universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Analysis of the role of transferrin receptor (CD71) on dendritic cell differentiation and function“

Verfasser

Andreas Staudigl

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, im Oktober 2012

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Diplomstudiengang Genetik- Mikrobiologie (A441)
Betreuerin / Betreuer:	Univ. Prof. Dr. Thomas Decker

Meinen Eltern und

Großeltern gewidmet

DANKSAGUNG

Besonders bedanken möchte ich mich bei ao. Univ. Prof. Dr. Johannes Stöckl (Medizinische Universität Wien) für die Integration in seine Arbeitsgruppe am Institut für Immunologie, der damit verbundenen Grundsteinlegung für das Entstehen dieser Arbeit sowie der Führung durch die hierfür vergangenen Monate.

Ein weiterer Dank geht an Dr. Maria Seyerl für die Begleitung und Lehre praktischer Arbeitsmethoden, sowie zahlreiche wegweisender Ratschläge.

Da meine Arbeit auf Antikörper basierenden Methoden aufbaut, bedanke ich mich rechtherzlich bei Ass.Prof. Dr. Otto Majdic und Petra Cejka für die laufende Herstellung und Versorgung mit Immunglobulinen, sowie für nette Unterhaltungen fernab der Laborarbeit.

Hierbei möchte ich auch meinen Kollegen/Innen Mag. Alexander Puck, Mag. Madhura Modak und Regina Aigner für labortechnische sowie pausenfüllende Unterhaltungen danken.

Mein Dank gilt auch der Arbeitsgruppe um Priv.-Doz. Dr. Peter Steinberger, die mich in meiner Arbeit mit der Bereitstellung muriner Zellen unterstützt hat, Claus Wenhardt, der viele Stunden mit dem Messen meiner FACS Färbungen verbracht hat, sowie Margarethe Merio für die Zytokinauswertungen diverser Zellüberstände.

Ein weiterer Dank richtet sich an den Leiter des Instituts für Immunologie, Univ. Prof. Dr. Gerhard Zlabinger.

Besonders bedanken möchte ich mich bei meinen Eltern, die mein Studium ermöglicht, sowie mich all die Jahre davor und währenddessen bestmöglich gefördert haben und niemals den Abschluss meiner Ausbildung bezweifelten.

Ein Dankeschön möchte ich auch an meine Schwester richten, die vor schweren Prüfungen immer aufbauende Worte gefunden hat.

ABSTRACT

Iron is a pivotal element for energy production and cell growth. In addition, iron is an important regulator of the immune system. Endocytosis of iron-loaded transferrin via its receptor (transferrin receptor, TfR) is a major route for the delivery of iron molecules into the cytoplasm. Dendritic cells (DCs) are prominent antigen presenting cells and key regulators of adaptive as well as innate immune responses. Yet, little is known about the functional role of TfR (CD71) on DCs. Therefore, the aim of this study was to analyze the impact of CD71 on differentiation and maturation of human, monocyte-derived DCs with four monoclonal antibodies (mAbs) directed against CD71. All CD71 mAbs used in this study – mAb VIP-1, 5-528, 15-221 and 13-344 – strongly reacted with $CD71^+$-transfectants and with proliferating cells including human lymphoma cells, which are known to express high levels of CD71. Epitope analysis performed by cross-inhibition experiments showed overlapping binding sites for mAb 5-528 and mAb VIP-1, whereas mAb 13-344 and mAb 15-221 were found to be directed against distinct epitopes on CD71. Analyses of TfR expression during DCs differentiation revealed only low levels on immature DCs but a strong upregulation upon activation of DCs with bacterial products such as LPS. However, addition of none of the four CD71 mAbs modulated or inhibited either the differentiation of precursor cells into DCs or the maturation process, which was analyzed by the expression of informative cell surface receptors (CD1a, CD83), by the cytokine production (e.g. IL-12) or by testing the T cell stimulation capacity of DCs treated with CD71 mAbs. However, when mAbs VIP-1 or 15-221 were added directly to a mixed leukocyte reaction (MLR), both mAbs markedly reduced T cell proliferation. Since development and activation was not directly influenced by a strong down-regulation of CD71 receptors from the surface of DCs upon mAb binding, we hypothesized that mAb VIP-1 and mAb 15-221 might inhibit the proliferation directly via T cells. To further investigate this, we activated T cells via CD3 mAbs alone or in combination with CD28 or CD63. T cell proliferation was inhibited in the presence of mAb VIP-1 or mAb 15-221. Beside T cell proliferation, there was also a clear reduction in the

production of IL-10, IL -13, IL-17 and IL-22 cytokines by mAb VIP-1 or mAb 15-221, whereas secretion of IL-2 was only diminished in the presence of mAb 15-221. In summary, CD71 is seemingly not critical for DC differentiation and maturation but is important during DC induced T cell proliferation at the T cell side.

Zusammenfassung

Eisen ist ein lebenswichtiges Element für die Energieproduktion und das Wachstum von Zellen. Endozytose von Eisen beladenen Transferrin über den jeweiligen Rezeptor (transferrin Rezeptor, TfR) ist die wichtigste Route für die Aufnahme von Eisenmolekülen in das Zytoplasma. Dendritische Zellen (DZ) sind bedeutende Antigen präsentierende Zellen und Schlüsselregulatoren sowohl für die adaptive wie auch angeborene Immunantworten. Bisher ist noch wenig bekannt über die funktionelle Rolle von Transferrin Rezeptoren (CD71) auf DZ. Daher lag das Ziel dieser Studie auf der Untersuchung der Auswirkung von CD71 auf Differenzierungs- und Reifungsprozesse von menschlichen Monozyten abstammenden DZ mittels vier monoklonalen Antikörpern (mAk), welche an CD71 binden. Alle in dieser Studie verwendeten CD71 mAk – mAk VIP-1, 5-528, 15-221 und 13-344- interagierten stark mit $CD71^+$ - Transfektanten, sowie mit proliferierenden Zellen, einschließlich menschlichen Lymhoma Zellen, von welchen bekannt ist, dass sie eine hohe Anzahl an CD71 exprimieren. Epitop Analysen mittels Cross-inhibierungs Experimente zeigten überlappende Bindungsstellen für mAk 5-528 und mAk VIP-1, wohingegen mAk 13-344 and mAk 15-221 an unterschiedliche Epitope von CD71 binden. Untersuchungen der TfR-Exprimierung während der DZ Differenzierung ergaben nur eine geringe CD71 Protein Verteilung auf unreifen DZ, hingegen aber eine verstärkte Exprimierung nach der Aktivierung von DZ mit bakteriellen Produkten wie LPS. Dennoch, keiner der vier mAk modulierte oder inhibierte die Differenzierung von Vorläuferzellen in DZ oder beeinflusste dessen Reifungsprozess, was durch die Untersuchung der Exprimierung von Oberlfächenrezeptoren (CD1a, CD83) und Zytokin Produktion (z.B.: IL-12) überprüft wurde, sowie durch das Testen der Fähigkeit T Zellen zu stimulieren nach DZ Inkubation mit CD71 mAk. Dennoch, wenn mAk VIP-1 oder 15-221 zu Beginn einer gemischten Leukozyten Reaktion (MLR) hinzugegeben wurden, reduzierten beide mAk deutlich die T Zell Proliferation. Da weder Entwicklung, noch die Differenzierung beeinflusst wurde durch eine deutliche Unterdrückung der CD71 Exprimierung auf der Oberfläche von DZ aufgrund der Bindung von

mAk, vermuteten wir eine direkte Inhibierung der T Zell Proliferation durch mAk VIP-1 und mAk 15-221. Um diese Möglichkeit zu untersuchen aktivierten wir T Zellen mittels CD3 mAk alleine oder in Kombination mit CD28 oder CD63, was in Verbindung mit mAk VIP-1 oder mAk 15-221 wiederum zu einer klaren Inhibierung der T Zell Proliferation führte. Neben der T Zell Proliferation detektierten wir auch eine klare Reduktion in der Zytokin Produktion von IL-10, IL-13, IL-17 und IL-22 während der Inkubation mit mAk VIP-1 oder mAk 15-221, wohingegen die Sekretion von IL-2 nur mit mAk 15-221 reduziert wurde. Zusammenfassend scheint CD71 nicht entscheidend zu sein für die Differenzierung und Reifung von DZ, jedoch dürfte es eine wichtige Rolle in der DZ induzierten T Zell Proliferation spielen, besonders auf T Zellen.

Table of contents

1. INTRODUCTION

1.1. Functional aspects of iron molecules in the cellular environment

Iron belongs to the group of essential trace elements and plays an important role for the survival of each cell. Especially in mammalians, the probably most well-known function of iron molecules is connected with the transport of oxygen via red blood cells [1]. An important function of non-heme conjugated iron is based on the formation of electron transport chains (ETC) within the membranes of chloroplasts and mitochondria and the functionality of enzymes of the citrate cycle [15]. Coordination complexes consisting of metal ion-protein associations and their conjugated organic ligands are the main components of ETC and finally permit the production of ATP through the progression of redox reactions. These redox reactions are facilitated by the electron transfer between electron donor Fe(II) and electron acceptor Fe(III) complexes [1]. Other prominent members of non-heme metalloproteins form iron-sulfur complexes and find applications in regulatory processes of the gene transcription and in the stabilization of protein structures [6, 10]. Catalytically active proteins with iron components are also important for DNA synthesis through the action of ribonucleotide reductase enzymes [1, 4] and in the disarming of free radicals via different dismutases [16].

Especially in the ferric form (Fe(III)), iron also has harmful effects for cells [1, 2, 10]. Combined with unsaturated fatty acids, iron participates furthermore in the appearance of alkoxyl radicals [16]. Under aerobic conditions, iron oxidation processes catalyze the production of hydroxyl radicals (also known as Fenton and Haber Weiss-chemistry [1, 2, 10]). $OH^{\cdot}$ Radicals produced in Haber Weiss reactions are also described as “reactive oxygen intermediates“ (ROIs). Accumulation of ROIs leads to mutation in the bases of nucleic acids, lipid structures and protein composition [1, 7]. These destructive effects are selectively used by phagocytes to avert bacterial spreading within the host tissue. Monocytes and phagocytes produce active ROIs via the membrane anchored NADPH oxidase complex [1, 2, 27]. Beside bone marrow cells, hepatocytes, enterocytes and myocytes, reticuloendothelial macrophages

participate also in the storage of iron particles [1, 10]. Based on these facts, iron absorption is essential for the growth and segmentation of cells, but also carries the risk of damaging host body cells.

1.2. Iron absorption from food by the human duodenum and further transport through blood circulation

Human duodenum apical membrane cells assimilate daily 1-2 mg iron from food to serve essential functions of the body [1]. High iron concentrations are detectable in liver, muscle cells (myoglobin) and in bone marrow, where iron atoms are integrated into porphyrin rings during the production of red blood cells [1]. After food intake, ferrous iron atoms (Fe(II)) are bound by divalent metal transporter 1 (DMT1, also referred as Nramp2 or DCT-1) and guided through the duodenal epithelium [1, 10]. Structural analysis of DMT1 resulted in 12 membrane spanning proteins which allow the transport of various ions across the double layer membranes [16]. Enterocytes permit not only the storage of iron in form of ferritin but also the further transport into the blood stream via ferroportin-1 (also termed as IREG1 or MTP1) membrane transport-proteins [1, 5]. Ferroportin-1 is just as DMT1 a membrane spanning protein consisting of 10 domains within of the membranes [16].

An iron metabolism control hormone, hepcidin, gets produced in the liver and has negative regulatory functions for the ferroportin-1 transport mechanism via reduction of iron absorption [5]. Before the ferroportin-1 export starts, ferrous iron atoms are oxidized by ferroxidase hephaestin. This oxidation process results in a ferric iron state (Fe(III)) and permits its fixation at apo- (serum) transferrin (Tf) glycoproteins [5, 16].

Tf is composed of two subunits (N- and C- lobe) and each half can carry one iron molecule within a hydrophilic gap between the upper and the lower part of each lobe [7]. Both fragments are connected via 13 disulfide bridges [16]. Iron attachment into the gaps causes a conformational change within of Tf proteins and permits the transport through the blood stream into the rest of the body [7, 9].

Fixation of Tf to its receptor is assumed to occur at least at two locations, one C-lobe and one fixation point on the N-lobe [7]. The binding affinity of Tf to its

receptor is noticeably high [7]. The Tf coding sequences have been changed only to a small degree in the course of the evolutionary development among different species [7, 46]. Tf peptides have been highly conserved as single-chain iron transport proteins and production in hepatocytes is amplified [7, 16]. In general, there are three known Tf variants: Serum Tf has been found in various body fluids of mammalians, ovotransferrin is mainly distributed in avians and reptilians and lactoferrin proteins are components of saliva and milk [7]. A further membrane anchored homologous protein to the Tf group is called melanotransferrin (MTf). It has been discovered on surfaces of mammalian and avian cells and seems to be connected with the iron transport through the blood- brain barrier [16]. MTf binds also other metals beside iron and get translated through a different mechanism compared to the Tf protein production [16]. However, MTf is also known as melanoma tumor antigen p97 based on its overexpression especially on melanoma cells [8].

1.3. Transferrin cell surface receptor- structure and function

1.3.1. Transferrin receptor 1 (CD71)

The transferrin receptor 1 (TfR1) can be found on nearly all nucleated cells and is composed of two identical glycoprotein trans membrane subunits [6, 7]. For the order of primates seven homologous receptors are described [17]. Each subpart has a molecular weight of 90 – 95 kDa and both monomers are connected via disulfide adhesions [6]. The disulfide bridges are located in the extracellular receptor part directly after the cell membrane and include cysteine residues 89 and 98 [6, 7, 16]. Based on short cytoplasmic N-terminal tails, TfR homodimers belong to the type-2 trans membrane proteins. The large C-terminal ectodomain of both monomeric subunits interacts with the iron loaded-Tf complex in at least two amino acid moieties (Tryptophan^{641} and Phenylalanine^{760} [6]).

The extracellular receptor part exhibits a trypsine attackable region and can be displaced into the serum [7]. Hence it lacks cytoplasmic TfR tails and single pass transmembrane domains in soluble serum TfRs [3]. The functions of the soluble receptor components are not completely clear, but probably its effectiveness relies on regulatory mechanisms. Approximately one third of all

produced TfR proteins reside on the cell surface, the other parts remain in the cytoplasm within endosomes or are delivered into the circulation [3].

In the membrane bound variant, three N-linked glycosylation sites and one O-linked glycosylation site have been characterized in the C-terminal region [6, 7]. These areas are essential for the correct functionality of the receptor, including the correct folding mechanism. A short hydrophobic single-pass trans membrane domain connects the N-terminal and C-terminal part of the respective units (Figure 1) [6]. Each monomer of the TfR complex encompasses 760 amino acids [9]. The cytoplasmic component of the receptor complex comprises the internalization sequence (2OYTRF23) that is required for endocytotic iron absorption mechanisms [16]. TfRs bind not only Tf but also HFE, a protein complex associated with the control of iron absorption [16]. The posttranslational regulation of receptor expression is further associated with the assembly of iron storage proteins and assures increased surface expression during low iron levels within the cytoplasm (Figure 3).

Beside the ingestion of metal ions, TfRs exhibit also regulatory functions for the immune system by attaching IgA1 antibodies as well as in the interaction with antigen receptors of immune cells [6, 24].

