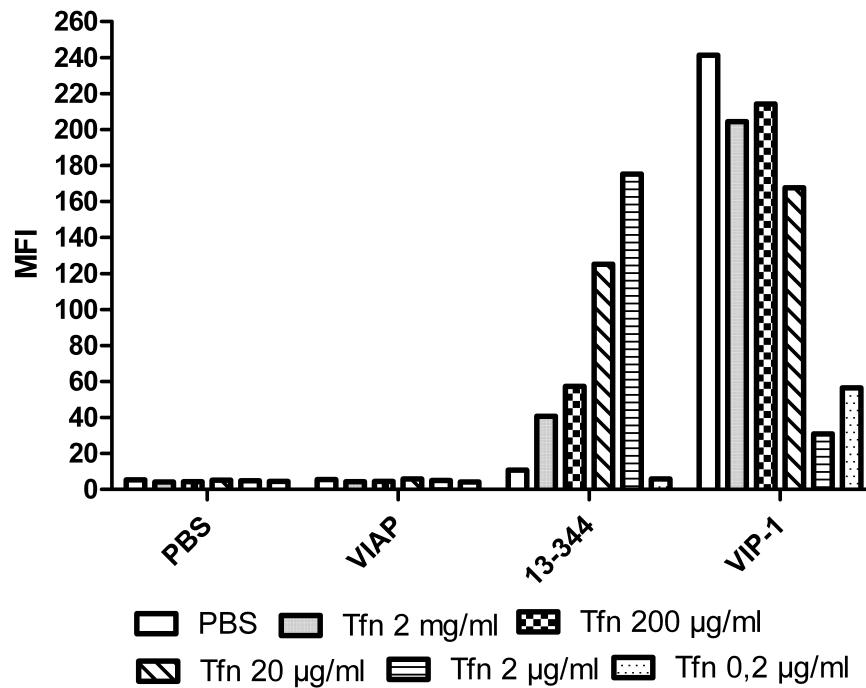


Figure 30 Binding affinity of 13-344 is induced by decreased Tf concentration. Jurkat T cells were pre-treated with human Tf at different concentrations and subsequently incubated with CD71 mAbs or VIAP for 30' on ice. Then mAbs were immuno-stained with OG to determine their binding affinity to CD71. The binding capability of 13-344 increases and VIP-1 decreases with the decline of Tf. Results of one experiment are shown.

4.6.5. The impacts of CD71 mAbs to transferrin binding

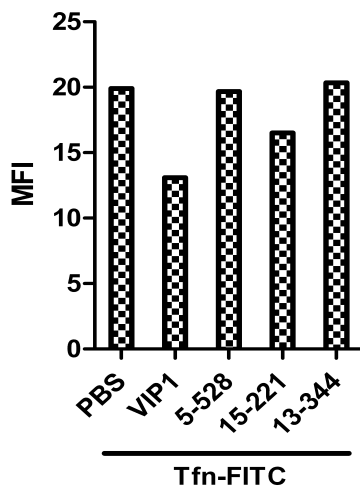


Figure 31 Inhibition of Tf binding due to VIP-1 and 15-221. Jurkat cells were pre-incubated with 20 µg/mL CD71 mAbs. At 0°C, binding of FITC labeled Tf is reduced in VIP-1 and 15-221 incubated cells. 5-528 and 13-344 have no impact on Tf binding. Results show one performed experiment.

Further, to analyze if CD71 mAbs block the interaction of Tf to the transferrin receptor, Jurkat T cells were tested through binding studies. Figure 31 shows the effects of CD71 mAbs on Tf binding. It was observed that VIP-1 blocked the binding of Tf much efficiently than mAb 15-221. However, the specificity of 5-528 and 13-344 mAbs to human tissue remained unaltered and represent no impairment of Tf binding on the cell surface of Jurkat T cells (Figure 31).

4.7. The impact of CD71 mAbs to Abraxane

The chemotherapeutic drug Abraxane is composed of albumin-bound paclitaxel particles, in which albumin is derived from human blood and used as a vehicle for the delivery and uptake of paclitaxel. In previous studies it has been reported that paclitaxel has a toxic effect on cells, leading to cell death via apoptosis and necrosis [79, 84].

To confirm the toxic effect of Abraxane, we cultivated Jurkat cells in presence or absence of Abraxane for three as well as for seven days. When Abraxane was added to cell culture the proliferation of Jurkat cells was suppressed on the third day followed by additionally reduction of cell number on the seventh day (Figure 32).

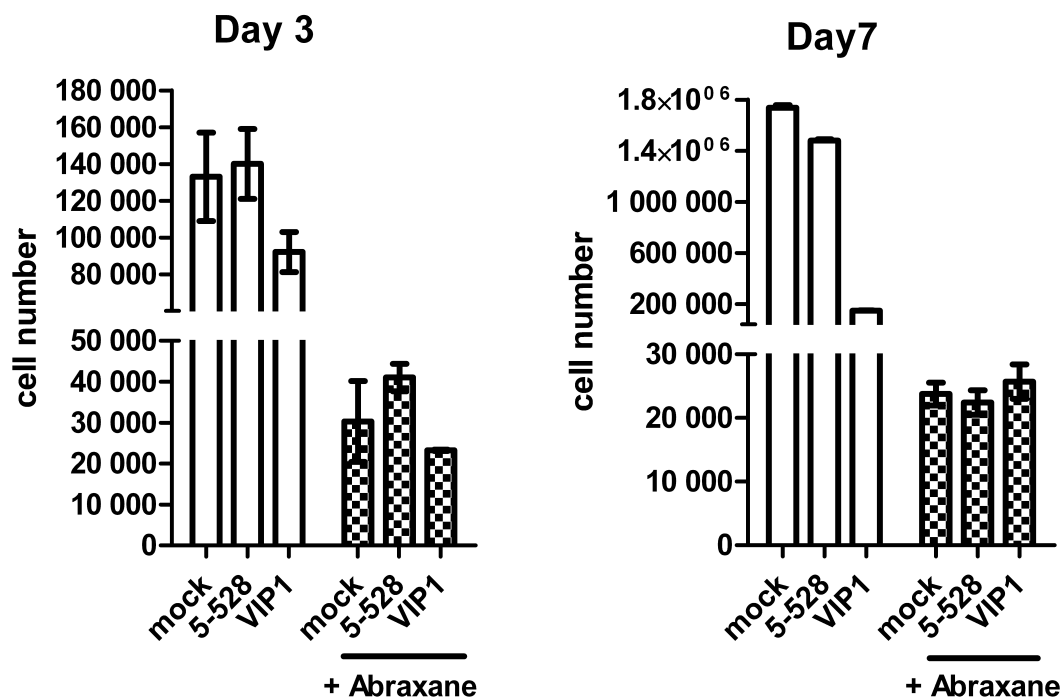


Figure 32 Toxic effect of Abraxane is not enhanced through CD71 mAbs treatment. 3×10^4 cells / well were seeded and cultivated for three and seven days in RPMI 1640 medium supplemented with 10% FCS in presence or absence of 100 ng/ml Abraxane. In addition, cells are incubated with 20 μ g/ml CD71 mAbs. Without Abraxane, cell proliferation is increased until day seven and retarded by VIP-1. After the seventh day, no enhanced Abraxane effect is observed during antibody treatment. Results, which are represented after three days, show the mean ($\pm$ standard deviation) of two independent experiments, whereas the data after seven days show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