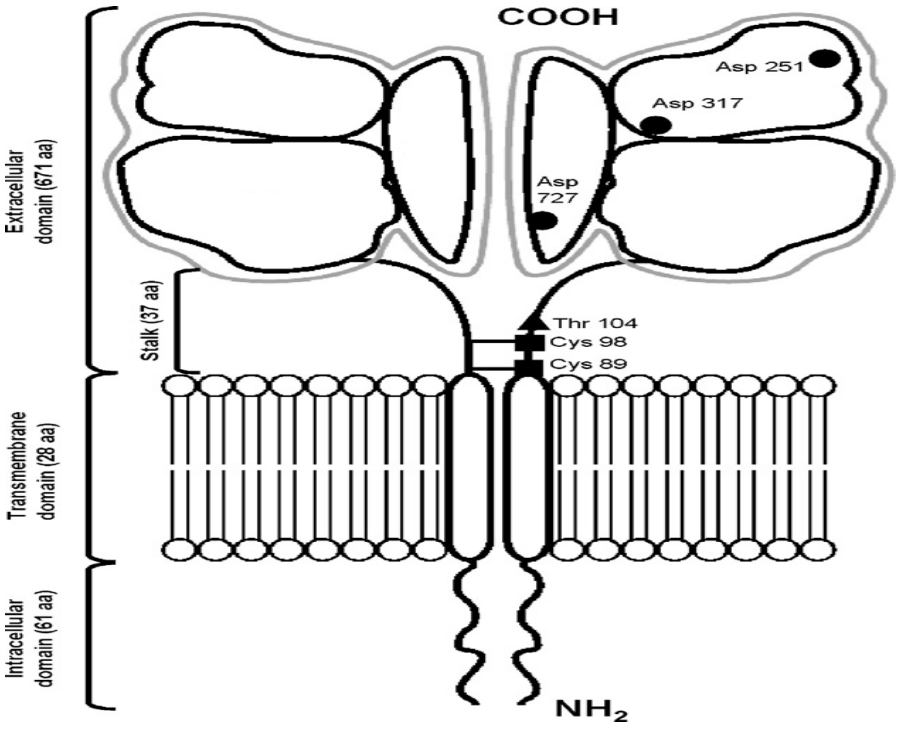

Figure 1: Structural composition of the human transferrin receptor 1 (TfR1). The homodimer is joined together via disulfide bridges arising from two cysteine residues at the

beginning of the extracellular domain. The large extracellular C-terminal domain is further categorized into a protease-like, apical and helical part. The latter one forms a binding cleft for iron loaded Tf proteins. Asp251, Asp317 plus Asp727 exhibit N- linked glycosylation sites (black circles) and Thr104 an O- linked gycolysation site (black triangle). All glycosylation sites are probably important for the correct shape of the receptor, but definitely for the internalization process. The extracellular domain can be cleaved in the stem area and delivered into the circulation. Each monomer encompasses 760 amino acids. Figure is adapted from Daniels et al. [6]

1.3.2. Translation of the TfR

The translation of the TfR is coupled to the actually available iron concentration of the cell. In humans, both TfR and Tf are coded on chromosome 3 [3, 16]. The 3` untranslated region (3`UTR) of the TfR mRNA includes 5 hairpin stem loop structured iron response elements (IREs) [6, 7]. The translation step of the IREs is inhibited by loop structures. Even if iron regulatory proteins (IRPs) bind to IREs, the loop structures are pulled apart and the TfR mRNA is available for the translation [1, 7]. The same regulatory system also plays an important role for the translation of DMT1 proteins [16]. IREs contain a 28-nucleotide consensus sequence whereas the remaining mRNA structures are species specific [16]. IRP-1 and IRP- 2 are the key proteins concerning the sensing of the intracellular iron concentration [10, 16]. IRP-1 enzymes are constructed of 4Fe-4S clusters and manifest sequence identity with the mitochondrial aconitase, which in its active form catalyzes the rearrangement of citrate into isocitrate [6, 10]. Low concentrations of iron atoms avoid the function of these enzymes and IRP-1 proteins bind to IREs. This process leads to the stabilization of the TfR mRNA and to its translation [1]. IRP-2 also binds TfR mRNA, but exhibit no catalytic activity [16]. Its sensing effect depends mainly on the oxygen and iron concentration and is neutralized in cells with low iron concentration via proteosomal degradation [1, 6]. Based on trials with IRP-1 deleted mice, it is assumed that IRP-2 may counterbalance IRP-1 deficiency [7]. Both IRPs equally act in the translation of ferritin iron storage proteins but contrary to the TfR mRNA, IRP- 1 and IRP- 2 prevent the translation start up to of the 5`UTR region [6].Synthesis of TfR1 occurs in the endoplasmic reticulum [7]. Oxidative stress, especially in form of extracellular H_2O_2 or nitric oxygen, has also positive consequences for the activation of IRPs and leads to its binding not only at IRE

sequences on the mRNA of TfRs, but also of ferritin [10]. Hence oxidative stress leads to stronger expression of TfRs but also to reduction of iron storage proteins whose IRP mediated translation is contrary regulated to TfRs [11, 16].

1.3.3. Hereditary hemochromatosis protein (HFE) allows the negative regulation of iron absorption

HFE participates in the regulatory mechanism of iron absorption by occupying the CD71 binding sites [16]. The hereditary hemochromatosis proteins (HFE) show a similar structure to the major histocompatibility complex 1 (MHC I) including the β2- microglobulin (β2m) structure [6, 9]. On the cell surface, it blocks the binding sites for Tf proteins within the helical domain and does not dissociate until the pH becomes acidified in the newly formed endosome [7, 9]. Hereditary hemochromatosis is a well-known defect in the HFE gene. In this case HFE proteins are not capable of inhibiting the permanent iron absorption and result in tissue and organ damages. However, there are also hemochromatosis diseases known independently of HFE gene mutations [16].

1.3.4. Transferrin receptor 2

LikeTfR1, TfR2 is also a type II transmembrane receptor [17]. Compared to TfR1, the translation process of TfR2 is not controlled via the attachment of IRP enzymes to IREs [6]. At the transcription level, two different receptor variants are produced by alternative splicing. TfR2α exhibits similar C- terminal ectodomains compared to TfR1, but completely different cytoplasmic and trans membrane regions [6].It is mainly distributed on liver cells and seems also to be important for the development of erythrocytes [7]. In TfR2β proteins, the trans-membrane and the cytoplasmic regions are completely absent [6]. The internalization sequence of TfR2α exhibits also slight modifications [16]. Tissue cells express higher levels of TfR1 proteins than TfR2 receptors, but it seems to play an important function in the development of liver tissue and erythroid cells [7]. Iron loaded Tf has a significant higher binding affinity for TfR1 than for TfR2 [7]. Higher levels of TfR2 are detectable in enterocytes of the small intestine and in hepatocytes [6]. Mutations in TfR2 proteins cause a HFE independent hemochromatosis variant and lead to the suspicion that iron excess charge is

related to TfR2 damage and that TfR2 exhibits important functions in the iron circulation [7, 17].

1.4. Receptor mediated iron absorption and transport into the cell interior

Shortly after the reduction of Fe(III) via duodenal cytochrome b (Dcytb), Fe(II) is gated into enterocytes via DMT1, again oxidized by the action of hepaestin and exported into the circulation via ferrroportin 1, where Tf proteins integrate up to two iron molecules for the transport through the circulation [1, 7]. Ferroportin 1 mediated exportation of iron molecules after passing the enterocytes is also employed by macrophages [10, 15]. In both cell types, the export process is negatively influenced by hepcidin, a hormone produced by liver cells [10]. Tf proteins charged with two iron molecules offer a higher receptor binding affinity than just single charged transport glycoproteins [6]. Based on the restructuring processes after ion attachment, Tf components without linked iron molecules bind not or only marginally to the receptor and are not integrated into the cell interior [10]. Absorption into the cell interior of Tf conjugated non-heme iron molecules is facilitated by their interaction with cell surface TfRs [1, 6, 9]. The Tf-TfR complex is integrated through endocytosis and clathrin- coated pits constitute the envelope of the newly forming endosome [6]. Beside the declining pH (from pH 7.4 on the cell surface down to pH 5.5) in the endosomal environment, the interaction between amino acid residues Tryptophan^{641} and Phenylalanine^{760} on the C-terminal ectodomain of the receptor as well as Histidine^{349} remnant on the Tf molecule itself causes a structural change of the Tf glycoproteins and enhance therefore the dissociation of the Fe(III) atoms [6, 9, 10]. Free ferric iron molecules are repeatedly reduced to Fe(II), exported from the endosome in a DMT1 dependent manner and as a result arrive at the cytoplasm of the cell. One part of the non-heme iron atoms are integrated in diverse enzymes such as in aconitases, cytochromes or RNA reductases [7]. Another part is kept in iron storage cells. Unconjugated Tf (Apo- Tf) and its receptor remain bound in the endosome and return in a further step back to the cell surface. Dependent on the higher pH (pH 7,4) at the cell surface, apo- Tf dissolves from the receptor and enters again the circulation [7]. The course of

one cycle needs approximately 10 minutes [3]. Receptor inclusions into endosomes occur often also without conjugated Tf and indicate that there is no correlation between the conjugation of Tf to its receptor and the internalization process [6, 13, 14].

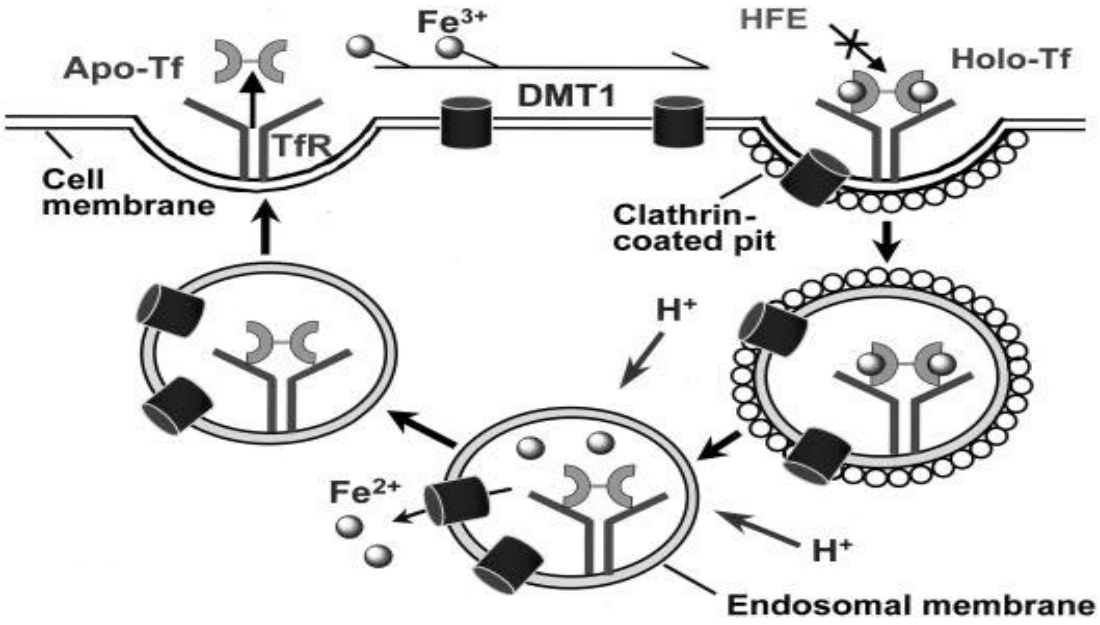

Figure 2: Each Tf protein transports maximally 2 iron atoms and is integrated into the cell via linkage to the TfR. HFE proteins compete with Tf for the TfR binding sites. Endocytosis occurs by the accumulation of clathrin coated pits on the inner cell membrane around the receptor integration area. The newly formed endosome is acidified via the influx of H^+ atoms, whereby the Tf molecules change their shape. Freed iron molecules are gated through the endosomal membrane via DMT1 proteins and cytoplasmic iron atoms are integrated into various enzymes or stored in ferritin protein complexes. Receptor and Tf remain conjugated and return back to the cell surface, where both components dissociate again. Schema is adapted from Qian et al. [7]

1.5. Cell internal iron storage via ferritin complexes

Most of the assimilated iron is not required immediately. Iron storage mechanisms have evolved evolutionary to prevent an excess of the harmful metal molecules within the mammalian body. Iron storage proteins, also referred as ferritin, form complexes with iron atoms and are accumulated mainly in enterocytes, hepatocytes and reticuloendothelial macrophages [10, 15]**.** Altogether the whole protein complex encompasses 24 subunits [1, 16]. The translation of the Ferritin protein complexes is based on the same principle as

mentioned before for the expression of the TfR, but contrary to the receptor translation, the functional IRP-1 enzyme based on iron integration into the enzyme complex prevents its binding to the ferritin mRNA and permits therefore the translation of ferritin proteins (Figure 3) [1, 6, 15]. Point mutations especially in the IREs of the L- ferritin lobe result in the development of diseases, such as hyperferritinemia [16].

Each ferritin complex can retain up to 4500 iron molecules [16]. Ferritin exists also in the cytoplasm in glycosylated form and exhibits higher levels during inflammatory processes [16].

Comparable to the opposite consequences of iron molecules on the translation process of TfRs and ferritin proteins, cytokines show comparable effects. Most of the ferritin translation enhancing cytokines exhibit simultaneously inhibitory effects on the expression of receptor proteins and vice versa [15].

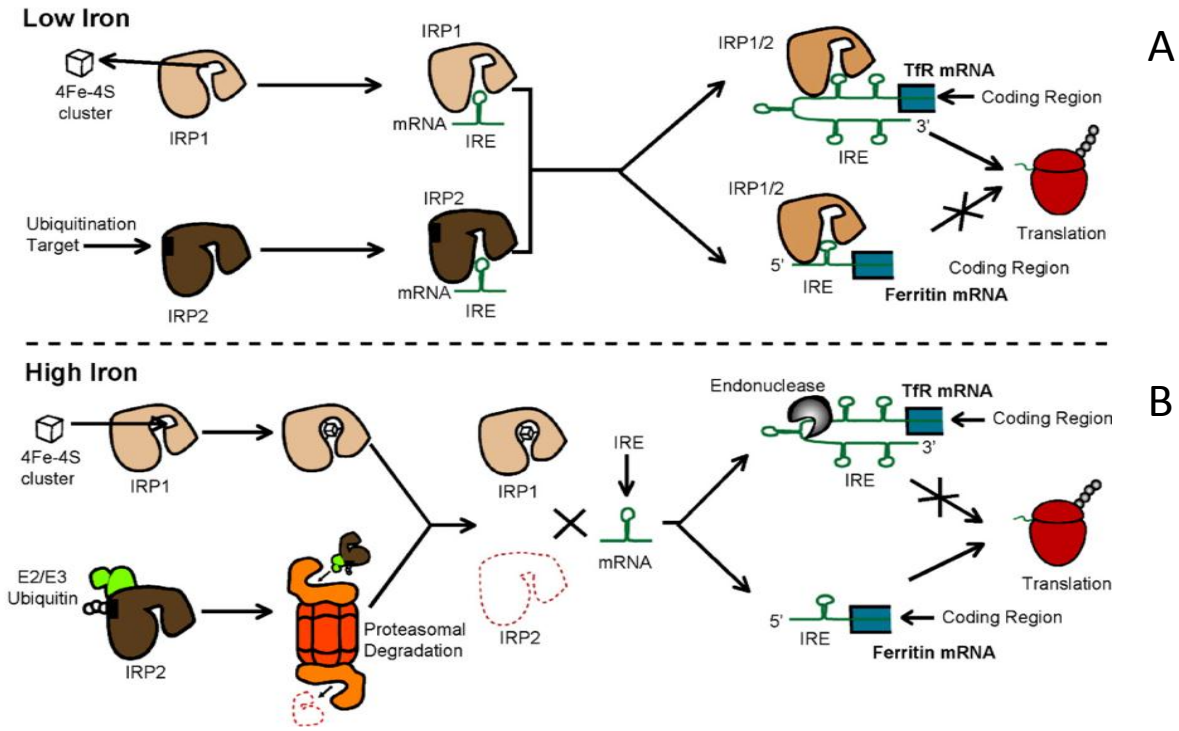

Figure 3: The regulatory mechansims of the translation of Tf and ferritin mRNA is based on the same principle, but ends up with opposite results. For the translation of both protein variants, iron regulatory proteins (IRP) bind to iron regulatory elements (IRE) in the 3`and / or 5`UTR of the TfR and ferritin mRNA. A) Low intracellular iron concentration leads to the dissociation of iron sulfur complexes from IRP-1 plus to the synthesis of IRP-2. IRPs bind to IREs in the 3`UTRs of the TfR mRNA as well as to IREs in the 5`UTR of the ferritin mRNA.

Binding of IRP stabilizes stem loop structures in the TfR mRNA und permit protein synthesis. On the other hand IRP attachment to IREs in the 5`UTR of the ferritin mRNA prevents its translation. B) Increasing intracellular iron concentration leads to a functional aconitase- like enzyme and inhibits the function of IRP1 proteins. Iron leads equally to the ubiquitination and consequently to the destruction of IRP2 proteins. Therefore the TfR translation remains inhibited through stem loop structures in the 5`UTR, whereas the coding regions of ferritin mRNA is available for its translation. Figure is adapted from Daniels et al. [6]

1.6. Iron has important immunoregulatory functions

The expression of Tf coupled with the iron absorption system has further important functions in the establishment and regulation of the mammalian immune system. In TfR attachment experiments Moura et al [18] observed an increased binding affinity for monomeric IgA1 antibodies in epithelial cells and mesangial cells. This antibody specificity may allow an immunoregulatory capacity beside the iron transport mechanism [6]. The absorption of iron is necessary for the activation process of nearly all immune cells. Therefore it is assumed that TfRs participate in the activation events of B- and T- cells [18-21] and participate further in the maturation process of DCs [5]. Especially for the T cell activation it is supposed that TfRs potentially exert influences on the signal transduction of the T- cell receptor (TCR) by the direct interaction on the ζ-chains and participate actively in the construction of immunological synapses [22].