Further, we wanted to investigate the impact of CD71 mAbs on the toxic effect of Abraxane. Therefore, Jurkat T cells were additionally incubated with CD71 mAbs and viable cells were measured on the third and seventh day. CD71 5-528 has the ability to retard the toxic effect

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of Abraxane until day three, whereas VIP-1 slightly increased cell death. However, it is mentionable that on the seventh day no differences between CD71 mAb treated and untreated cells in the presence of Abraxane were observed, resuming that 5-528 cannot generally inhibit the toxic effect of Abraxane (Figure 32).

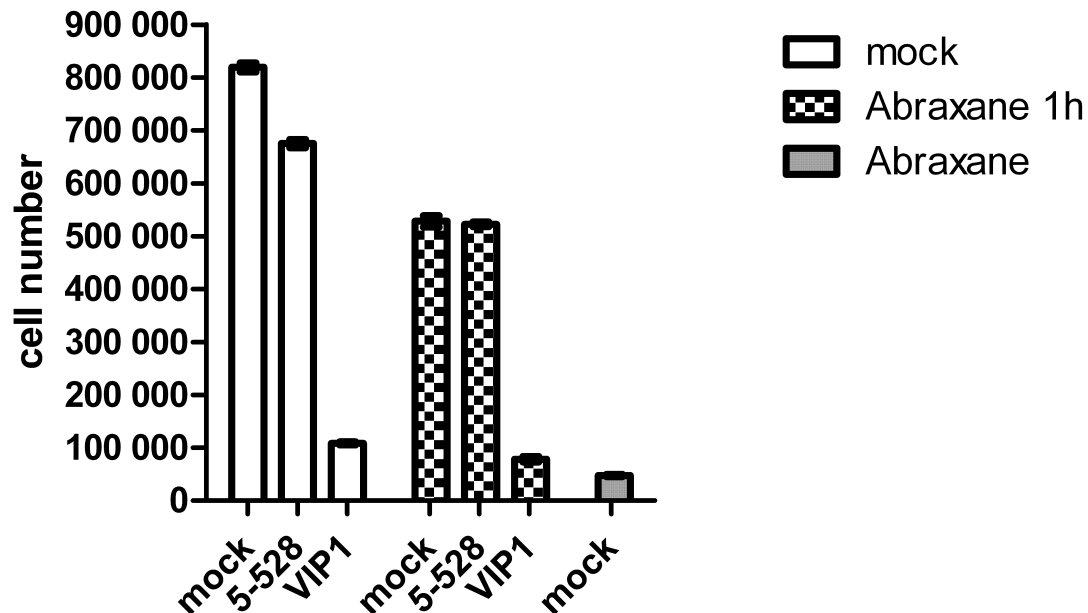


Figure 33 The toxic effect of Abraxane. On the one hand, Jurkat T cells were pre-treated with 100 ng/ml Abraxane for 1 hour in presence or absence of 20 μ g/ml CD71 mAbs and on the other hand permanently incubated for six days with 100 ng/ml Abraxane. The toxicity of Abraxane to cells for a shorter incubation time is not as severe as the permanently exposure to Abraxane. Results illustrate mean values ($\pm$ standard deviation) of one experiment measured in triplicates.

Moreover, to determine the intensity of the toxic effect of Abraxane, a proliferation assay of Jurkat T cells was performed. T cells were either treated with 100 ng/ml Abraxane for 1 hour or permanently, followed by cell cultivation in RPMI 1640 medium supplemented with 10% FCS with or without CD71 mAbs until day six. A weak inhibitory effect in cell growth was observed in Abraxane pre-treated Jurkat T cells. VIP-1 was found to inhibit cell growth in Abraxane pre-treated as well as untreated cells. Jurkat T cells, which were permanently incubated with Abraxane, represented a much higher toxicity than pre-treated cells for 1 hour. However, in the presence of mAb VIP-1 and Abraxane we could not determine if the antibody or the drug mediated the impairment of cell growth (Figure 33).

The previous experiment showed that 5-528 slightly diminish the toxic effect of Abraxane. Therefore, we wanted to confirm in particular if the binding of 5-528 mAb can effectively

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influence the toxic effect of Abraxane. By culturing Jurkat T cells in the presence of Abraxane for 24 hours and 72 hours with or without CD71 mAbs, the effect of CD71 mAbs on the cell growth of Abraxane treated cells was examined. Death cells were stained with propidium iodide to determine the percentage of viable and death cells. In addition, we wanted to analyze if the toxic effect of Abraxane declined by fractionation. On the first day, CD71 mAbs increased the growth-inhibitory effect of Abraxane treated cells. This observation was similar to cells, which were treated with fractionated Abraxane. After 72 hours, 5-528 was detected as an antagonist of Abraxane, which was reflected by reduced cell death of 30 %. Furthermore, CD71 mAbs VIP-1, 15-221 and 13-344 did not have the ability to remove or enhance the toxicity of Abraxane, irrespective of Abraxane fractionation. However, neither Abraxane nor fractionated Abraxane had significant effects on leukemic cell growth (Figure 34).