1.7. Dendritic cells and their function in the human immune system

Dendritic cells (DCs) belong to the important group of antigen presenting cells (APCs) of the mammalian innate immune system [23]. The function of APCs relies on the recognition and phagocytosis of foreign peptides as well as in the detection of damaged self- tissue. Antigen detection and admission occur via C-type lectin receptors (mostly langerin or mannose receptors), scavenger receptors and most often via Fc and pattern recognition receptors [23, 24]. DCs are categorized into the innate immunity, but they are also characterized as important transitional immune cells based on their ability to activate cells of the adaptive immune system. Phagocytic peptides are processed and loaded onto

MHCs to afford their presentation to naive T- cells [24]. Classical peripheral DCs emerge from bone marrow precursor cells, mostly from $CD34^+$ hematopoietic stem cells inside of bone marrow [25]. Dendroid appendages facilitate the capture of possible pathogens. Antigen catching occurs by the recognition of evolutionary conserved molecular patterns, also described as pathogen- associated molecular patterns (PAMPs). Detection of PAMPs is performed by pattern recognition receptors (PRRs) [23]. Absorption of antigen leads to the startup of a standardized programme, which includes the maturation to professional APCs resulting in a strong expression of MHC class II molecules as well as to the strong expression of costimulatory B- and T- cell activation proteins (CD40, CD54, CD58, CD80, CD86) on cell surfaces [5, 23]. In the same context, DCs start to migrate to secondary lymphoid organs to allow an efficient antigen presentation [23]. Receptor mediated antigen recognition additionally activates signal transduction pathways for the initiation of cytokine production [23]. Functional aspects of cytokines on other cells are discussed below. In vitro studies often use $CD14^+CD11c^+$ blood monocyte derived DCs. $CD14^+CD11c^+$ monocytes can be kept in liquid nitrogen for a long time period and monocyte derived DCs are obtained five days after treatment with GM-CSF and IL-4 [5, 25]. Actually two more main groups of dendritic cells are defined: Plasmacytoid DCs are important type 1 interferon producers and are specialized for the defense against viral infections, whereas Langerhans cells (LC) are positioned in epithelial layers to absorb fast infiltrating pathogens [23].

1.8. Iron molecules are important cofactors for dendritic cell maturation

Analysis of freshly isolated monocytes demonstrated only hardly detectable numbers of TfRs on the surface [24, 27]. Cultivation in an iron containing medium leads to an increase of the receptor proteins within of 3 days and is in contrast to the previously described TfR expression regulatory mechanism for proliferating cells [4]. Expression of higher protein levels of TfRs and DMT1 leads to the accumulation and storage of iron molecules within the DCs. TfR and DMT1 proteins are oppositely regulated to low expressed Ferroportin-1, the

iron export molecule [5]. Ferroportin-1 appears up to 70% lower in DCs than in other cells [5]. This protein arrangement is intensified during the maturation process to professional antigen presenting cells (APCs) [5]. The variety of stimuli seems not to be important for the up- regulation of CD71 proteins. Pasquier et al. reported 2004 [24] that TfR proteins on the surface of DCs achieve additional functions in the detection of IgA immunoglobulins and mediate the endocytosis in immature and activated DCs. Dimeric and also polymeric IgA is delivered in an amplified fashion by plasma cells mainly residing in the intestinal laminia propria, transported via the blood stream and further emitted by mucosal cells to detect antigens on mucous membranes [26]. The monomeric variant of this immunoglobulin isotype (IgA1) predominant within the human body is frequently encountered in blood serum and is recognized by CD71 and other receptors [24].

1.8.1. Macrophages belong to the iron storage cells

Beside enterocytes and hepatocytes, monocyte derived macrophages are the most important iron storage cells [1, 10]. Therefore macrophages express mostly high levels of surface TfRs. However, macrophages gather most of the iron from the destruction of senescent erythrocytes [2, 10]. Macrophages fulfill this process more efficient than monocytes, which is also referred as erythrophagocytosis. Most of the recovered iron molecules are integrated into protein complexes (hemoglobin, myoglobin or Tf) and are exported back into to the cytoplasm [2]. Macrophages and also many other immune and non-immunogenic cells of mammals express additional to TfRs also lactoferrin receptors [15]. Lactoferrin also belongs to the Tf family and offers another possibility also for iron absorption. Lactoferrin has additional regulatory functions for the augmentation of NK- cells, lymphocytes and monocytes and supports the immune system to prevent the spreading of bacteria [2, 7].

Beside the toxic components of iron molecules, the excessive charge has also negative effects for the immune function of macrophages, because it blocks IFNγ- mediated pathways [2, 15]. For that reason the production of NO, the secretion of IFN-α as well as the translation of MHC II proteins does not work [2, 15]. An increased iron concentration in the body affects also the production of

granules through neutrophile granulocytes and monocytes based on a malfunction of the inducible nitric oxide reductase (iNOS) [1, 15]. Malfunction of the regulatory system for the iron absorption diminishes therefore the defense mechanism for the killing of invaders. Similar to the previous described effects of cytokines referring the TfR and ferritin storage protein translation, IFN-γ pretreatment of macrophages prevents the surface appearance of TfR on cell surfaces as well as the translation of ferritin proteins [28, 29]. Cytokines derived from the T_H2 subgroup neutralize the effect of IFN-γ treated and activated macrophages and enforce the iron absorption and attachment to ferritin proteins [15].

1.8.2. Tf and TfR independent iron absorption mechanisms

Experiments demonstrated a sufficient iron supply in a Tf free cell environment particular for liver cells, HeLa and Raji cells [64]. Beside the conventional iron absorption with CD71, endocytosis of iron molecules without intermediate TfR proteins are assumed for cells of healthy tissues, e.g. for reticulocytes and hepatocytes [66]. Melanoma cells express p97 proteins similar to Tf and are known to gate iron molecules into the cell [65]. The integral membrane protein SFT is also supposed to transport iron into the cell interior and further to stimulate its uptake via Tf dependent and independent mechanism [7, 16]. Furthermore experiments with B Cell lymphoma have shown that cells incubated in a Tf- free, but iron salt containing medium exhibited no effects on the iron dependent cell growth [64]. The alternative iron absorption system seems to permit also the uptake of other metal elements and is strongly expressed at low iron salt concentrations [64, 68]. Previously studies on fibroblasts demonstrated an increasing concentration of intracellular Ca^{2+} level after binding of transition metals to its belonging receptors. Therefore Kaplan and collegues [68] assume that strong intracellular Ca^{2+} concentration may influence the intensity of the non- iron transport mechanism. Further observations of various cell types showed also a temperature and time dependency for the alternative iron absorption [64, 68]. Actually some models attempt to explain the iron absorption mechanism independently of Tf conjugation or receptor attachment [64-66]. However, the mechanisms of Tf or TfR independent iron absorption are not completely understood and seem to

depend on the respective cell type. Yet it is obvious that iron uptake exclusively via Tf independent mechanisms alone are insufficient to sustain erythropoiesis and important cellular functions within the mammalian body [16].

1.8.3. Iron influences the progression of infections and cancer diseases

The intracellular iron concentration affects the mammalian immune system in many ways. A low total iron concentration causes an uncontrolled development on B and T lymphocytes, especially in the proliferation step between the already functional first receptor chain and the incompletely arranged second one [15]. The missing proliferation step results in minimal or only in a strongly limited number of antigen specific immune cells [20, 62]. Further degradation of the immune system concerns the defense mechanism of phagocytes, macrophages and monocytes. Iron is necessary for the function of the NADPH complex to produce ROIs [1, 2, 15]. Iron deficiency affect also the activation process in most of the other immune cells. On the other side it is known that iron loaded macrophages lose their killing function based on the transcriptional inhibition of iNos as well as their qualification of cytokine producing cells [2, 15]. The strongly weakened immune system makes it easier for invaders to propagate and to spread in the whole host. On the other hand, bacteria and other extracellular invaders, except for viruses, are equally dependent on the iron concentration and are therefore also inhibited in their growth [2].

Increased iron concentrations offer invaders an optimal initial point for the assimilation of this trace element. The immune system developed various strategies to remove iron and to inhibit the growth of pathogens. One mechanism relies probably on the strong production of Hepcidin, the liver derived hormone, which prevents the iron export from enterocytes and macrophages by interacting with ferroportin 1 proteins [10]. High iron concentration affect the immune system in a similar way, lead to malfunctions of many immune cells and furthermore to a strong adjustment of the T_H1 and T_H2 cytokine dispersal [15]. Both low and high iron concentration reduce the monitoring function of the immune system concerning diverse invaders but also in regard of malfunctions of host body cells [15]. The reactivity of unconjugated iron molecules to form hydroxyl radicals and the increased availability for the

integration in enzymes of proliferating cells combined with a suppressed immune system probably enhances the formation of diverse cancers [15].

1.8.4. Therapeutic application linked to the iron absorption mechanism

Since the end of the last century, the TfR was considered for use its specific form of transport in the drug therapy for the treatment of different diseases, especially in cancer therapy. Already in 1981 Trowbridge and Domingo [69] observed inhibitory effects on the growth of human lymphatic tumor cells by the application of Ricin A conjugated mAb B3/25 (IgG isotype) directed against the TfR. Further experiments showed a strong tumor cell growth inhibition by the utilization of mAbs alone or in combination with other mAbs directed against TfR and led to the assumption that iron deprivation is the most effective mechanism to reduce tumor growth [49, 72]. A notably inhibitory effect has been achieved via the application of mAb 42/6 (IgA isotpye) that is assumed to compete efficiently for the Tf binding site on the external part of the TfR [70, 71] or to cross- link several surface receptors [72]. Both mAb variants reduce Tf binding sites, but only mAb 4/25 efficiently inhibits the receptor dependent uptake of iron-Tf complexes [73].

Other trials are based on the fact that Tf also carries metal ions different from iron, such as diagnostical, therapeutic (BI^{3+}, Ru^{3+}, Ti^{4+}), platinum complexes or toxic metal ions [7]. In the circulation of the human body, only 30% of the human Tf proteins are charged with iron and the remaining proteins are available to bind different metal ions. The biggest benefits of drug transport via TfRs rely on the non-immunogenic and nontoxic characteristics of the protein components as well as the high appearance on rapidly and often proliferating cancer cells [7]. Further approaches for more site specific delivery of pharmaceutical products are based on the conjugation to the Tf protein complex. Chemical linkage and protein engineering are two promising methods to assure the directed delivery of drugs into the cell interior [7]. The Tf protein is also a promising tool to insert DNA sequences into target cells [7]. The transfection via Tf mediated endocytosis is neither toxic nor infectious contrary to some of the common transfection methods. Probably most approaches in the

cancer therapy referring to this use the linkage of mAbs directly on surface epitopes of TfRs [7].

1.9. T- cell activation via APCs

T cell precursor cells are produced in the bone marrow and arrive in the thymus for maturation. During the developmental steps in the thymus, T cells are also called thymocytes [23]. Mature T cells are categorized into the 2 main groups based on the different expression of coreceptors, which are necessary for the activation process [86]. APCs evolve equally 2 MHC variants to permit the interaction with the main groups of T cells. MHC molecules exhibit highly polymorphic antigen binding sites and affix to only one specific antigen combination specific [23].

Differentiated $CD4^+$ effector T cells are especially required for the activation of diverse immune cells such as B cells, macrophages and $CD8^+$ T lymphocytes through the generation of diverse cytokines and chemokines [2, 23]. Based on specific cytokine profile and the effects of the soluble proteins on the total immune system, $CD4^+$ T helper cells are categorized in further subgroups. Blocking studies of the TfR via mAbs on the two main $CD4^+$ T cell subgroups exhibit a clearly easier and stronger inhibition of the T_H1 line as for the T_H2 subgroup [2]. The second major group of T effector cells express $CD8^+$ costimulatory surface molecules conjugated to the T cell receptor (TCR) and are specialized to prevent the spreading of intracellular pathogens via secretion of cytotoxic granules [23]. The recombination process of genes required for various TCR chain building, the translation of the two mentioned main receptor types and the earlier positive and negative selection process to avoid self-reactivity are described elsewhere [23]. Mature, but naive T cells migrate to secondary lymphoid organs such as lymph nodes. Already antigen stimulated DCs encounter naive T cells in corresponding zones of lymph nodes, where APCs start to present antigens via MHC molecules [30]. Consequently activated T cells start to migrate to the peripheral tissue, where they achieve their respective effector function.

Interaction between APCs and T cells occurs at distinct surface areas, which are directly connected with the cell interior via adapter proteins [23]. The immunological synapse (IS) on T cells is formed by two “supramolecular

activation clusters" (SMACs) [30, 32]. The central SMAC (cSMAC) contains the TCR complex in combination with the respective coreceptor and additional costimulator proteins. Expression of costimulatory molecules on both APC and DC surfaces are required for the initiation of T cell responses additional to the recognition of processed antigen [23, 30, 31]. The peripheral SMAC (pSMAC) contains integrin ligands for sufficient adhesion forces between APCs and T cells such as LFA-1 and ICAM [31].

Resting T lymphocytes express only a limited number of surface TfRs. This number is strongly increased after detection of mitogen stimuli [31]. T lymphocyte activation is associated with IL-2 receptor and cytokine production which results in the clonal expansion of specific T cell clones. Neckers and Cossman [20] figured out that surrogated components for monocytes with or without additional mitogen activating substances stimulate T cells to manifest TfR expression one day after IL-2 cytokine receptor occurrence. IL-2 detection by type I receptors is required for the appearance of TfRs on the cell surface [20]. In the natural environment, the interaction between TCR and MHC complex combined with costimulatory surface molecules triggers the translation of IL-2 genes [21]. Inhibition of TfR expression by mAbs resulted in weakened cell growth probably based on nonfunctional DNA synthesis during the S-phase of the cell cycle [20]. Blocking of DNA replication based on iron deficiency can be explained by the replication machinery whose enzymes require iron for its functionality. Further analysis demonstrated a direct context of TfRs with the cytoplasmatic signal transduction motifs of ζ- [22] and / or CD3 chains [31] associated with the TCR complex. The appearance of surface TfR in ISs is coupled to the TCR. During the formation of the IS, surface TfRs migrate to the pSMAC while TCRs remain in the cSMAC [23, 31]. Inhibitory antibodies targeted on the Tf attachment site on surface receptors which are migrated to the pSMAC show a clear negative effect on the formation of the IS. Furthermore TfRs in endosomes are accumulated directly in front of the forming APC- T cell contact site and integrate probably into lipid rafts of the cSMAC [31].

Already in 1988 it was reported that CD71 mAbs FG 1/5 and FG 1/6 bind to TfR of NK cells in a different area than the Tf binding site [87]. Concerning the T cell activation process there is only a marginally reduction detectable for mAb FG

1/5 [31]. The weak reduction is in contrast to the strong inhibitory effect for the formation of ISs by mAb FG 2/12 that compete with transferrin for the binding site [31, 88]. Anyway addition of mAb 1/6 lead to T cell activation, tyrosine phosphorylation by TfRs [22] is detectable and probably activate costimulatory molecules [31]. The observation of TCR ζ and CD3 chain tyrosine phosphorylation by TfRs showed that TfRs participate in the communication with the TCR [31]. Furthermore, mAbs inhibiting the Tf binding to their receptors inhibit synapse formation potentially through the overlapping of a special sequence area proximate to Tf binding site, which may offers an important function for the generation of IS. T cell treatment with mAb FG 2/12 results in the reduction of Lck recruitment and therefore to a nonfunctional signaling through CD3 chains [31]. Lck belongs to the Scr family of Protein tyrosine kinases (PTKs) and is associated with the TCR complex [33]. It offers important roles in the T cell activation via tyrosin phosphorylation of Immunoreceptor tyrosine-based activation motifs (ITAMs) on basis of CD3 and ζ chains (Figure 4B) [23]. Therefore it is assumed that TfR may possess important signal transduction functions already before IS formation and participates actively in its formation process [31]. Beside the homologous heterodimers of the cytoplasmic CD3 receptor parts, both ζ chains transmit signals from TCR to activate transcription factors mostly of cytokine genes [23].

TfR surface receptors are particular strongly expressed to begin of the physical contact between APC and TCR and associate with ζ- chains [22]. By reason that PTK Zap70 is activated after tyrosine phosphorylation of TCR ζ- chains, Salmerón et al. [22] showed that active TfRs exhibit initiating effects on the TCR signal transduction into the cell interior. Direct stimulation of TfR showed comparable signal transduction effects as described for TCR activation via antigen [22].