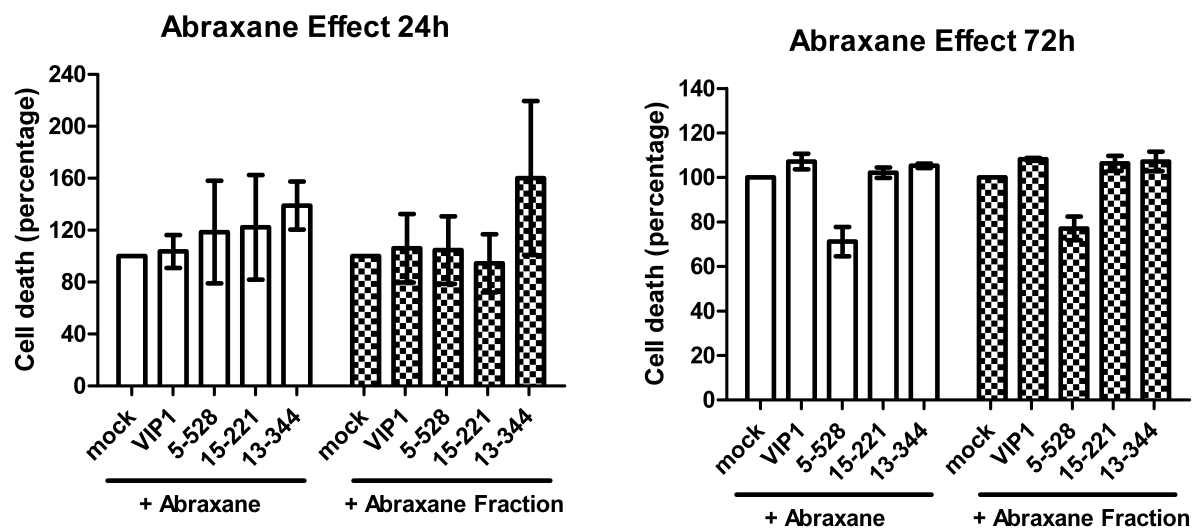


Figure 34 Toxic effect of Abraxane and fractionated Abraxane diminished by 5-528. 1×10^5 cells were cultivated in triplicates in 96-well plates using RPMI 1640 supplemented FCS medium and pre-incubated with 100 ng/ml Abraxane in combination with 10 μ g/ml CD71 mAbs for 24 hr and 72 hr. Cell death was analyzed through DNA staining with propidium iodide. After three days the cell death of Jurkat cells is reduced by 30% in case of 5-528 treatment. Results represent the percentage of cell death, showing mean values ($\pm$ standard deviation) of three independent experiments.

4.7.1. Abraxane induces 13-344 binding to CD71 in human lymphoma and erythroblast cells

The finding that human serum albumin blocks the binding of CD71 mAb VIP-1 as well as strongly enhances the binding of 13-344 to the cell surface receptor on leukemic T cells (Figure 20), the question was if Abraxane has the same impact on the binding affinity of CD71 mAbs due to comprised albumin molecules. Thus, binding assays were performed with lymphoma and erythroblast cells. T cells were pre-incubated with Abraxane and subsequently treated with CD71 mAbs.

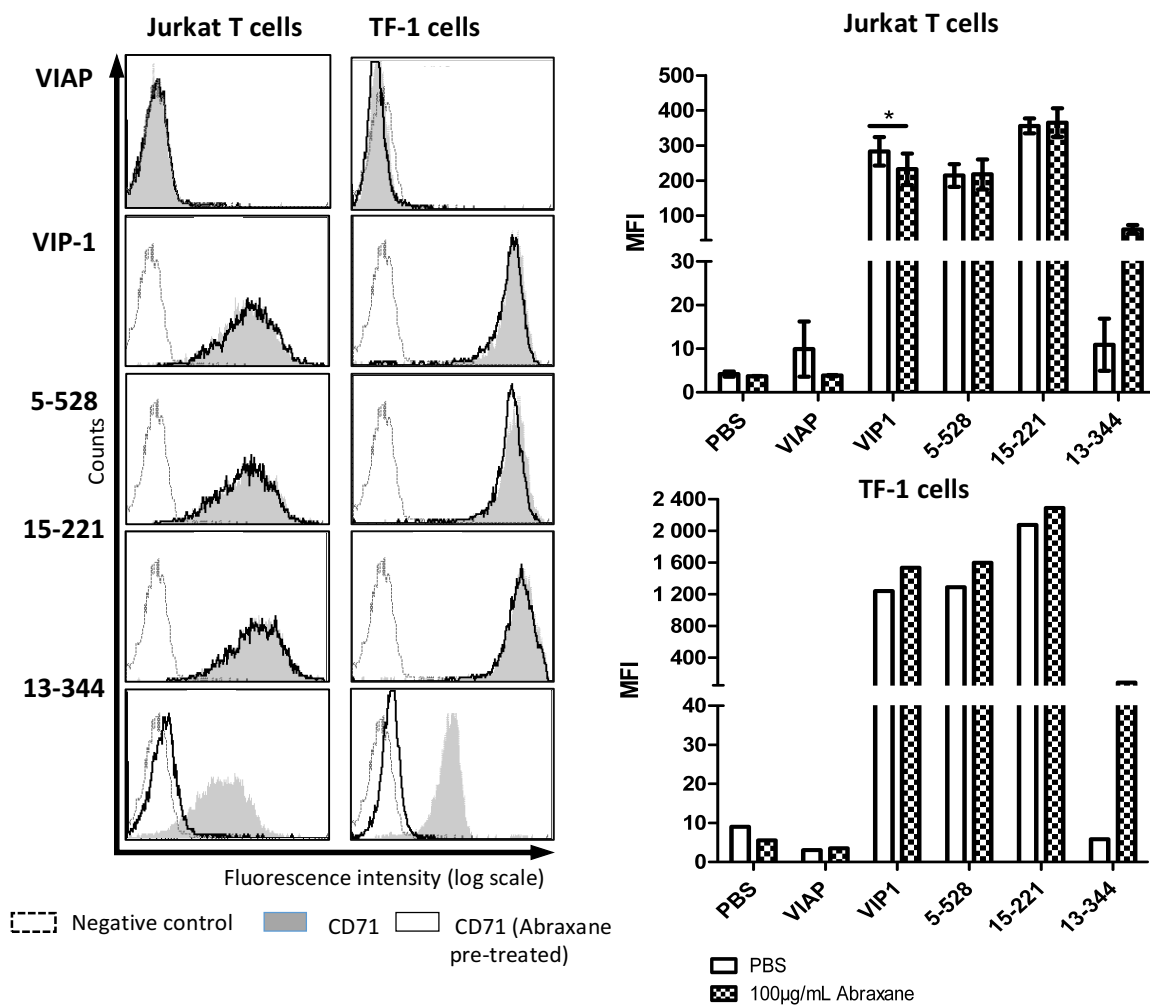


Figure 35 Human lymphoma and human erythroblast cell lines were used to determine the binding affinity of CD71 mAbs to CD71 on the cell surface after pre-treatment with human serum albumin. Cells were pre-incubated either with 200 µg/ml Abraxane or nothing, Membrane staining was accomplished as described earlier for CD71 binding on Jurkat cells. VIAP was used in this study as isotype control. Data represent one of three independent experiments of Jurkat T cells, but only one experiment in case of TF-1 cell line.