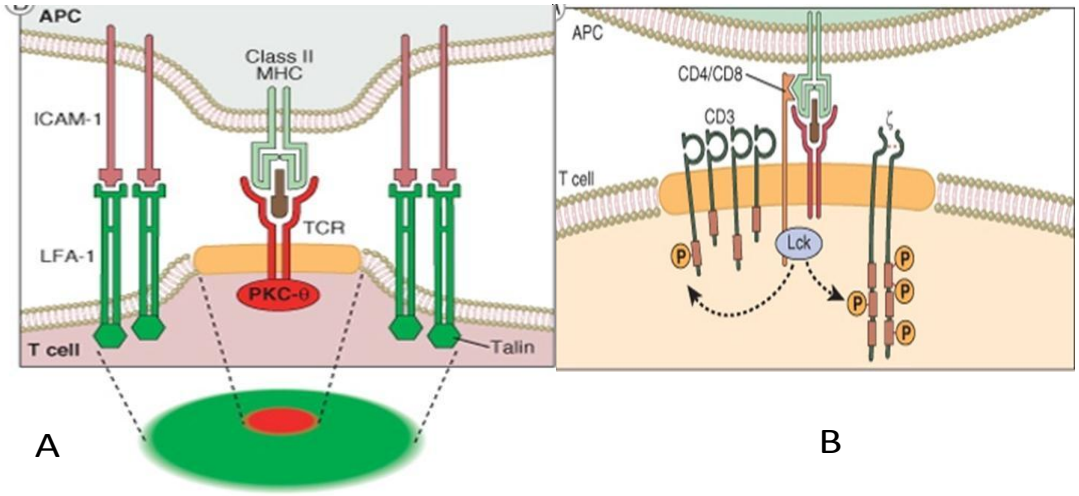

Figure 4: The interaction between APCs and T cells require the formation of immunological synapses (IS). Formation of IS requires the participation of TfRs probably in signal transduction processes. A) Migration of surface TfRs into the periphery (green circle) as well as the integration of previous endosomal TfRs into lipid rafts in the cSMAC (red circle) are essential for the formation of the IS and further for the signal transduction via CD3 chains. B) TfRs are probably necessary for the recruitment of PTK Lck to the TCR and in the next step for the tyrosine phosphorylation of CD3 and ζ chains. Adapted from [23]

1.10. T helper cell differentiation into T_H1 and T_H2 subtypes is also influenced by cellular iron concentration

Based on the variance of their effector function, mammalian T helper cells are classified into T_H1, T_H2, T_H17 plus regulatory T cells [34, 35]. T_H1 cells secrete mainly IFNγ and TNFβ and activate thereby not only macrophages of the innate immunity [15, 35]. Contrarily the T_H2 subgroup enhances via cytokines IL-4, IL-5, IL-10 and IL-13 production the B cell isotype switch to IgE secreting plasma cells and additionally represses the effector function of macrophages [15, 35]. The cell subtype differentiation occurs early in the development and is dependent on the respective predominant cytokine environment. The exposition to IL-12 or INFs during the $CD4^+$ T cell development advances the differentiation to the T_H1 lineage, while IL-4 cytokines induce the determination into the T_H2 subdivision [35]. It is further assumed that iron influences the specialization of T helper cell subdivisions. Iron unloading studies via mAbs directed against the TfR verified a stronger iron requirement for the development of the T_H1 subgroup than for T_H2 cell line [15].

1.11. B lymphocyte growth requires the expression of Tf surface receptors

Similar to T cell proliferation, TfR accumulation on B cell surfaces is also required for B cell propagation. Resting B lymphocytes express only a low number of TfRs. Stimulation with B- cell growth factors (BCGFs) plus T cell derived mitogen stimuli result in increased fusion of TfR loaded endosomes with the plasma membrane [62]. Inhibition of iron ingestion by the application of monoclonal antibodies directed against the Tf binding sites prevent the DNA copy mechanism probably in the same way as already described for T cells. Production and disposal of diverse antibodies require stimuli by mitogen proteins but seem not to depend on the iron assimilation process [62]. TfR knock out assays in mice hypothesize that iron plays an important role already in early steps of the erythropoiesis and lymphopoiesis [63]. Iron deprivation after Immunoglobulin heavy chain expression during B cell development leads in some cells to functional IgM^+ antibodies [63]. However, only a limited number of B lymphocytes were allowed to mature to effector cells and is probably associated with deficient proliferation of precursor B cells. Nevertheless, the strongest effect of mural TfR deletion was noted for T cell processing, because thymocytes lacking iron atoms arrest the development between the triple negative and the double positive growth stage [63].

1.12. Cytokines- communication between immune cells

Cytokines are soluble proteins produced by both cells of the innate and adaptive immunity and play a major role in the host defense. During an infection process, cytokines are necessary to stimulate lymphocyte proliferation and mediate the activation and conversion into effector cells as well as the reconstitution of natural B- and T- cell homeostasis after deletion of pathogens [23]. Further important functions include embryogenesis and cell survival. Signal transduction within the target cell occurs after cytokine ligation to its surface cell receptor and results in alternate gene expression. Cytokines share pleiotropic characteristics and redundant effects [36]. The main categorization encompasses interleukins, lymphokins and chemokins [23]. Cytokines are often produced as precursor proteins and posttranslationally converted into the active

form, but regulation occurs also at the RNA transcription level [37]. Actually there are five known cytokine groups [23].

1.12.1. Cytokines mainly produced by cells from the innate immunity

The innate immunity demonstrates the first defense against the spreading of pathogens. Contact with antigens leads early on to the initiation of cytokine transcription and thereafter to the activation of cells from the adaptive immunity.

- Tumor necrosis factors (TNFs) -

are important cytokines in the induction of cell apoptosis, reduplication and inflammatory reactions [39]. TNFs, earlier also named as cachectin, have further important functions in the regulation of the leukocyte movement to the site of inflammation [40]. Especially the latter one is caused by TNFα variants. In mammalians there are two known isoforms (TNF α and β) [23]. Nuclear factor κB (NF- κB) proteins are activated by TNF receptor-associated factors (TRAFs) and participate in the TNF transcription [23]. Secretion occurs mostly from neutrophils, mast cells and NK cells, but also from activated T cells [39]. TNF exists in two variants: the trans membrane bound type forms a heterotrimer and interacts with type II TNF receptors (TNFRs) [23]. Interaction of TNF with another receptor variant such as type I results in cell apoptose [23]. Both receptor variants are available in nearly all cell types. Splitting of TNFR leads to soluble receptor variants and permits the binding especially to TNFα cytokines [23, 39]. It is not completely clear if this conjugation step supports the TNFα protein conglomeration or inhibits the binding mechanism to membrane anchored TNFRs. The exact genetic localization is described by Goetz and MacKenzie [39].

- IL-1β

IL-1 gene codes for three protein variants, IL-1α and IL-1β and IL-1 receptor antagonists (IL-1RA) [88]. Processing of IL-1β by cysteine proteases produces its active form [23]. Both cytokine alternatives play important roles in inflammatory reactions, whereas IL-1RA inhibits coupling to the IL-1 receptor and reduces therefore reactions against inflammatory processes [88].

- IL-6-

manifests itself in both innate and adaptive immunity functions during infection processes. Transkription via Jak-STAT transcription factors leads to the production of acute phase proteins (innate immunity) as well as to the differentiation of antibody producing B cells (adaptive immunity) and proinflammatory cytokines [41].

- Type I Interferon -

production is the direct consequence of viral infections. Interferon (IFN) α and β are the two main members of the type I interferon group, but IFN-ε,- κ, and –ω belong also to it [23, 42]. Its main function relies on the activation of the innate immunity especially by upregulation of MHC I proteins on surfaces of APCs [42]. IFNs are mainly synthesized by leukocytes, phagocytes and DCs.

- IL-10

The immunosuppressive properties of IL-10 are well studied. Beside the inhibition of TNF, IFNγ and GM-CSF, reduced levels of IL-1, IL-2, IL-3, IL-6, IL-8 and IL-12 were observed [43]. Receptors of IL-10 belong to the type II cytokine receptors [43]. Based on the inhibition of cytokines, IL-10 suppresses immune responses against inflammatory processes. Dependent on the cell type, IFNγ prevents the synthesis of IL-10 or reciprocal [43]. IL-10 synthesis occurs in activated T_H2 T helper lymphocytes and B cells as well as in cells of the innate immunity such as monocytes, macrophages and mast cells [23, 43]. Actively IL-10 is composed of two identical monomer subunits and can favor both the formation of IgA, IgG or IgM producing B cells as well as the proliferation of leukocytes [43]. High levels of IL-10 are related to higher periods of viral latency but also participate in the development of diverse diseases [43].

- IL-12

Equal to many other cytokines, IL-12 is an important immunoregulatory factor. The main producers are APCs activated by intracellular pathogens [44]. IL-12 stimulates cells of the innate and adaptive immunity to produce INFγ and has thereby important functions in the activation of macrophages [44]. Il-6 and IL-12 cytokines exhibit a high redundancy. The structure of IL-12 consists of covalently bound p35 and p40 heterodimer [44]. The p35 part is identical to that in IL-6 cytokines and the structure of p40 exhibits similarities to the extracellular part of the IL-6 receptor [45]. IL-12

binds to heterodimeric type I cytokine receptors. The IL-12 cytokine family encompasses further IL-23, IL-27 and IL-35 cytokines [45].

1.12.2. Soluble mediator proteins made by lymphocytes affect innate and adaptive immunity

- IL-2

T helper cells are the main producers of IL-2 cytokines [50, 51]. IL-2 cytokines, categorized into the type I cytokine group, are important key factors in the formation and distribution of effector and T regulatory cells [50]. High affinity binding of IL-2 to its receptors (IL-2Rs) requires the combination of three main receptor components, which are used differently by other immune cells: the α- chain (IL-2Rα, CD25) appears on the cell surface of stimulated lymphocytes and binds its cytokine only with low affinity, whereas β (CD122) and γ (CD132) chains form a separate complex and are mainly distributed on cell surfaces of memory T cells and NK cells [50, 51]. The latter two chains are also integrated in other cytokine receptors. According to the cell type, IL-2 detection starts-up the PI- kinase, RAS-MAP kinase or JAK STAT pathway and results in activation- induced cell death (AICD), cell growth, differentiation and survival [50].

- IL-4-

is mainly produced by the T_H2 subset of $CD4^+$ T cells [53]. More sources are basophile granulocytes and mast cells [53]. Hematopoietic cells, endo- and epithelial tissues, fibroblasts and hepatocytes express type I receptors for the IL-4 cytokine detection [53]. The main consequences of its detection concern the switch of immunoglobulins into IgE and IgG4 isotype and protect therefore against extracellular parasites [53]. Il-4 advances also the development of the T_H2 subcategory of T helper cells, the secretion of T_H2 cytokines and the increased expression of MHC II proteins on cell surfaces [52].

- IL-13-

cytokines cause high MHC class II expression on surfaces of monocytes and B cells and additionally exhibit anti-inflammatory properties by reducing the cytokine secretion of various cell types [54]. Furthermore IL-13 supports the IgE production during class switch processes in B cells and controls the cell mediated immunity against intracellular bacteria [54].

- IL-17-

cytokine family contains 6 members (IL-17A, IL17B, IL-17C, IL-17D, IL-17E and IL-17F) [55, 56]. T helper cells stimulated by IL-15 or IL-23 are the major producers of IL-17 cytokines. The activation of T cells via TCR is not necessary for the secretion of some members of the IL-17 cytokine family [55]. All IL-17 cytokines achieve important pro- inflammatory functions. The first discovered IL-17 family member, IL-17A, initiates the production of cytokines by different cell types mainly from the innate immunity whereas IL-17E is known to activate the production of T_H2 cytokines and multiplication of eosinophils [56].

- IL-22-

Secretion occurs by activated DCs and T- cells and affects mainly epithelial cells to emit pro-inflammatory and anti- microbial products [57]. IL-22 cytokines belong to the IL-10 superfamily and bind to a complex consisting of IL-10 and IL-22 components for the complete effector function [57].

- IFN-γ-

also referred to as type II interferon- are produced by both cells of the innate and the adaptive immunity [23]. IFN-γ plays an important part in early phases of the immune defense and is highly produced by NK cells, $CD8^+$ lymphocytes and T_H1 subgroup of T helper cells [58]. IL-4, IL-10 and TGFβ clearly reduce type II IFN secretion. The biggest effects of IFN-γ relies in the activation of NK cells and macrophages as well as in the enhanced appearance of MHC surface molecules [58], T helper cell differentiation into T_H1 subgroup and B cell switching into the IgG isotype [23].

- TGF-β

The immmunoregulatory properties of TGF-β affect lymphocytes, Natural killer cells (NK cells), DCs, macrophages, mast cells as well as granulocytes [60]. Actually there are three known mammalian TGF- β cytokines (TGF- β 1-3) and each of them is produced in nearly all leukocytes [61]. Thymocyte proliferation and differentiation plus T cell survival and expansion is strongly influenced by TGF- β polypeptides. This effect holds equally for all T cell subgroups such as regulatory T cells (Tregs), cytotoxic T cells and T helper cells [60, 61]. TGF- β influence on B cell development is comparable to the inhibitory consequences on T cells and results in non- activation and apoptosis of immature or resting B cells. In addition to the apoptotic effects, TGF- β blockades the B cell receptor (BCR) isotype class switch except for IgA

heavy chain variants [61]. It has also been reported that TGF- β inhibit the appearance of TfRs on B cell surfaces [61]. Inhibitory effects are also noticeable for NK cells (effector function and cytokine production), macrophages (phagocytosis and antigen presentation) and neutrophil granulocytes (movement across the endothelium by reducing adhesion molecules) [60]. Pro- inflammatory regulation by the action of TGF- β is observable for macrophages, mast cells and monocytes (upregulation of attachment molecules on cell surfaces) [60].

2. AIM OF THE STUDY

The transferrin receptor (TfR) is important for import of transferrin-iron complexes into cells and is known as a critical regulator of the function of immune cells. Dendritic cells (DCs) are prominent antigen presenting cells and are key regulators of adaptive as well as innate immune responses. Yet, little is known about the functional role of TfR (CD71) on DCs. Therefore, the aim of this study was to analyze the impact of CD71 on differentiation and maturation of human, monocyte-derived DCs with four monoclonal antibodies (mAbs) directed against CD71, which have been generated in our laboratory (mAb VIP-1, 5-528, 15-221 and 13-344), as demonstrated in Figure 5.

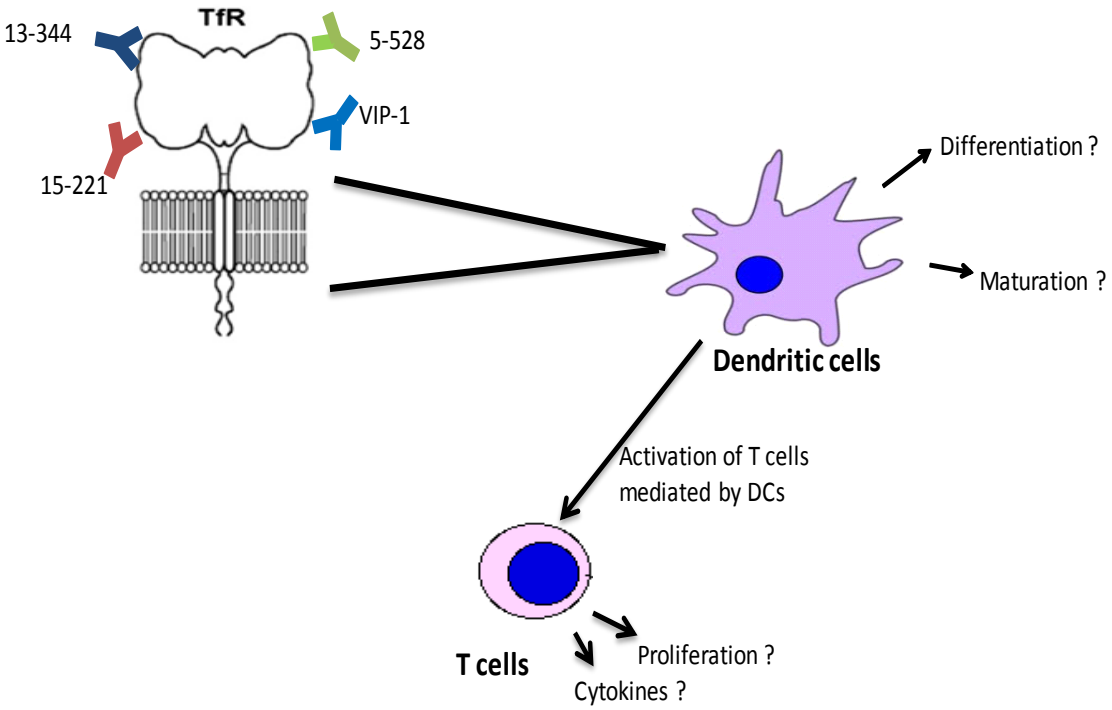

Figure 5: Analysis and characterization of 4 mAbs that recognize epitopes on the extracellular part of the human TfR (CD71).