RESULTS

In case of Jurkat T cells, Figure 35 shows a significant inhibition of VIP-1 binding to CD71. However, no growth inhibition was visible in TF-1 cells. In contrary, a potentially increased fluorescence signal for 13-344 was recognized for both T cell types, suggesting that albumin molecules of Abraxane mediate 13-344 binding.

4.7.2. Impact of ferric ammonium citrate on the toxic effect of Abraxane

To determine if Abraxane influences T cell proliferation in a dose-dependent manner, Jurkat T cells were cultured in presence or absence of different Abraxane concentrations. Furthermore, we wanted to find out if iron influences the T cell growth during Abraxane treatment. Performing a proliferation assay, Jurkat T cells were cultivated for three days with different concentrations of Abraxane along with or without FAC. The highest concentration of Abraxane led to cell death, whereas the cell growth is less affected by decreased Abraxane concentrations (1 ng/ml – 0.01 ng/ml). However, the toxic effect of Abraxane is weakly abolished due to iron treatment (Figure 36).

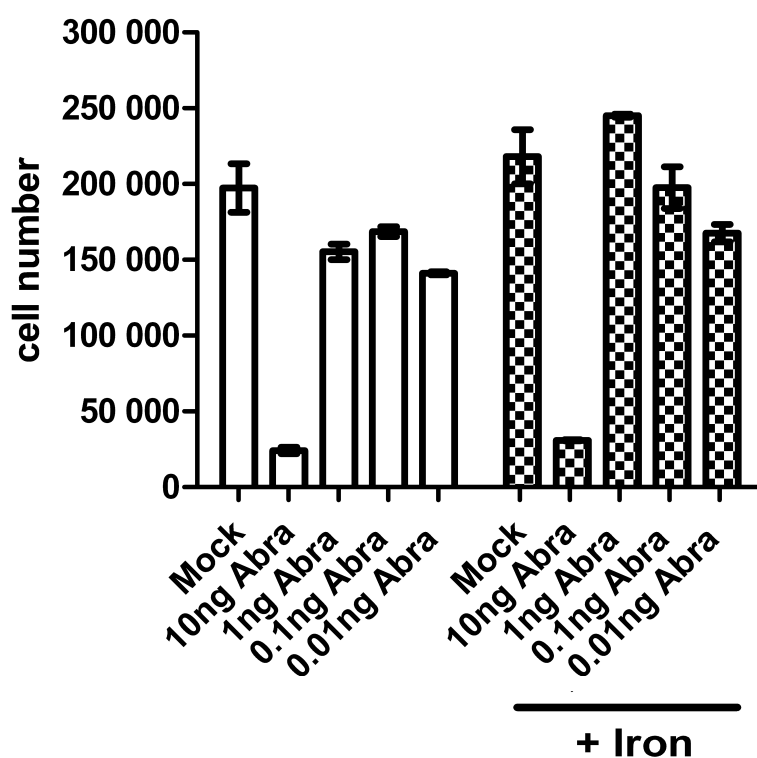


Figure 36 Iron abolishes the cytotoxic effect of Abraxane. Jurkat T cells were incubated with different Abraxane concentrations (10 ng – 0.01 ng/ml) in presence or absence of 25 µg/ml FAC for three days. The major toxic effect is shown during the incubation with the highest Abraxane concentration. Cells, which were incubated additionally with FAC, indicates better growth behavior than without FAC. Data show mean values ($\pm$ standard deviation) of one experiment, which was measured in triplicates.

5. DISCUSSION

The transferrin receptor is an important iron uptake mechanism, whereas serum Tfn is the crucial vehicle for cellular iron delivery. In this project we used four CD71 mAbs, including the well-defined VIP-1, against TfR, which is highly expressed on the cell surface of leukemic cells. We could verify that targeting of specific epitopes on CD71 via CD71 mAbs can determine the fate of T cell expansion under different conditions. In particular, the aim of this project was to determine the stimulatory factors of HSA for leukemic T cell proliferation as well as the impact of CD71 mAbs on iron intake.

Previous studies of our group have indicated the binding of CD71 mAbs to CD71 on the surface of proliferating T and B lymphocytes such as Jurkat, K-562, Daudi, and Raji cells via surface staining [87, 96]. Thus our study wanted to confirm the specific binding affinity of CD71 mAbs to CD71 on leukemic cell lines such as Jurkat and TF-1 cells as well as their effect on cell proliferation. Our results validated the specific binding of VIP-1, 5-528 and 15-221 by binding assays in two different T cell lines. However, in regard to 13-344 our findings are not consistent with the above mentioned results because 13-344 was not detectable on the surface of leukemic cells in the absence of Beriglobin, suggesting that 13-344 binding is mediated by immunoglobulins. Since we have observed that 13-344 needs certain interaction partners, we further determined no detectable amount of 13-344 in CD71 mAb treated leukemic cells. In contrary, we assume that 13-344 binding to CD71 is mediated by conformational changes of CD71, which is induced by HSA, because direct interaction between 13-344 and HSA was disproved via indirect antigen detection. Remarkably, apo- and holo-Tfn showed direct interaction with 13-344 as well as Tfn pre-treated lymphoma cells influenced 13-344 binding to CD71, suggesting that Tfn is a relevant factor. Our results represent that decreased Tfn concentration mediated 13-344 binding, whereas less is insufficient and excessive amounts led in turn to decreased binding. According to this observation, we suggest that Tfn is apparently available in HSA, but only a very low concentration is sufficient to enable 13-344 binding. Therefore, it is important to determine the optimal HSA concentration with the most efficient Tfn amount. Together, we can exclude that CD71 mAbs as well as albumin variants are potential factors, but it seems like that HSA and Tfn acquire the ability to induce 13-344 binding to CD71 on leukemic T cells. Furthermore, we determined the impact of Tfn to albumin-CD71 interaction, resulting in decreased fluorescence signals of HSA or rHSA binding,

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whereas BSA represented only slightly reduced binding affinity in presence of Tfn. Together, we can hypothesize that human Tfn and albumins may exhibit overlapping binding sites, followed by higher binding affinity of Tfn to CD71 because we investigated no impairment of Tfn binding in HSA pre-treated cells.