3. MATERIALS AND METHODS

3.1. Antibodies

Specifity	Clone	Isotype	species	Source
T cell stimulation				
CD3	OKT 3	IgG2a	mouse	Jansen-Cilag, Vienna
CD28	15E8	IgG1	mouse	Caltag Laboratories, Burlingame, CA
CD63	CD63-11C9	IgG3	mouse	Otto Majdic,IFI, Vienna
Straining and Treatment				
Calf intestine alkaline phosphatase	VIAP-2D5	IgG1	mouse	Otto Majdic,IFIy, Vienna
CD1a	VIT6b	IgG1	mouse	Otto Majdic, IFI, Vienna
CD3	UCHT1	IgG1	mouse	Otto Majdic, IFI, Vienna
CD4	VIT4	IgG2a	mouse	Otto Majdic, IFI, Vienna
CD8	LM2	IgG1	mouse	ATCC, Rockville, Tenesse
CD14	VIM13	IgM	mouse	Otto Majdic, IFI, Vienna
CD40	G28-4	IgG1	mouse	ATCC, Rockville, Tenesse
CD45	VIT200	IgG2a	mouse	Otto Majdic, IFI, Vienna
CD45	8-301	IgG1	mouse	Otto Majdic, IFI, Vienna
CD69	Leu-23	IgG1	mouse	Becton Dickinson, Palo Alto, CA
CD71	13-344	IgG1	mouse	Otto Majdic, IFI, Vienna
CD71	5-528	IgG1	mouse	Otto Majdic, IFI, Vienna
CD71	VIP-1	IgG1	mouse	Otto Majdic, IFI, Vienna
CD71	15-221	IgG2b	mouse	Otto Majdic, IFI, Vienna
CD80	7-480	IgG1	mouse	Otto Majdic, IFI, Vienna
CD83	HB15	IgG2b	mouse	Caltag Laboratories, Burlingame, CA
CD86	BU63	IgG1	mouse	Caltag Laboratories, Burlingame, CA
CD169	7-239	IgG1	mouse	Otto Majdic, IFI, Vienna
CD169	7-526	IgG1	mouse	Otto Majdic, IFI, Vienna
CD274	1-550	IgG1	mouse	Otto Majdic, IFI, Vienna
CD274	5-496	IgG1	mouse	Otto Majdic, IFI, Vienna
B7-H1	5-272	IgG1	mouse	Otto Majdic, IFI, Vienna
B7-H3	7-517	IgG1	mouse	Otto Majdic, IFI, Vienna
HLA-class I	W6/32	IgG2a	mouse	ATCC, Rockville, Tennesse
HLA- DR class II	L243	IgG2a	mouse	Otto Majdic, IFI, Vienna

3.2. Buffers, chemicals and media

List of reagents and chemicals

Beriglobin	20 mg/ml (in PBS/BSA)	Aventis Behring, Vienna, Austria
GM-CSF	130 µg/ml	Fermentas, Burlington, Canada
IL-4	100 U/ml	Novartis Research Institute, Vienna
methyl-3H-thymdine	1m Ci/ml	Perkin Elmer, Waltham, MA
Ionomycin	200 nM	Sigma-Aldrich
L-Glutamin	2 mM	Sigma-Aldrich
LPS, serotype O127-B8, *E.coli*	1µg/ml	Sigma-Aldrich
Penicillin	100 µg/ml	Sigma-Aldrich
Peptidoglycan	10µg/ml	Sigma-Aldrich
PMA	200 nM	Sigma-Aldrich
Poly I:C	20 mg/ml	Sigma-Aldrich
Streptomcyin	100 µg/ml	Sigma-Aldrich
zymosan A, S. cerevisiae	30 µg/ml	Sigma-Aldrich

- *Heparin Medium:*

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

- *Freezing medium:*

RPMI1640 supplemented with 25% FCS and 10% DMSO

- *MACS- buffer (stored at 4°C):*

1000 ml 1x PBS def. + 25 ml Human Serum Albumin (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0,5 M); sterile filtration

- *10x PBS stock solution:*

5,8 g KH_2PO_4

16,6 g $Na_2HPO_42H_2O$

72 g NaCl

Dissolve in aquabidest (=ddH_2O), fill up to 10 litres and adjust to pH 7,2

- PBS/BSA stock solution (20%):

100 g BSA

10 g NaN_3

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

- *0,5M Tris-HCL pH 6.8:*

6 g Tris (Biorad, Hercules, CA) were dissolved in ddH_2O and pH was adjusted to 6.8 with 6 N HCL. Then the solution was brought to 100 ml with ddH_20.

PBST (PBST 0,5%)

PBS with 0,5% Tween 20

- *1,5M Tris-HCL pH 8.8:*

18,2 g Tris (Biorad, Hercules, CA) were dissolved in ddH_2O and pH was adjusted to 8.8 with 6 N HCL. Then the solution was brought to 100 ml with ddH_2O.

- *10% n-dodecylsulphate sodium salt,*

SDS (Sigma, St. Louis, MO): 5g were dissolved in 50 ml ddH_2O and stored at room temperature.

- *10% Ammoniumpersulfat*, APS (sigma, ST. Louis, MO): 100 mg/ml
- *Running buffer (4x)*

Tris 12 g

SDS 4 g

Glycin 57,6 g

Fill up to 100 ml with ddH_2O

- *Blotting buffer*

250 ml of 4x running buffer

200 ml methanol

ad ddH_2O= to 1000 ml

- *Dry milk solution*

5% dry milk powder dissolved in PBST buffer

3.3. cultivation of cell lines

Human tumor cell lines

Cell line	Cell type	Immunology	Source
K562	Human chronic myeloid leukemia in blast crisis (erythriod, no MHCs)	CD3-, CD13+, CD19-, CD34-, CD41(+), CD42+, CD45+, CD71+, GlyA(+)	ATCC (American Type Culture Collection)
B and T cell lines			
DAUDI	Human Burkitt`s lymphoma (EBV+)	CD3-, Cd10+, CD13(+), CD19+, CD20+, CD34-, CD37+, HLA-DR+	ATCC
KM-H2	Human Hodgkin`s lymphoma	CD3-, CD13(+), CD14(+), CD15+, CD19-, CD21+, CD25-, CD30+, HLA-DR+	DMSZ
JURKAT	Human T cell leukemia	CD2+, CD3+, CD4+, CD5+, CD6+,CD7+, CD8-, CD13-, CD19-, CD34+, TCRα/β+, TCRγδ-	ATCC

Murine tumor cell lines

Murine AKR, BW 5417 as well as BW cells expressing the human CD71 were kindly provided by Dr. Peter Steinberger (Institute of Immunology, Medical University of Vienna).

For cell culture of monocytes, dendritic cells and both human and murine cell lines RPMI1640 (NBK, Novartis Research Institute, Vienna) supplemented with 10% fetal calf serum (HyClone, Utal), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml strepamycin was used. Cells were cultured at 37°C with 5% CO_2.

3.4. PBMNC extraction

Peripheral blood mononuclear cells (PBMNCs) were separated from fresh blood samples (Buffy coats, Red Cross Vienna) of healthy donors. Blood was diluted 1:2 with Heparin medium (Gibco Ltd., Paisley, Scotland). The diluted blood was then overlaid above 15 ml Ficoll-Paque plus (Axis- Shield PoC AS, Oslo Norway) to a final volume of 50 ml. For density gradient centrifugation the tubes were centrifuged at 900 g for 30 minutes at 25°C without braking. In the FIcoll gradient blood cells are separated due to their different density. The pellet

contains mainly erythrocytes and leukocytes, whereas the white interphase contains PBMNCs consisting of lymphocytes and monocytes and the upper phase contains serum. PBMNCs were then collected into fresh tubes and washed twice with MACS buffer.

3.5. Monocyte and T cell isolation

For monocytes up to 1 x 10^9 PBMNCs were incubated with 15 µg/ml of biotinylated (CD14 (VIM13, MEM18). Therefore PBMNCs were resuspended in 750 µl MACS buffer and incubated with 250 µl streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 min at 4°C. In the meantime a CS column (Miltenyi Biotec) that can be loaded with a maximum of 10^9 cells, was placed onto a VarioMACS apparaturs and equilibrated with 40 ml MACS buffer. Monocytes were positively enriched. The PBMNCs incubated with the CD14 mAb and the streptavidin MicroBeads were loaded onto the CS column. $CD14^+$ monocytes remained in the column, $CD14^-$ cells were washed out with 40 ml MACS buffer. The flow through was collected as monocyte negative fraction containing T cells, B cells and NK cells (fraction 1). The column was removed from the magnetic field and the MACS buffer was added in 10 ml portions alternating through the side syringe or loaded on the top of the column. After each addition step the column was put back into the magnet field and about 10 ml were allowed to run through. These steps were repeated until a volume of 50 ml had passed through (fraction 2). For monocyte collection the column was removed from the magnet field and the liquid was taken off through the lateral valve using a syringe. These steps were repeated until the collected volume came to 40 ml (fraction 3). The third fraction was centrifuged for 5`at 900 g, resuspended and the number of monocytes was determined. The purity (> 95%) of monocytes was controlled by immunofluorescence analysis. Double-staining with directly labeled antibodies using CD4-PE (CD3-FITC, CD8-PE/CD3-FITC, CD19-PE/CD3-FITC, CD56-PE/CD3-FITC, HLA-DR-PE/CD3-FITC, CD14-PE/HLA-DR-FITC.

To isolate T cells from up to 1x10^9 PBMNCs the MACS technique was used. T cells were isolated by collecting the flow through of PBMNCs depleted by using

different mAbs at a concentration of 10 µg/ml containing CD14 (MEM18) for collecting monocytes, CD16 (3G8) for binding granulocytes and NK cells, CD19 for B cells (BU 12) and CD33 (4D3) for monocytes, thrombocytes and myeloid progenitors (negative selection). Freshly isolated PBMNCs were resuspended in 750 µl MACS buffer and incubated with 250 µl biotinylated antibodies for 15 min at 4°C. To remove unbound antibodies the cells were washed twice with MACS buffer and again resuspended in 750 µl buffer. Then 250 µl streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 min at 4°C. In the meantime a CD column (Miltenyi Biotec) that can be loaded with a maximum of $1*10^9$ cells, was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. PBMNCs were loaded onto the column, T cells were eluted and washed until a volume of 50 ml was reached. The collected T cells were centrifuged for 5`at 900g, resuspended and the number of cells was determined.

3.6. Freezing and thawing of cells

3.6.1. Freezing of cells

Mammalian cells can be stored in liquid nitrogen for prolonged periods of time with minimal loss of viability in a subsequent cell culture. For that purpose, cells were centrifuged, counted, resuspended in freezing medium (RPMI1640 supplemented with 25% FCS and 10% DMSO and adjusted to a cell number ranging from 10^7 – 5 x 10^7 cells/ml. 1 ml aliquots were filled into cryotubes (Nalgen Nunc International, Roskilde, Denmark) and kept overnight at -80°C in a freezing box filled with isopropanol before being transferred to liquid nitrogen.

3.6.2. Thawing of cells

Cryotubes were thawed in lukewarm water bath. To avoid overheating we left a rest frozen and put the tube on ice for 10 min. Afterwards the cells were transferred to a 15 ml tube and droplets of supplemented cell culture medium (RPMI1640 plus 10% FCS (Sigma-Aldrich), L-glutamine, penicillin, streptomycin) were added in an interval of 1 min: Starting with 3 drops, 6 drops after the first minute, 12 drops in the second minute etc. up to 48 drops. The cells were washed twice with RPMI1640, counted and cultured in medium.

3.7. Generation of dendritic cells derived from monocytes

DCs were generated by culturing purified monocytes for 6 days with a combination of 50 ng/ml GM-CSF and 100 U/ml IL-4 in RPMI1640 supplemented with 10% characterized FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (sigma-Aldrich). Maturation of DCs was initiated by adding 1 µg/ml LPS for 24 hours.

3.8. Mixed leukocyte reaction (MLR)

In an allo – MLR lymphocytes from a potential donor are mixed with stimulator cells from a potential recipient, i.e. APCs like DCs. If the two cell populations are not compatible in their MHC, which means they are allogeneic, proliferation of T cells occurs. $5*10^5$ DCs pretreated with 5 µg/ml CD71 mAbs for 48 hours were cultivated with $1*10^5$ responder T cells in RPMI1640 (10% FCS (Sigma-Aldrich), L-glutamine, penicillin, streptomycin) for 5 days on a final volume of 250 µl under standardized cell culture conditions (37°C, 5% CO_2, humidified atmosphere). Each experiment was performed in triplicate in 96-well plates (Costar, Sigma Aldrich). On day five 25 µl of 1:20 diluted methyl- [3]thymidine (1 µCi/well) (Perkin Elmer, Waltham, MA) and after adding 25 µl of Microscint scintillation mix (Packard, Meriden, Connecticut) in each well radioactive emissions quantified on a Packard microplate scintillation counter.

3.9. T cell proliferation assay

96-well plates (Costar, Sigma Aldrich) were coated with CD3 (OKT3), CD3/CD28 (OKT3/15E3) and CD3/CD63 (OKT3/11C9) mAbs at a concentration of 2 µg/ml. The plates were coated with a volume of 25 µl of antibody-solution overnight at 4°C or for about 2 hours at 37°C. Then the antibody solution was removed and the plates were washed twice with PBS. 1×10^5 T cells in RPMI1640 medium supplemented with FCS were added into each well and incubated in combination with 5 µg/ml CD71 mAbs. Proliferation of T cells was monitored by measuring (methyl—H)-TdR (Perkin Elmer, Waltham, MA) incorporation on day 4 of the culture. Cells were harvested 18 hours later, and radioactivity was determined on a microplate scintillation counter (Perkin Elmer, Wellesley, MA). Assays were performed in triplicate.

3.10. Cell analysis via flow cytometry

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

3.11. Membrane staining with unconjugated mAbs

Unspecific binding of mAbs to Fcγ-receptors was blocked by incubation of cells with human immunoglobin Beriglobin (Aventis Behring GmbH, Vienna). As isotype control VIAP, a calf intestine alkaline phosphatase- specific antibody was applied. For secondary labeling Oregon Green- conjugated goat anti-mouse antibody was used.

- PBS/BSA: 1xPBS + 1% BSA
- Beriglobin (final concentration 20 mg/ml) diluted 1:8 in PBS/BSA (1% v/v BSA) (Aventis Behring GmbH, Vienna)

- Primary antibody: 20 µg/ml
- Secondary antibody: Oregon Green-conjugated goat-anti-mouse IgG 20 µg/ml in PBS/BSA (Molecular Probes; Eugene, Oregon)

First the cell suspension (5 x 10^5 / assay) was centrifuged for 5 minutes at 300 g and the pellet was resuspended with 50 µl Beriglobin/assay and kept 10 minutes on ice. Then 20 µl of the antibody were prepared in Mirconic-tubes and 50 µl of the cell suspension was added, mixed and incubated 30 minutes at 4°C. Each assay was washed twice with PBS/BSA (resuspended in PBS/BSA, centrifuged (5 min at 300g), the supernatant was discarded). 20 µl Oregon Green were added to the cells, and again incubated for 30 min at 4°C. Each assay was washed twice with PBS/BSA and the cells were resuspended in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow cytometer (Becton Dickinson, Palo Alto, CA).

3.12. Cell surface staining with conjugated mAbs

The cell suspension (5 x 10^5 / assay) was centrifuged for 5 minutes at 300 g and the pellet was resuspended with 50 µl Beriglobin/ well and kept 10 minutes on ice. 20 µl of the respective antibody was prepared in Micronic-tubes and 50 µl of the cell suspension was added, mixed and incubated for 30 minutes at 4°C. Each assay was washed twice with PBS/BSA (resuspended in PBS/BSA, centrifuged (5 min at 300 g), the supernatant was discarded) and the cells were resuspended in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow-cytometry.

3.13. Epitop mapping

Epitop mapping was applied to examine whether different mAbs have overlapping binding sites. For this experiment we used strongly proliferating KM-H2 cells (Human Hodgkin`s lymphoma). The cells (5 x 10^5 KM-H2 cells/assay) were spun down (5 min at 1750 rpm). The pellet was resuspend with 50 µl Beriglobin/assay and kept 10 min on ice. 25 µl of an unlabeled antibody (20 µg/ml) were prepared in Micronic tubes and 50 µl of the cell suspension was added, mixed and incubated 30 min at 4°C. 20 µl of a

conjugated antibody were added to the cells, mixed and incubated 30 min at 4°C. The tubes were kept in ice until they were analyzed by flow cytometry.

3.14. Protein extraction and detection

3.14.1. Protein sample preparation

KM-H2 and K562 cells were cultured for 4 days with PMA and ionomycinein RPMI1640 + 10% FCS or without stimulation. Subsequently, cells were harvested, washed 2 times with ice cold PBS and solubilized in lysis buffer (20 nM NaH_2PO_4; 2 mM ETA; 2 mM EGTA; 2 mM NaF, pH8,4) containing 1% IGEPAL CA-630 (Sigma) and supplemented with Complete Protease Inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 20 minutes on ice. Lysates were spun down for 20 min at 13.000 rpm at 4°C and immediately frozen.

3.14.2. Immunoprecipitation

For immunoprecipitation 10 µg/ml anti CD71 mAbs (CD71 mAbs VIP-1, 15-221, 5-528, 13-344) were loaded onto $7x10^8$ sheep anti-mouse IgG Dynabeads (M-280, Streptavidin) with 2,8 µm diameter according to the manufacturer`s instructions. After washing twice in PBS, the beads were incubated with cell lysate of cell- culture supernatant for 48 h at 4°C. Subsequently the beads were washed twice and bound protein was eluted into reducing sample buffer (Biorad, Richmond, CA) by boiling for 5 min and centrifugation (20200 rcf; 5 min). Samples were analyzed using gel electrophoresis combined to silver staining and western blotting.