In the primary part of our project we addressed the question if various albumins are able to induce leukemic cell proliferation. HSA is extensively studied over many years and is used in various systems due to its stability and availability. However, rHSA is rather used in clinical fields due to avoiding contaminations. Minami *et al* demonstrated that HSA and rHSA differs in its thermal stability and cell growth activity, however the structural properties are similar [65]. Thus, we wanted to determine the growth behavior of leukemic T cells under certain conditions. Even though HSA might not be directly the interaction partner between 13-344 and CD71, experiments show that HSA is important in a dose-dependent manner for malignant T cell growth stimulation in serum-free medium. However, treatment with commercially available albumin products, including rHSA and dHSA, decreased the number of cells grown in serum-free medium. It could be possible that due to different manufacturing and purification processes the structure and function of mentioned albumins alter, leading to different growth behavior of T cells. However, it is obvious that leukemic T cell growth depends on the availability of specific factors such as iron and/or Tfn, which are may be available in HSA but not in other albumin proteins, because it is well known that iron is an essential cofactor for tumor cell proliferation [97]. This hypothesis was reinforced with simultaneous treatment of Jurkat cells with albumins and FAC or Tfn, enhancing the cellular proliferation. However, excessive iron availability led to the conversely effect, suggesting that iron overload antagonizes growth stimulation of leukemic cells. Since Tfn showed also growth stimulatory effects it has to be further questioned if HSA contains human Tfn. Thus, it is necessary to examine HSA in more details may be by separation via gel electrophoresis or chromatographic processes to determine and purify binding partners of the heterogeneous molecule, which could be additionally added to albumins to analyze the responsible components for cell growth stimulation in serum-free medium. According to the growth stimulatory effect of iron, we determined the influence of albumin-hemin on T cell growth. Interestingly, albumin-heme was also found to be able to strongly stimulate leukemic T cell proliferation. In a similar way we have observed the same stimulatory effect by culturing cells in dHSA-hemin, which seems like that hemin is crucial for cellular expansion and the

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responsible factor in HSA for growth stimulation. Further experiments showed no significant growth effects in presence of hemin, biliverdin or rHSA-biliverdin, which gives rise to the assumption that only bounded hemin has the ability to induce cell growth. Thus, we can assume that hemin as well as iron play a crucial role in T cell proliferation, suggesting that iron is released from hemin via HO-1 which, is subsequently transported through Tfn to CD71 followed by receptor-mediated endocytosis. Since it is well-known that HO-1 is responsible for the initiation of iron release, it is tempting to speculate that the same effect would occur in EBV cell lines, but not in HO-1 defect B cells. Therefore, further experiments have to be performed with B cell lines as in comparison to already described experiments with T cells. To determine if albumin-hemin is able to stimulate cell growth in EBV as well as HO-1 defect B cell lines, proliferation assays have to be performed.

As previously mentioned, VIP-1 and 15-221 have the ability to inhibit T cell growth in leukemic cells, cultured in FCS-medium. In regard to this observation we our data represent the same inhibitory effects of VIP-1 and 15-221 on leukemic T cell growth in presence of HSA. Moreover, 5-528 and 13-344 showed higher interference of cell growth as in FCS cultured Jurkat cells. As we have seen that Tfn is may be a mediator for 13-344 binding to CD71, it could be possible that 13-344 negatively influences the iron liberation from HSA and subsequent iron uptake may be due to downregulation of CD71. The same hypothesize could be possible in case of 5-528 because we observed higher fluorescence signals of 5-528 in HSA pre-treated cells by membrane-staining assays.

Noticeable, Jurkat cells, which are cultured in FCS or albumin supplemented medium in presence of iron or Tfn not only enhanced T cell proliferation, but also antagonize the growth-inhibitory effect of VIP1 or 15-221. Other studies investigated the regulatory mechanism of CD71 in human erythroblast cells during iron treatment and deficiency [98]. Abe *et al* has reported that a high level of cellular iron as well as hemin did not influence the expression of CD71 on the surface of human erythroblasts *in vivo*, but still the level of mRNA. According to these findings, it has been suggested that growth-inhibitory effects by CD71 mAbs are not abolished through increased CD71 expression, but may be due to the competition between CD71 mAbs and iron-Tfn, interacting with identical epitopes, which is determined by higher binding affinities of iron-Tfn. We agree with this prediction because we observed on the one hand weaker fluorescence signals of Tfn on the cell surface of VIP-1 or 15-221 pre-incubated T cells and on the other reduced binding affinity of VIP-1 in presence of Tfn. However, in more

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detail our data showed that only lower amounts of Tfn can strongly suppress VIP-1, may be due to CD71 internalization, whereas overload seems to have slight effects. But it is difficult to conclude this observation. Thus, it would be relevant to further investigate VIP-1 and Tfn on the basis of molecular analysis.

With the knowledge that CD71 mAbs are capable to inhibit cell growth in presence of FCS or HSA, we wanted to verify this impact on rHSA-hemin treated T cells. In contrary to the previous experiments stronger inhibitory effects were only noticed during VIP-1 or 15-221 incubation, but were not as effective as in HSA or FCS cultured cells. It could be that growth inhibition by VIP-1 or 15-221 is reduced due to hemin followed by the availability of higher iron concentration, which could interfere with CD71 mAb binding. Further, binding assays showed slightly reduced fluorescence signals of VIP-1 in presence of rHSA-hemin. In order to gain insight in the binding behavior of rHSA-biliverdin and dHSA to CD71 we conducted binding assays especially with CD71 mAbs. These data showed no significant differences in binding patterns, detecting no 13-344 binding.

Conversely, we analyzed the capability of CD71 mAbs to influence the binding of various conjugated albumins to CD71 in lymphoma cells. 15-221 or 13-344 pre-incubated lymphoma cells indicated no impact to HSA binding, but remaining CD71 mAbs negatively influenced HSA binding to CD71. The observation that VIP-1 has the ability to block HSA binding as well as HSA leads to the inhibition of VIP-1 binding, which was shown in previously described experiments, provided us with the knowledge that VIP-1 as well as HSA exhibit overlapping binding sites. According to the observation that 5-528 binding to CD71 was significantly enhanced in HSA pre-incubated cells, but in contrast HSA binding slightly inhibited by CD71, we suggest that 5-528 reduces the number of CD71 in such a way as to limit the binding of HSA to CD71. A study has reported that the affinity of bovine Tfn is not as effective to CD71 as of human Tfn [95], which could agree with our results, showing no influence of CD71 mAbs to BSA binding. In addition, we assessed the impact of CD71 mAbs to the binding of rHSA and dHSA in absence or presence of bounded hemin. However, CD71 mAbs were found to be unable to influence the affinity of commonly available albumins as well as hemin bounded proteins, which could be explained by lacking Tfn. However, 13-344 did not interfere with either HSA nor remaining albumins because 13-344 binding has to be induced by proteins, which is not well-defined yet.