3.14.3. Gel electrophoresis

SDS Polyacrylamide Electrophoresis is a useful system to separate proteins according to their size. SDS is a powerful detergent, which has a very hydrophobic end (the lipid like dodecyl part) and highly charged part (the sulfate group). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures, transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups masking the actual charge of proteins. After

boiling in SDS proteins therefore become elongated with negative charges arrayed along their length, so they will move towards a positive electrode more or less fast according to their respective size. The reason why β-mercaptoethanol is usually included in the sample buffer is to cleave disulfide bonds within or between molecules, allowing molecules to adopt an extended monomeric form. Lysates were 1:2 mixed with reducing Laemmli sample buffer containing 5% β- mercaptoethanol (Biorad, Richmond, CA) and boiled at 95°C for 5 min. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels using the Hoefer Mighty Small system (Amersham, Little Chalfont, UK). As running buffer 25 mM Trise base, 192 mM glycine, 0,1% SDS was used.

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

As protein standard SeeBlue Plus 2 prestained marker (Invitrogen) was used.

3.14.4. Western blot

Western blotting allows to determine, with a specific primary antibody, the relative amounts of the proteins present in different samples. Separated samples are transferred to a membrane for detection. The membrane is incubated with a generic protein to bind to any remaining sticky places on the membrane and therefore blocking unspecific antibody adherence on the membrane itself. A secondary antibody is then added to the solution which recognizes the primary antibody to find locations where the primary antibody has bound.

Proteins that were separated by SDS-Page were blotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA) using the Hoefer Semiphor TE77 system (Amersham, Buckinghamshire, UK) for 1 hour at 15 V. As blotting buffer 25 mM Tris base, 192 mM glycine, 5% Methanol was used. Membranes were blocked with 5% dry milk / 0,1% Tween20 and incubated with primary antibody (1 µg/ml) in the same solution (7 rpm, overnight, 4°C). After washing (5 x 5 min) with 1% PBS / Tween20, membranes were incubated with the secondary HRP conjugated anti-mouse-Ig antibody dilution (1:10000 in blocking buffer) in 50 ml falcon tube (7 rpm, 1 hour, 25°C or overnight at 4°C). Enhanced chemiluminescence (ECL) was used for the detection of proteins. Therefore membranes were incubated with ECL solution (20 ml 1 M tris pH 8.8, 500 µl p-coumaric acid 340 mg in 26 ml DMSO, 1 ml Luminol 2.26g in 51 ml DMSO, 180 ml aqua dest.), placed between two plastic overhead sheets, covered with a Kodak XAR film (Sigma-Aldrich, ST. Louise, MO) and placed into a film cassette.

Sample buffer

Laemmli sample buffer (Biorad, Hercules, CA), before use 5-10 % β-mercaptoethanol was added

Protein standard

Benchmark Prestained Protein Ladder from Invitrogen, Carlsbaden, CA

3.14.5. Silver staining

50 ml methanol, 12,5 ml acetate and 2,5 ml reagent A were mixed and brought with ddH_2O to 100 ml (solution 1). Gels were shaken in that solution for 30 min. After discarding of solution, 10 ml methanol and 12,5 ml acetate were brought with ddH_2O to 200 ml (solution 2). Gels were shaken for 15 min, then the solution was changed to solution 2 and gels were shaken for another 15 min. The gels were washed for twice for 5 min in ddH_2O. For enhancement 25 ml reagent B and 50 ml ddH_2O were mixed and gels were shaken for 10 min in that solution. The gels were washed again in ddH_2O. For staining 2,5 ml reagent C were brought to 100 ml with ddH_2O and gels were incubated in this solution for 10 min. After the last washing step with ddH_2O (1 min), 1,5 g of reagent D were dissolved in ddH_2O, 1,25 ml reagent B were added, brought to 50 ml and

incubated for 10-15 min. The reaction was stopped by immersing the gel into reagent F.

4. Results

4.1. MAbs VIP-1, 15-221, 5-528 and 13-344 recognize human TfR proteins (CD71)

It is common that the high proliferation rate of lymphoma cells is dependent on an efficient iron uptake mechanism. Therefore we verified the binding of our tested CD71 mAbs using a murine BW lymphoma cell line expressing human TfR proteins. Each mAb recognizes only particular sequence areas of the surface proteins and ensures the high specificity of the staining. Depending on the number of expressed surface proteins, the fluorescence intensity increases or decreases. In 1989, Knapp et al [74] described mAb VIP-1 to recognize epitopes of the human TfR. Therefore mAb VIP-1 was used as a positive control antibody to verify the detection of TfR surface proteins.

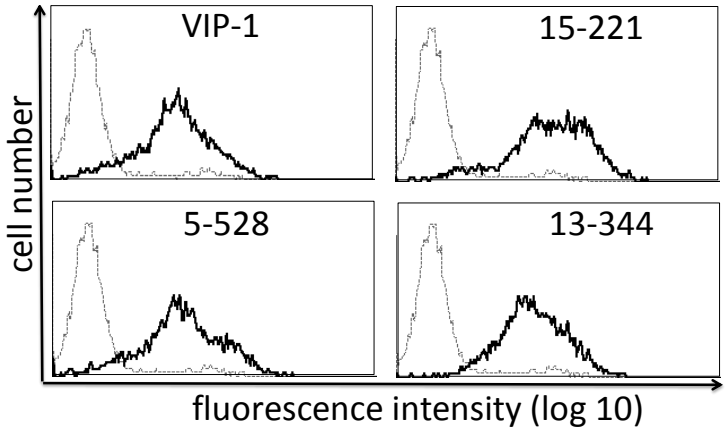

Figure 6: A murine cell line (BW) has been transfected to express human TfRs on the cell surface. The mAb VIP-1 serves as control antibody for the detection of the human TfR proteins. All antibodies have been used in the fluorochrom conjugated form. Black lines demonstrate the linkage of diverse CD71 mAbs (names within the respective boxes); gray and spotted lines show the negative control. One representative experiment of three independent experiments is shown.

As shown in Figure 6, mAb 15-221, 5-528 *as* well as mAb 13-344 show similarly increased levels of fluorescence intensity compared to mAb VIP-1. These data leads to the validation that all of our tested antibodies recognize the same surface proteins.

4.2. mAbs bind to Tf surface receptors (CD71) on various cell lines

Since all of the tested antibodies appear to bind to human TfRs expressed on murine cells, we were interested in the binding capacity to other cell lines exhibiting a high number of CD71 molecules. Based on the fact that growing and fast proliferating cells need iron to a higher degree, we analysed human lymphoid leukemia cells for the surface staining of CD71 receptors. The species specificity of our mAbs was tested by the following staining of murine and insect cell lines.

4.2.1. MAbs VIP-1, 15-221, 5-528 and 13-344 react with human B- and T- cell leukemia cells

Daudi B cell lymphoma belongs to the major group of non-Hodgkin`s-lymphoma. Already in 1985 Besancon et al. [75] used this cell type to demonstrate that treatment of Daudi cells with INFα lead to the downregulation of Tf surface receptors. The publishing of further TfR inhibition data supported the theory of iron requirement for the functionality of enzymes and therefore the importance of highly expressed TfRs on cell surfaces of fast proliferating cells. All four tested antibodies bound with high affinity to the surface of the Daudi cell line (Figure 7).

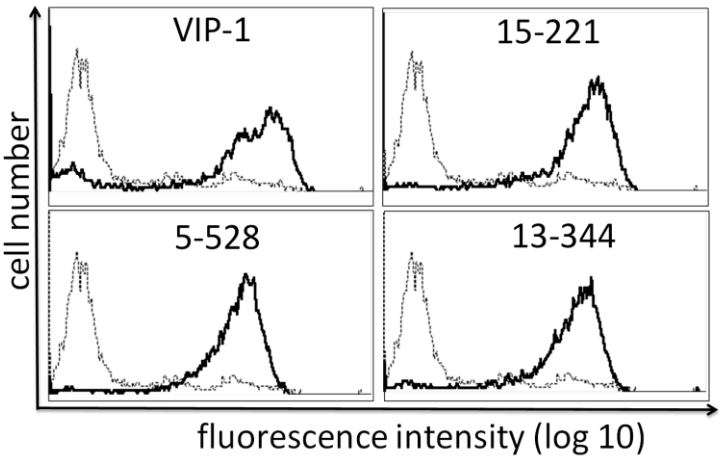

Figure 7: Daudi B lymphoma cell line was tested for the expression of TfR proteins via the conjugated anti- CD71 mAb VIP-1. The high expression of TfRs (black line) reflects the increased necessity of iron during the DNA syntheses process. Protein surface staining occurred via direct conjugated (FITC or PE) mAbs. Antibodies 15-221, 5-528 and 13-344 show

a similar strong binding pattern compared to the CD71 control antibody VIP-1. One representative experiment of two independent experiments is shown.

We used furthermore a second type of B cell lymphoma to test the binding affinity of CD71 mAbs on human lymphocytes. The Raji cell line had originally been taken from a Burkitt`s lymphoma patient [76] and exhibits a high division rate under standard cultivation conditions. Simliar to the previously shown non-Hodgkin B cell lymphoma (Figure 8), all tested mAbs interact with protein areas on the surface of the immortalized B cell line. The high fluorescence level correlates directly with the number of bound antibodies and indicates therefore an increased appearance of surface proteins. CD71 mAb VIP-1 bind to a TfR epitope in a similarly intensity compared to the other three mAbs and agree with the data from the staining of the Daudi cell type (Figure 8).

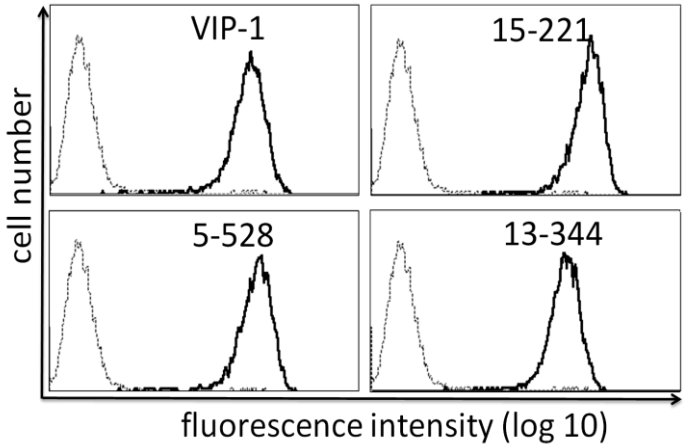

Figure 8: Raji, a Burkitt`s lymphoma cell line including Epstein- Barr virus sequences, was used to analyze the expression of CD71 receptors. Comparable to Daudi cells (Figure 8), Raji lymphomas grew under standard conditions and were subsequently stained for TfR expression. For the staining process we used identical mAbs as already used for the staining of Daudi cells. One representative experiment of two independent experiments is shown.

Based on the results of Daudi and Raji cell stainings, we analyzed the CD71 expression on surfaces of non- B cell lymphoma. K562 are immortalized cells derived from an acute myeloid leukemia patient. Jurkat lymphoma, a further analyzed cell line has been isolated in the 1970`s from a T cell lymphoma patient and is known for the high IL-2 secretion after treatment with mitogen stimuli. Jurkat cells are an important tool to analyze T cell receptor signaling and processes in correlation with cytokine production [77]. Both the human

erythroleukemia cell line K562 and Jurkat cells exhibit an elevated number of TfR surface proteins in which we observed a much stronger CD71 expression on Jurkat lymphoma. Beside the increased fluorescence signal for CD71 mAb VIP-1, mAbs 15-221, 5-528 and 13-344 manifest again comparable strong affinities for receptor recognition at both cell types (Figure 10 and 11).

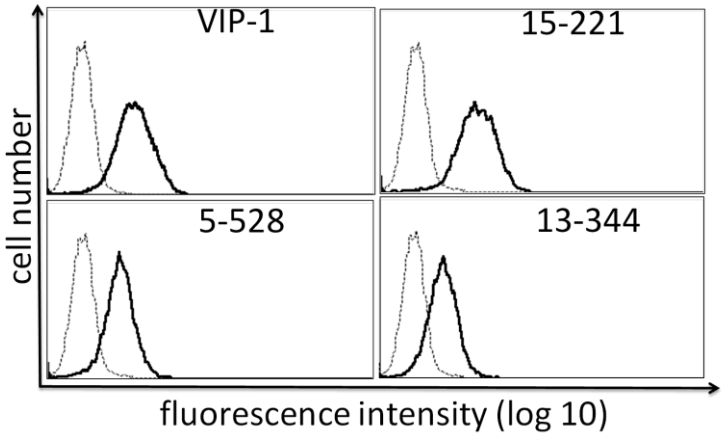

Figure 9: Antigen recognition of conjugated mAbs VIP-1, 15-221, 5-528 and 13-344 at the cell surface of K562 cells. Cultivation of the human erythroleukemia cell line was established in an identical manner as already described. One representative experiment of two independent experiments is shown.

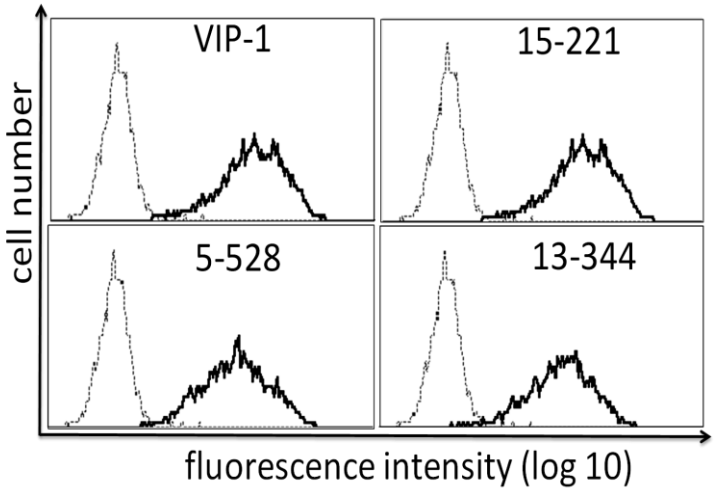

Figure 10: Human CD4+ Jurkat T cell lymphoma cells were cultivated in the same way like other cells described before and analyzed concerning the CD71 surface protein expression. The number of epitope linked antibodies is reflected by the increased fluorescence intensity (black graphs) detected via flow cytometry. The gray dotted lines show the isotype control.

4.2.2. mAb 13-344 cross-reacts with murine TfR proteins

In order to define the species specificity of mentioned mAbs, we performed staining approaches for murine cell lines in the same way as described previously for human lymphoma. The binding affinity of mAb VIP-1, 15-221, 5-528 and 13-344 to murine CD71 antigen was investigated on a AKR mice strain derived T cell lymphoma as well as on murine B cell lymphoma cells.

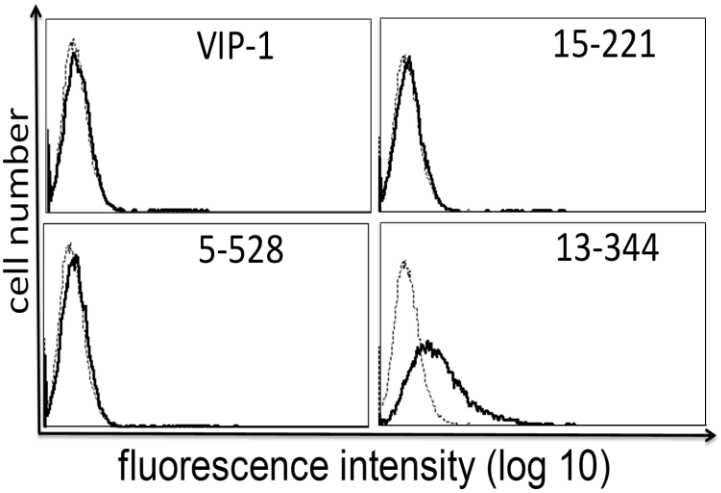

Figure 11: Analysis of the correlation between the four CD71 mAbs and murine T cell lymphoma extracted from AKR mice. Murine cells grew under identical conditions as described for human cells. In contrary to human cell lines, only mAb 13-344 binds the murine TfR. One representative experiment of three independent experiments is shown.