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Since in this project we have observed that Tfn as well as bounded hemin to albumin is needed for cell proliferation, it is of interest to analyze if other components like FAs have the ability to stimulate T cell expansion. It is well-known that HSA bind endogenous and exogenous substances [65]. As seen in a former study, unsaturated FAs such as oleic and linoleic acid induced apoptosis and necrosis of lymphocytes, which was detected by FACS analysis [93]. To determine further potential factors for cell growth, we cultured lymphoma cells in serum-free medium, which was supplemented with various albumins in presence or absence of unsaturated FAs, including oleic and linoleic acid. Notably, we observed impaired proliferation phenotype by culturing dHSA or dHSA-hemin pre-treated lymphoma cells with linoleic or oleic acid, leading to a reduction of T cells in linoleic acid treated cells. However, data represented less growth inhibition by oleic acid, whereas a stronger effect was observed by simultaneously treatment. These findings agree with a previous independent study by Cury-Boaventura *et al* [93]. Thus, we suggested that linoleic acid possess a higher toxicity than oleic acid, causing increased cell death. Moreover, the same experiment was carried out for a longer time period, clearly observing a higher amount of cell death in combination with both FAs.

Abraxane is composed of paclitaxel, which is surrounded by albumin and is used for the treatment of different types of cancers. In the last part of our project we wanted to investigate if albumin enhances the uptake of Abraxane via CD71. Firstly, the extent of the toxicity of Abraxane was determined, which resulted in significant suppression of T cell expansion, showing increased cell death in permanently incubated cells, may be due to apoptosis and necrosis. This observation correlates with a study by Myrick *et al* performed with Jurkat T cells *in vitro*, which demonstrated that paclitaxel induces cell arrest in G2-M phase of the cell cycle followed by cell growth inhibition. Conversely, short-term treatment of T cells with Abraxane seems to be not as effective as permanently incubation. Referring to the effects of iron we have seen in CD71 mAbs treated T cells, we hypothesized a reduction of cell death in Abraxane treated cells. We have indeed observed that cells showed only a slight attenuation of the toxicity of Abraxane via iron. Moreover, to determine the interference of CD71 mAbs on the growth rate of Abraxane treated cells we conducted a proliferation assay. We observed increased cell death in Abraxane treated cells in absence as well as presence of VIP-1. However, it is still unclear, if VIP-1 or Abraxane is the crucial factor, which blocks cell proliferation because both components exhibit antiproliferative effects on T cells as mentioned before. More importantly, treatment of T cells with 5-528 retarded the toxic effect

DISCUSSION

of Abraxane, which was subsequently abolished. This observation was confirmed via membrane-staining assays in more detail, representing that 5-528 has the ability to retard the induced cell death by Abraxane, which agrees with the results we have previously obtained via proliferation assays, demonstrating that 5-528 decelerated the toxic effect, whereas after two days the effect was abolished. In particular, we found no differences in growth behavior of T cells, which were either cultivated in presence of Abraxane or fractionated Abraxane, suggesting that it is very hard to remove the toxicity of Abraxane by fractionation of particular components via MW.

In addition to determine the influence of Abraxane regarding to the binding of CD71 mAbs we performed binding assays as done before in case of HSA. Mentionable, Abraxane pre-treatment seems like to have the same impact on VIP-1 and 13-344 binding as we have seen in HSA pre-treated Jurkat T cells. However, no binding differences could be detected within the remaining CD71 mAbs. In contrary, it was observed that Abraxane pre-treated TF-1 cells only positively influenced CD71 mAb binding to CD71, with respect to 13-344 showing the highest fluorescence signal. The results of the two assays indicate that Abraxane reacts differently in comparison to HSA. Our results could agree with the statement that a moderate amount of Tfn present in HSA inhibits VIP-1 and the other way round enhances 13-344 binding because we have previously investigated that decreased Tfn concentration causes inhibition of VIP-1 as well as mediates binding of 13-344 to CD71. However, other components, which are may be included in HSA can mediate and block mAb binding. Another possibility could be that HSA undergo modifications during assembling paclitaxel with HSA resulting in alteration of protein folding, stability and function, which could interfere with CD71 mAb binding differently to HSA. Further, this data could indicate that the uptake of Abraxane is also mediated by a CD71-independent mechanism. It has been reported that conjugation of paclitaxel with Tfn lead to increased cell death demonstrating stronger antitumor activity [99]. According to this observation, we could further establish Tfn- as well as albumin-conjugated Abraxane, in order to analyze their impact to lymphoma T cell proliferation.

In summary, we have found that hemin is one of the potential factor in HSA and has the ability in its bounded form to stimulate the expansion of leukemic cell lines in serum-free medium. It is reported, that HSA is important for the protection against free heme-Fe, which causes oxidative damage as well as is a useful biomarker for many diseases [64]. However, under physiological conditions only 20 % of free heme-Fe binds to HSA and hemopexin in the

DISCUSSION

bloodstream followed by the clearance of heme-Fe in the liver [64, 100]. Some studies have already reported that the plasma level of heme-albumin in healthy patients is very low (about 1×10^{-6} M), whereas under pathological conditions the concentration of heme-albumin increases to 4×10^{-5} M, which is mediated by excessive intravascular hemolysis because heme binds to HSA after hemopexin gets saturated [64, 68, 100]. Moreover, the heme-albumin level is used for the prediction of severity of pancreatic diseases as well as is strongly available in serious intra-abdominal conditions [101]. With the knowledge that heme-albumin is upregulated under pathological conditions, albumin-heme is may be useful for diagnosis and treatment of various diseases such as severe hematologic diseases, pancreatitis or analbuminemia as well as for the development of therapies for patients with heme toxicity [64]. Furthermore, it might be relevant for pharmacological therapies because it is known that increased concentration of heme-Fe in plasma induced the release of HSA-bound drugs. Moreover, cells with iron-deficiency could be additionally replete with heme-albumin as iron source, to reinforce cell regeneration and fight against infections. Further, as our actual evidence point to a stimulatory role of albumin-heme in leukemic cells, we assume that it is not only an additionally iron uptake mechanism, but also an antiviral system, which could support our immune system against pathogens. This statement could be confirmed by Santangelo *et al*, because it is well-known that during heme metabolism HO-1 converts heme to CO, iron and biliverdin, which is rapidly reduced to bilirubin. Bilirubin is known to have antiviral functions, particularly interferes with enterovirus 71 and human herpes simplex type 1 virus. Therefore, it is obvious that during pathological conditions heme-albumin could not only stimulate T cell proliferation but also antagonize viral infections by the release of bilirubin through HO-1 and biliverdin reductase (BVR) [102]. Due to the efficiently suppression of T cell proliferation by VIP-1 and 15-221 in HSA treated T cells, leading to cell death as a consequence of lacking iron-Tfn, CD71 mAbs could facilitate the fight against acute T cell leukemia and enable the development of various therapeutic approaches.