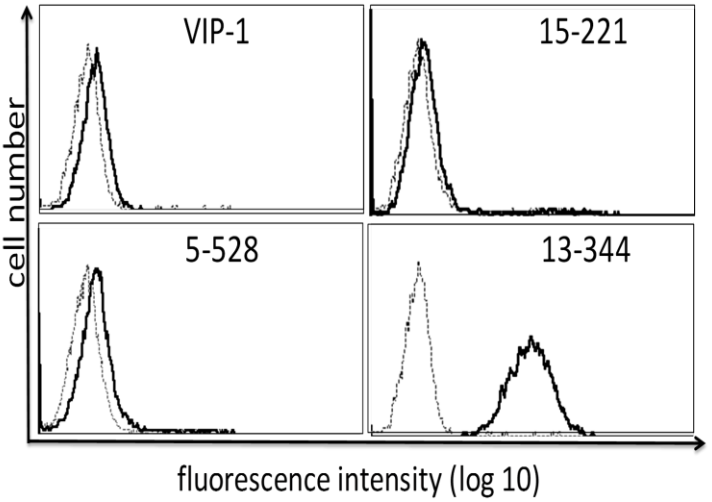

Figure 12: BW 1C8, a B cell lymphoma in mice, was used to determine the binding affinity of 4 mAbs to the TfR proteins on the cell surface of murine cells. For the receipt of the fluorescence signal the same staining procedure has been followed as described earlier for

AKR cells and human lymphoma. One representative experiment of two independent experiments is shown.

Formerly studies on mAb VIP-1demonstrated a binding specificity exclusively for TfR proteins on surfaces of human cells [78-80]. This result is confirmed by staining experiments of murine CD71 surface proteins (Figure 11 and 12). In the same way as mAb VIP-1 but contrary to stainings of human cell lines presented above, neither mAb 15-221 nor mAb 5-528 shows any reactivity with murine TfR proteins. However, a higher binding activity is clearly visible for mAb 13-344 in both murine T and B cell lymphoma (Figure 11 and 12).

4.2.2.1. Activated murine primary B and T lymphocytes show also an upregulation of TfRs.

Similar to lymph nodes, the mammalian spleen provides important functions in blood filtration, antigen detection and renewing of erythrocytes. The white pulp of the spleen mostly contains a collection of various monocytes, macrophages, B and T lymphocyte populations [23]. Immune cells inside the spleen are also described as splenocytes. Based on the fact that mAb 13-344 recognized TfR epitopes on fast proliferating murine B and T lymphoma cells, we analyzed the surface receptor expression pattern on non- leukemia cells. Freshly isolated murine splenocytes are barely recognized by any of our tested antibodies (Figure 13). However, activation of these immune cells via PMA / Ionomycin causes an increased iron requirement and results in clearly higher fluorescence intensity for the staining exclusively with mAb 13-344. As with lymphoma cells, none of the other CD71 antibodies bind to murine surface receptors.

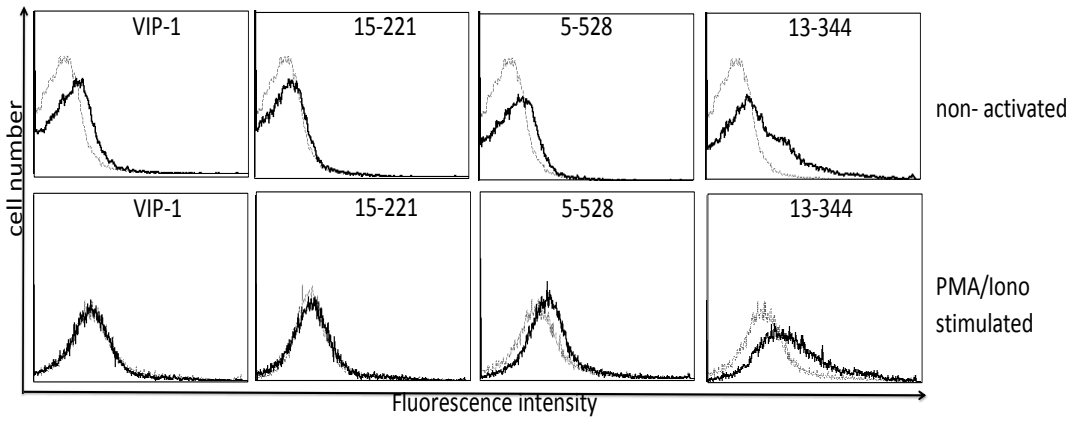

Figure 13: Murine immune cells were isolated from the spleen, activated and tested for TfR expression, stained with CD71 mAb conjungation and analyzed via flow cytometry. Non- stimulated cells have been stained immediately after isolation with the mAbs presented before. Activation of immune cells occurred with PMA / Ionomycin and at 37°C. After 48 hours of stimulation, cells were stained and analyzed via flow cytometry. One representative experiment of two independent experiments is shown.

Given that fast proliferating cells require more iron for the DNA synthesis plus the observed stronger conjugation of these antibodies on leukemia cell lines in a similar intensity to the already described antibody mAb VIP-1 support the assumption that all presented antibodies recognize epitopes on the TfR. However, mAbs VIP-1, 15-221 and 5-528 are specialized to recognize proteins only on human tissues, whereas mAb 13-344 also recognize presumable TfR proteins on murine cells.

4.2.3. None of the tested antibodies react to insect cell surface Proteins

Not only for drosophila but also for other insect organisms, diverse functions have been described as being associated with the iron transport protein Tf. The N-terminal part of mammalian compared to insect Tf proteins possess highly conserved sequence areas, but completely lack the C-terminal part [81]. Interestingly, for insects no TfR molecules are known. However, proteins for the absorption of the Tf- iron complex must exist. Therefore we tested the binding affinity of our 4 mAbs on *spodoptera litura* (SL) cells. *Spodoptera litura* belongs to the family of Noctuidae insects that cause enormous damages in agriculture [12]. Flow cytometry analysis of CD71 mAb VIP-1 labeled SL cells result in no detectable aberrance of fluorescence signal compared to the isotype control. Similarly, the other mAbs did not bind to any epitopes on the insect cell surfaces (Figure 14).

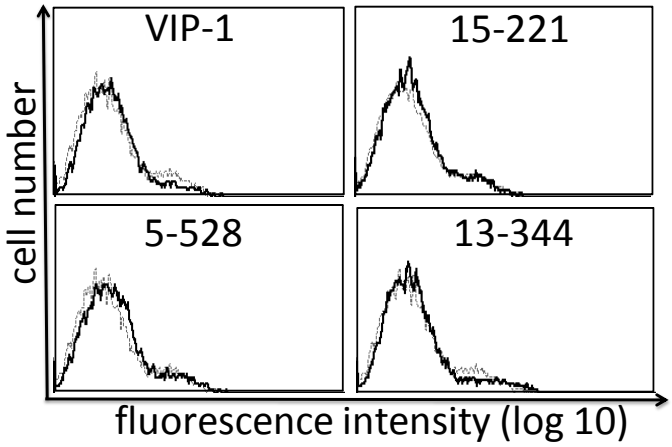

Figure 14: Cells of the insect species *spodoptera litura* have been taken as a representative to test the binding capacity of mAbs VIP-1, 15-221, 5-528 and 13-344 in a non- mammalian cell approach. The staining occurred according to the standard protocol.

4.3. Human leukemia cell line proliferation is not influenced by CD71 mAb treatment

The staining ability of our TfR antibodies confirms the effective conjugation especially on surfaces of diverse human lymphoma variants. The important function of iron in the cell growth turned the TfR into an important target in the cancer therapy. Numerous mAbs directed against the iron absorption mechanism have already been described [69, 70, 72]. In order to test the presented antibody in this regard, we incubated treated B and T cell lymphoma with 3 different concentrations of CD71 antibodies. In regard to the proliferation, neither the erythroleukemia cell line K562 nor the Jurkat T cell or the Hodgkin`s lymphoma KM-H2 cells was inhibited by antibody treatment (Figure 15). Concerning the incorporation of methyl-3-thymidine into the DNA during the cell population growth, the scintillation detector recorded similar counts for antibody incubation and non-treated cells. The spreading of the Burkitt lymphoma Raji offered in the same way no effects related to the antibody treatment (data not shown).

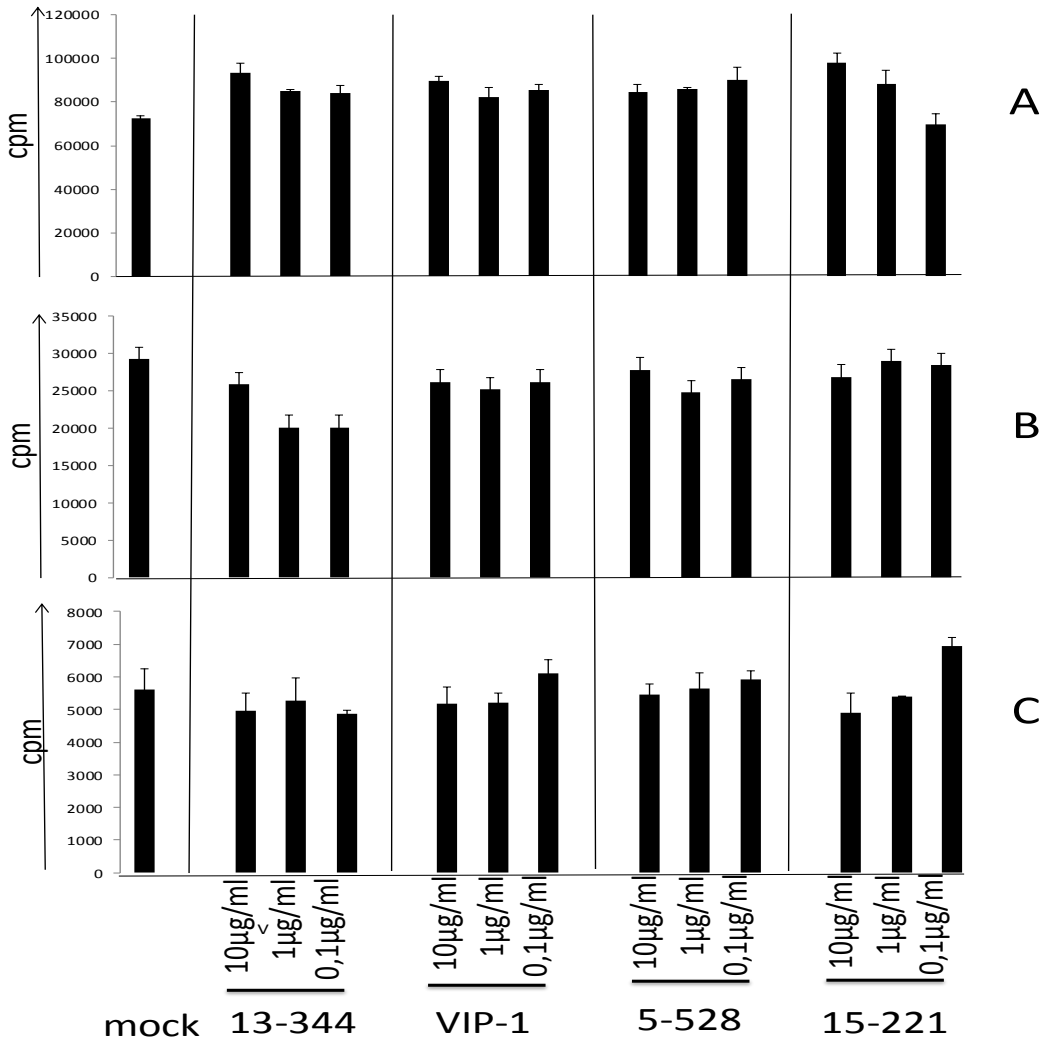

Figure 15: Human lymphoma cell lines were cultivated in 1640 RPMI medium including 10% FCS. For the growth analysis in the presence of CD71 antibodies $3*10^4$ Jurkat (A) (n=1), KM-H2 (B) (n=1) and K562 cells (C) (n=3) were cultivated in 24- well plates. Jurkat cells grew for 72 hours, KM-H2 cells and K562 cells for 24 hours. The influence of antibodies on the lymphoma accretion was tested in 3 concentrations: 10 µg/ml, 1 µg/ml and 0,1 µg/ml. For each condition, antibodies were pipetted into triplets and the cell growth monitored via the incorporation of methyl-3H-thymidine. Mean ± SD of triplicate measurements is represented.

4.4. Molecular characterization of epitope specifity

4.4.1. mAbs VIP-1 and 5-528 bind to overlapping epitopes

In order to characterize the epitopes of our CD71 mAbs, cross inhibition assays were performed. Based on the fact that previously presented antibodies bind to external parts of TfRs, we used the fast dividing Reed- Sternberg cell type KM-H2 for the accomplishment of epitope mapping analysis. For the prevention of

unspecific antibody fixation via Fc receptors, cells were preincubated with Beriglobin. The addition of CD71 mAbs to cells lead to the occupation of antibody specific binding sites. Disguised epitopes are therefore not detectable for conjugated antibodies possessing identical or overlapping binding sites. For the visualization of conjugated antibodies we used flow cytometry analysis technology.

Beside the epitope occupation by its respective conjugated antibodies, mAb 13-344, as well as mAb 15-221, shows no effect on the binding intensity for any of the other mentioned antibodies (Figure 16 A and 16 B). Our cross inhibition studies showed a strong reduction in the linkage of conjugated VIP-1 antibodies to cells pretreated with mAb 5-528 (Figure 16 C). In similar way KM-H2 cells pre-incubation with VIP-1 immunoglobulin resulted in a clearly inhibited binding capacity of conjugated 5-528 mAbs (Figure 16 D).

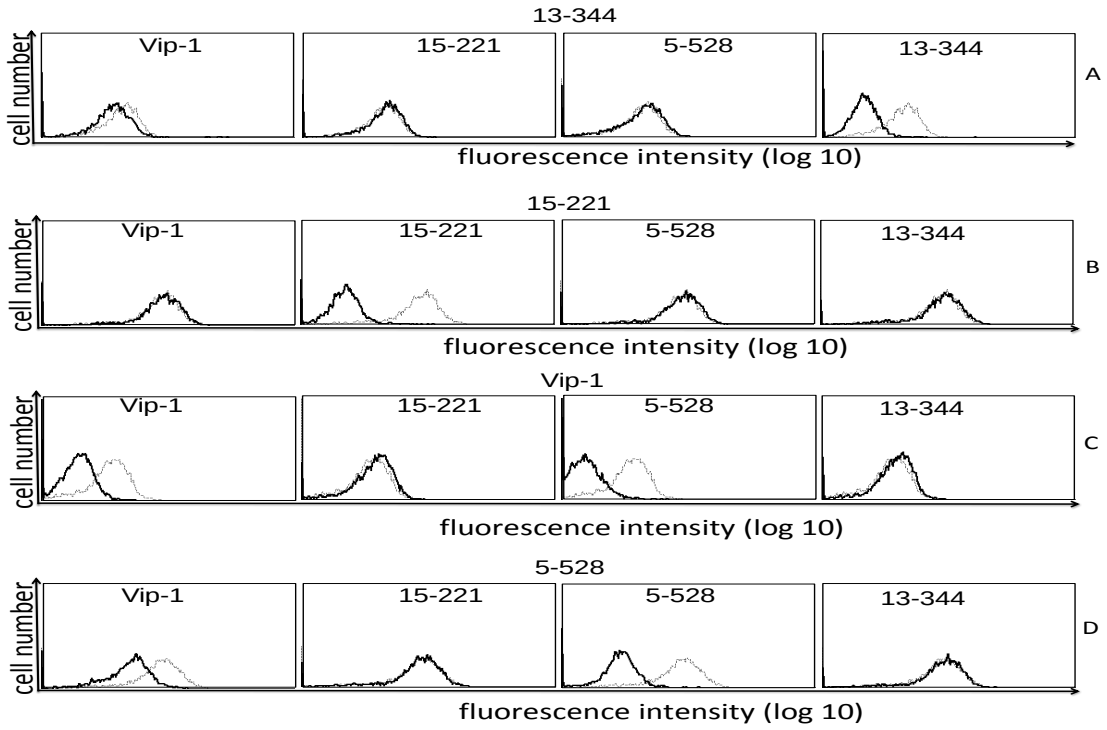

Figure 16: Combination of mouse anti- human TfR (CD71) mAbs to identify overlapping epitope regions. For the accomplishment of the cross inhibition assays, KM-H2 cells were pretreated with various CD71 mAbs for 30 minutes at 4°C (antibody names noted inside of the boxes) and thereupon stained using the fluorochrome conjugated (FITC or PE) variants of each CD71 antibody (A: 13-344; B: 15-221; C: VIP-1; D: 5-528). Detection of conjugated antibodies bound to TfR epitopes occurred via flow cytometry analysis. Black lines: conjugated CD71 mAbs, gray dotted lines: negative control. One representative experiment of 10 independent experiments is shown.

However, the strong intensity of VIP-1 binding site inhibition through mAb 5-528 pretreatment is not exactly reflected by mAb VIP-1 pre-incubated cells stained via conjugated 5-528 antibodies. These results lead to the assumption that mAbs VIP-1 and 5-528 bind to overlapping epitopes, but mAb 5-528 covers TfR sequences shared by both antibody variants with higher efficiency. The non-overlapping characteristics of the half of our tested antibodies (15-221 and 13-344) reflect the enormous range of 671 amino acids of the extracellular receptor domain [7].