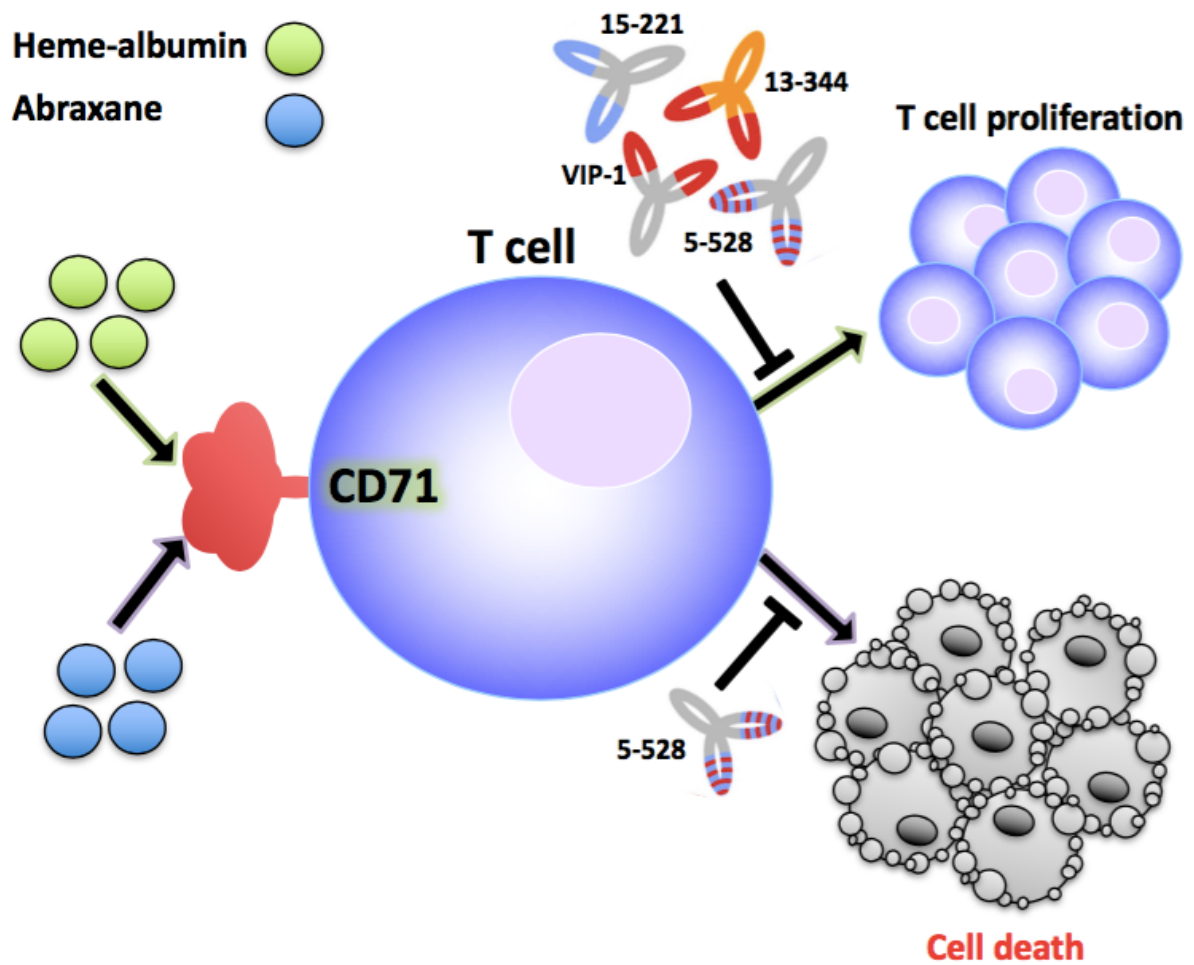


Figure 37 Schematic representation of the conclusion of our study. Heme-albumin interacts with CD71, which stimulates T cell proliferation. However, this effect is inhibited by CD71 mAbs. In contrary, paclitaxel-bound albumin molecules (Abraxane) cause cell death, which could be retarded for a short-term by 5-528.

6. ABBREVIATIONS

APC (s)	Antigen-presenting cell (s)
Apo-Tfn	Iron-free transferrin
ATCC	American Tissue Culture Collection
BSA	Bovine serum albumin
CD71	Transferrin receptor
CTL	Cytotoxic T lymphocytes
DC (s)	Dendritic cell (s)
Dcytb	Duodenal cytochrome-b
dHSA	Fatty acid-free human serum albumin
DMT1	Divalent metal transporter-1
ELISA	Enzyme linked immunosorbent assay
ETC	Electron transport chain
FA(s)	Fatty acid (s)
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Fe II / Fe III	Iron oxidation state II / III
FITC	Fluorescein isothiocyanate
FLVCR1	Feline leukemia virus subgroup C cellular receptor 1
FPN-1	Ferroportin-1
Ftmt	Mitochondrial ferritin
HCP1	Heme carrier protein-1
Hb	Hemoglobin
HFE	Gene encode for hereditary hemochromatosis protein
Hpx	Hemopexin
hr (s)	Hour (s)
HO	Heme-oxygenase
Holo-Tfn	Iron-bound transferrin
HSA	Human serum albumin
Ig	Immunoglobulin
IL	Interleukin
INF-γ	Interferon
IRP (s)	Iron-regulatory protein (s)
IRE (s)	Iron-responsive element (s)
ISC (s)	Iron-sulfur cluster (s)
kDA	Kilo Dalton
LAT	Linker for activation of T cells
LIP	Labile iron pool
LCFS	Long chain fatty acids
LPS	Lipopolysaccharide
mAb (s)	monoclonal antibody (s)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mg	Milligram
min	minutes

ABBREVIATIONS

ml	Milliliter
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
NK cell (s)	Natural Killer cell (s)
OG	Oregon Green
PBS	Phosphate buffered saline
PCFT	proton-coupled folate transporter
pH	Power of hydrogen
PTK	Protein tyrosin kinase
rHSA	Recombinant human serum albumin
ROS	Reactive oxygen species
SA	Serum Albumin
S.c.	Saccharomyces cerevisiae
SD	Standard deviation
SMAC	Supramolecular activation cluster
STEAP3	Epithelial antigen of the prostate 3
sTfR	Soluble transferrin receptor
TCR	T cell receptor
Tfn	Transferrin
TfR	Transferrin receptor
Th	T helper cells
TNFα	Tumor necrosis factor
Treg cell (s)	T regulatory cell (s)
UTR	Untranslated region
ZAP-70	ζ chain associated protein kinase 70
β2m	beta-2 microglobulin
μg	Mikrogramm

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7.1. LIST OF FIGURES

- a. Lipinski, P., A. Stys, and R.R. Starzynski, *Molecular insights into the regulation of iron metabolism during the prenatal and early postnatal periods*. Cell Mol Life Sci, 2013. **70**(1): p. 23-38.
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A. ABSTRACT

Iron plays a pivotal role in cell biology. Iron bound to transferrin is delivered to proliferating cells via the transferrin receptor (TfR, CD71) and represents the classical iron uptake mechanism. Human serum albumin (HSA), the most abundant protein in human blood, is an important transporter of endogenous and exogenous components including scavenging of heme. We demonstrate in this study that addition of heme-HSA from human plasma or recombinant HSA, loaded with hemin *in vitro*, is sufficient to promote proliferation of human cell lines in otherwise serum- or protein-free medium. In contrast, HSA loaded with biliverdin, which is the heme-analogue without iron, or HSA loaded with lipids failed to induce proliferation in Jurkat T cells or in erythroblast TF-1 cells. In order to further elucidate if CD71 is involved in the binding heme-HSA to cells we used four murine anti-human monoclonal antibodies (mAbs) 5-528, 15-221, VIP-1 and 13-344, directed against 4 different epitopes on CD71, to inhibit binding of heme-HSA to Jurkat cells. Binding of heme-HSA was indeed inhibited with mAb 5-528 and VIP-1. Accordingly, mAbs against CD71 were able to block proliferation of Jurkat cells in response to heme-HSA. Binding of heme-HSA did not compete with TfR binding and vice versa. Moreover, uptake of HSA via CD71 is obviously also involved in the drug-carrier function of HSA as demonstrated in this study for the anti-cancer agent Abraxane. Abraxane is composed of paclitaxel surrounded by HSA nanoparticles and is a potent growth inhibitor. We could show in this study that the anti-proliferative capacity of Abraxane was diminished by CD71 mAb 5-528. In summary, the results of this thesis demonstrate that binding and uptake of heme-HSA via CD71 is an efficient and alternative mechanism to provide iron for proliferating cells. Our data further show that this novel pathway may be also involved in the drug-delivery function of HSA.

B. ZUSAMMENFASSUNG

Eisen spielt eine lebenswichtige Rolle in der Zellbiologie. Eisengesättigtes Transferrin wird durch den Transferrin Rezeptor in proliferierenden Zellen aufgenommen und repräsentiert den klassischen Mechanismus für die Eisenaufnahme. Das menschliche Albumin (Human serum albumin, HSA) ist das meist vorkommende Protein im menschlichen Blut, welches einen bedeutsamen Transporter für endogene und exogene Komponenten repräsentiert als auch eine zentrale Rolle in der reversiblen Bindung von Hämin spielt. In dieser Studie haben wir gezeigt, dass die Beladung von menschlichen Albumin als auch rekombinanten Albumin mit Hämin Molekülen im Gegensatz zu serumfreien oder proteinfreien Medien das Zellwachstum menschlicher Zellen fördert. Andererseits konnte HSA beladenes Biliverdin, welches das Gegenstück zu eisenfreien Hämin repräsentiert, als auch HSA beladene Fettsäuren das Zellwachstum in Jurkat T-Zellen oder in erythroblastischen TF-1 Zellen nicht fördern. Um in weiterer Folge zu erklären, ob CD71 an der Binding von Hämalbumin an Zellen involviert ist, verwendeten wir vier monoklonale Antikörper (mAk) 5-528, 15-221, VIP-1 und 13-344, welche gegen vier unterschiedliche CD71 Epitope gerichtet sind, um die Bindung von Hämalbumin an CD71 in Jurkat Zellen zu blockieren. Die Bindung von Hämalbumin wurde durch 5-528 und VIP-1 inhibiert. Dementsprechend wurde auch in Bezug auf Hämalbumin das Wachstum von Jurkat Zellen durch CD71 Antikörper gehemmt. Es wurde gezeigt, dass Hämalbumin als auch Transferrin nicht um dieselbe Bindungsstelle konkurrieren. Außerdem, ist es sehr wahrscheinlich, dass die HSA Aufnahme über CD71 im Transport von Medikamenten involviert ist, wie bereits in unserer Studie mit dem Anti-Krebs Medikament Abraxane dargestellt wurde. Abraxane besteht aus Paclitaxel, welches von HSA Molekülen umgeben ist, und ist ein wirksamer Hemmstoff für das Zellwachstum. Wir konnten in unserer Studie zeigen, dass die antiproliferierende Fähigkeit von Abraxane durch den CD71 Antikörper 5-528 vermindert wurde. Zusammenfassend kann berichtet werden, dass die Bindung und Aufnahme von Hämalbumin durch CD71 einen effizienten als auch alternativen Mechanismus darstellt, um proliferierenden Zellen Eisen zur Verfügung zu stellen. Weiteres zeigten unsere Daten, dass dieser neue Übertragungsweg ebenfalls an der Verteilung von Medikamenten mit Hilfe von HSA beteiligt sein könnte.

C. CURRICULUM VITAE

PERSONAL INFORMATION

NAME Jennifer Brell

NATIONALITY Austria

EDUCATION

12/2015 – 2016 Master thesis at the Institute of Immunology, Division Immune Regulation at the Medical University of Vienna

Since 10/2014 Master study of Molecular Microbiology, Microbial Ecology and Immunology at the University of Vienna

7/2014 - 8/2014 Bachelor thesis at the University of Veterinary Medicine, Vienna, Department of Microbiology

10/2010 – 8/2014 Bachelor study of Microbiology at the University of Vienna

16/6/2010 Matura passed with good success

2002 - 6/2010 Secondary school "Ella Lingens", Gerasdorferstraße, Vienna

1998 - 2002 Primary school, Rittingergasse, Vienna

SCIENTIFIC AND OTHER WORK EXPERIENCE

11/2015 Internship at Polymun Scientific GmbH, Klosterneuburg

9/2014 – 11/2014 Internship at Boehringer Ingelheim RCV GmbH & Co KG, Vienna

LANGUAGE SKILLS

German (native language)

English

Spanish (basic knowledge)