4.4.2. Immunoprecipitation of CD71 protein from the Raji cell line recognized by CD71 mAbs

Since we observed overlapping epitope regions recognized via mAbs VIP-1 and 5-528 on the cell surface of KM-H2 cells, we were interested in a more detailed characterization of the sequence areas bound by mAbs VIP-1, 15-221, 5-528 and 13-344. For epitope precipitation from the human Raji B cell lymphoma line we used sheep anti mouse dyna beads coated with human CD71 mAbs. The immune staining of extracted cell proteins reflected the data obtained with cross inhibition assays. A strong reduction of fluorescence intensity was detectable for conjugated variants of identical antibodies that were used prior to this staining for the protein isolation process. TfR fragments assigned with mAb VIP-1 resulted in an obvious weaker fluorescence signal for mAb 5-528 isolated proteins as well as for the precipitation using mAb VIP-1 (Figure 17 A). Similarly, when mAb 5-528 was used for the protein staining, we observed a reduced fluorescence signal also for protein precipitation via mAb 5-528 but also for mAb VIP-1 (Figure 17 C). Immunostainings with mAbs 15-221 or 13-344 showed no decrease in the fluorescence intensity for protein isolates accomplished with any of the other three antibodies.

The similarity between the results of the previously described epitope mapping and immunostaining of precipitated proteins of the latter confirmed that mAb 5-528 and VIP-1 share overlapping epitope sequences. Nonetheless the stronger inhibition for the binding of antibody mAb VIP-1 while the epitope occupation with mAb 5-528 observed in epitope mapping analysis and therefore the higher sequence recognition affinity for mAb 5-528 as for mAb VIP-1 were not confirmed in the immunostaining of precipitated antigens. Protein extraction by

using mAb 5-528 resulted in similarly fluorescence intensity values for the staining with conjugated antibody mAbs VIP-1 and 5-528. Similar fluorescence data were obtained for the TfR isolation with antibody mAb VIP-1.

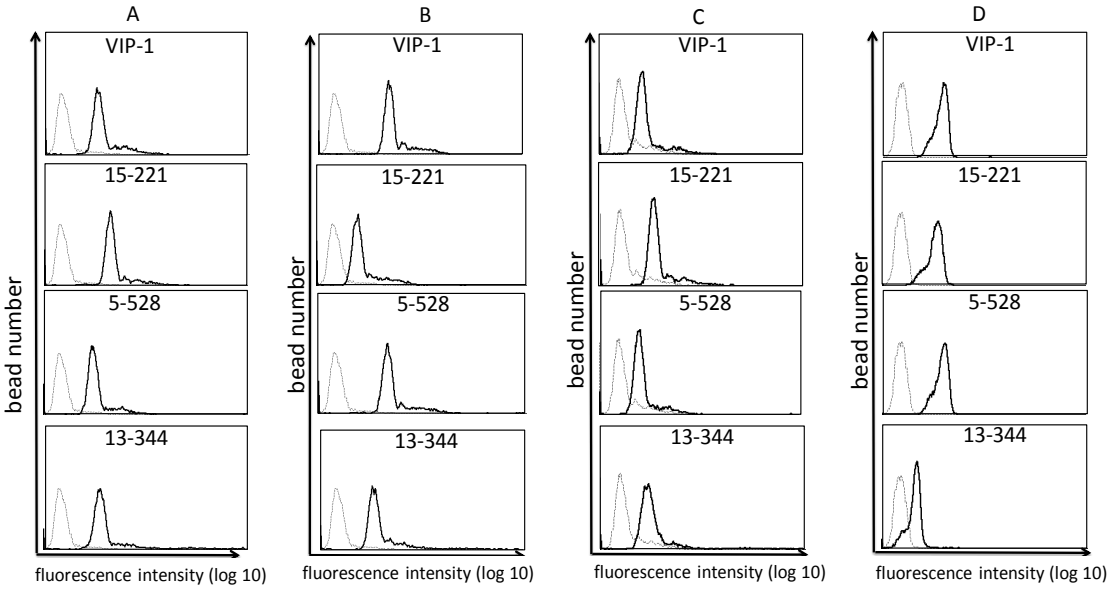

Figure 17: Extraction of Raji protein sequences detected by TfR mAbs. Lyses of cultivated Raji cells resulted via mixture with RIPA buffer for 1 hour at 4°C. The lysate was centrifuged, cell debris discarded and the supernatant kept ready for the incubation with prepared dyna beads. Sheep anti- mouse dyna beads were coated with 10 µg/ml CD71 mAbs, washed and rotated with extracted Raji cell proteins overnight at 4°C. Beads were coated with: A: VIP-1, B: 15-221, C: 5-528 and D: 13-344. Protein parts bound to the coating antibodies were stained using conjugated CD71 mAbs (antibody names inside the rectangles). The fluorescence intensity is compared to the negative control staining (gray, dotted graphs). One representative experiment of two independent experiments is shown.

In order to characterize CD71 mAbs linked proteins, we determined the molecular weight of the antigens coupled to the beads/ antibody complex. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS—PAGE) technique enables the separation of proteins in regard to their size and charge and therefore the identification of varying peptides. To gain specific information about the proteins recognized by all four mAbs, we separated antibodies from the beads via high speed centrifugation and analyzed bound proteins via SDS gel electrophoresis. Figure 18 shows the peptide distribution visualized through the silver staining method. Protein extracts of CD71 mAb VIP-1 coated beads (second lane from the right border) plus peptides isolated through the other

three tested mAbs possess a molecular weight of 95 kDa (Figure 19, rectangle framed). A second peptide fraction was identifiable at the 75 kDa band (Figure 19, circular framed) on the gel. The 95 kDa comes up with the molecular weight of the membrane anchored variant of TfR monomers, whereas the 75 kDa bands refer to the soluble form of TfR proteins. The soluble TfR variant is described as a truncated variant of CD71 proteins and has been detected at both 75 kDa [67] and 85 kDa [3]. The band pattern at the same size for all of the tested CD71 mAbs confirms the recognition of the same peptide. The precipitation process was further achieved with an antibody that does not bind to human proteins and served as isotypecontrol.

For the characterization of the total assemblage of the TfR protein expression of diverse human and murine B or T cell lymphoma, cells were lysed before TfR proteins were separated and blotted onto a nitrocellulose membrane by using the SDS-PAGE technique. To monitor whether the proteins transfer worked efficiently, we used mAb 8-301 that is directed against the cytoplasmic region of the prototypic transmembrane tyrosine phosphatase CD45 [89]. As a second control antibody we detected MHC I proteins. However, membrane incubation with murine antibody VIP-1, 15-221, 5-528 or 13-344 did not lead to the detection of any CD71 proteins within any of the cell lysates.

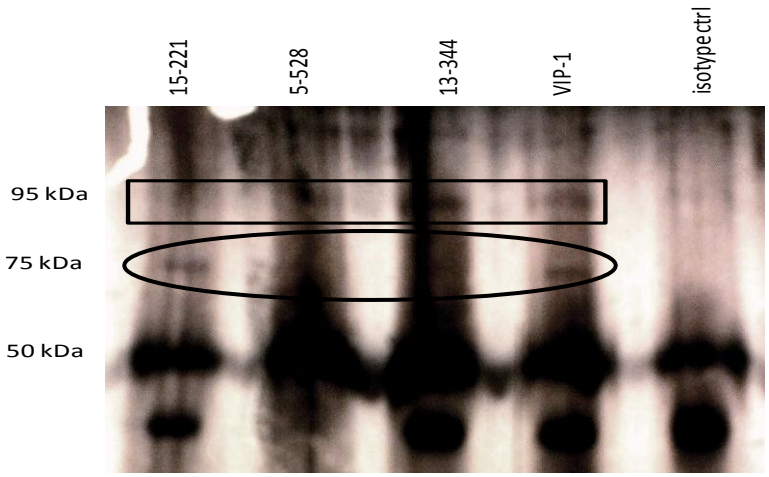

Figure 18: Antigen conjugated to antibody coated dyna beads were separated, loaded on a 10% SDS gel and visualized via silver staining reagents. Protein extractions were electrophoresed under reduced conditions. Precipitates of the already characterized mAb VIP-1 act as positive control whereas proteins isolates with VIAP, a calf intestine alkaline

phophatsase- specific antibody, served as negative control. The strong distinguished bands at 50 kDa show the heavy chain fragments of respective antibodies.

4.5. CD71 is upregulated on activated dendritic cells

As shown before, mAbs VIP-1, 15-221, 13-344 and 5-528 bind strongly to activated and fast proliferating human lymphoma cell lines. Stimulated cells derived from myeloid progenitors are important mediators of the innate and adaptive immunity and some of them are known to accumulate iron. Brinkmann et al. reported 2007 [5] that non- activated CD14+ dendritic cells (DCs) freshly isolated from human blood samples show higher TfR expression than comparable tissue cells. However, stainings via our tested CD71 mAbs resulted in only limited TfR detection on the cell surface of non-activated monocyte derived DCs (Figure 19 A). To analyse the TfR upregulation during the activation of human DCs, the bacterial components peptidoglycan (PGN) or lipopolysaccharide (LPS) or the human rhinovirus 14 (HRV14) were provided as respective stimuli. Labeling of conjugated antibodies targeted to the TfR happened 48 hours after stimulation. On the strength of antigen detection (e.g.: LPS, PGN…) as well as the linkage of cytokines (TNFα, IL-1…) to its respective receptors lead to a structural reorganization of DCs. This reorganization concerns mainly the conversion of antigen detecting cells into professional APCs and in correlation with this to an increased surface expression of lymphocyte co- stimulatory ligands, adhesion molecules and antigen presenting complexes [83]. Human leukocyte antigen- DR (HLA-DR) is the human homolog to the murine MHC class II peptide complex and is strongly expressed on surfaces of professional APCs. The strong appearance correlates with an increased production and minimized inclusion of these proteins as well as the nearly 100 fold expanded half life time [83]. None- stimulated DCs exhibit a relative high level of HLA-DR mRNA in the cytoplasm, but only a limited number of surface proteins [83].

This statement is also confirmed by the reduced HLA-DR receptor detection on the surface of DCs in contrast to LPS or PGN stimulated and structural modified APCs (Figure 19, A-C). Similar data were obtained for the surface staining of the TfR, whereupon the fluorescence signal for CD71 proteins was much lower

than for the expression of HLA-DR peptides. Activation of DCs via LPS and PGN (Figure 19 B + C) resulted in strong expression of TfRs detected by all tested CD71 mAbs as well as from the antigen presenting peptide HLA-DR. Contrarily, when DCs were mixed with HRV particles, mAb VIP-1, 15-221 and 5-528 showed only a very weak reactivity with antigens on DC surfaces. Equally, the activation marker HLA-DR shows no upregulation on the cell surface. Compared with other tested CD71 mAbs which are added under the same stimulatory conditions, the fluorescence intensity of mAb 13-344 increases only minimal.

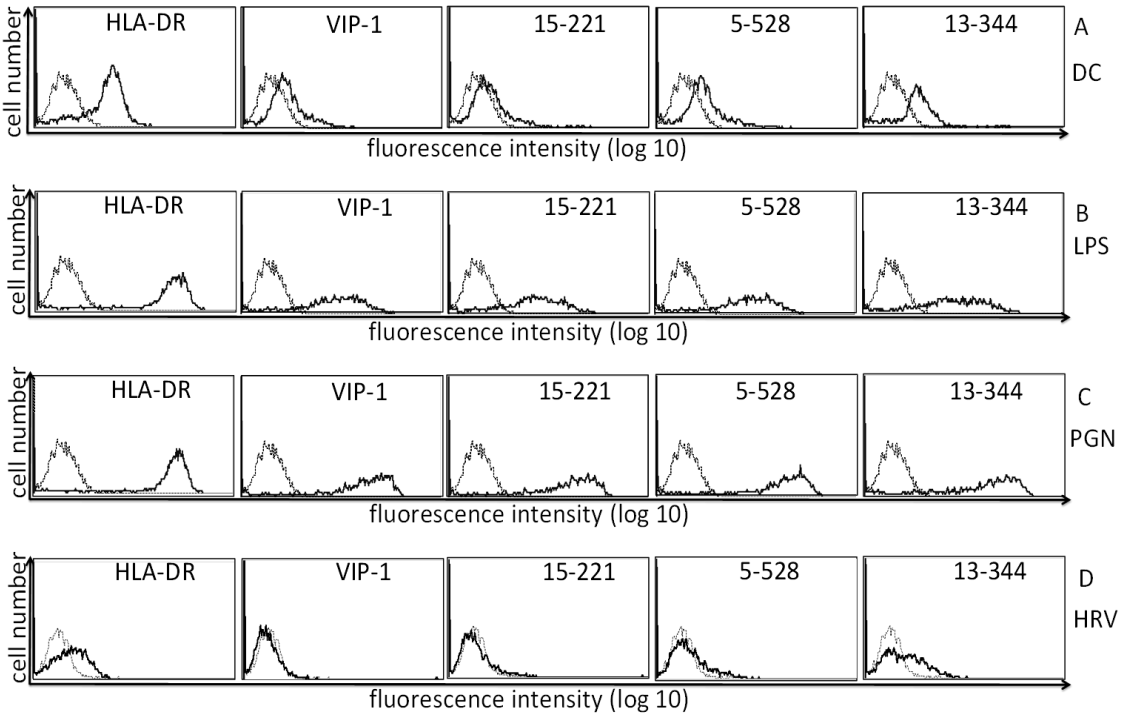

Figure 19: Comparison between non-stimulated (A) and activated (B-D) DCs concerning the expression of surface TfRs. DCs were maintained by cultivation of monocytes with IL-4 and G-CSF in 1640 RPMI medium supplemented with 10%FCS for 5 days. Activation followed by addition of LPS, PGN or HRV for two days, before DCs were stained and analyzed via flow cytometry. One representative experiment of two independent experiments is shown.

4.5.1. The activation process of dendritic cells is not influenced by any of the tested mAbs

Because of the evidence of the high appearance of CD71 mAbs on the cell surface of stimulated DCs, we were interested to verify whether the activation state is affected by any of the CD71 antibodies. For that purpose DCs were repeatedly stimulated with LPS, PGN or HRV14 and simultaneously treated with

respectively one immunglobuline variant of our tested mAbs (Figure 21). The stimulation via PGN or LPS resulted in a strong expression of activation molecules CD1a, CD40 and HLA-DR on the cell surface (Figure 20). CD40 is known to be highly expressed on APCs and its attachement to ligands on B or T cells is associated with the expression of costimulatory molecules on both APCs and lymphocytes [84]. Costimulatory molecules again are important to prevent the anergic behavior of lymphocytes. CD1a and HLA-DR surface molecules are both highly expressed on APCs with similar functions in the presentation of antigens. CD1a belongs to the CD1 group of MHC like structures and present mainly lipid components of the cell membranes of diverse microorganisms [85].

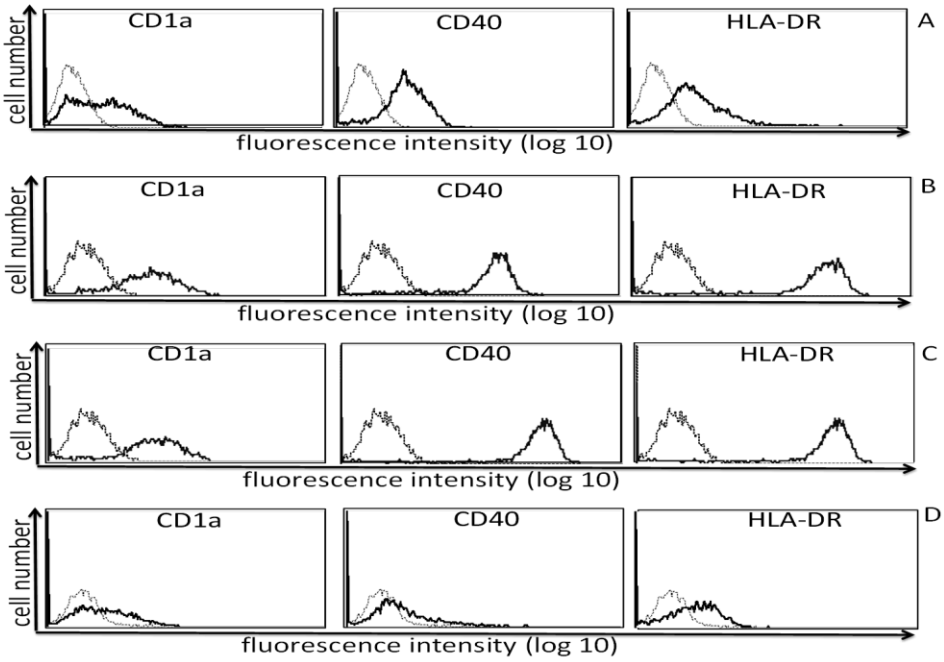

Figure 20: Quality control of DC stimulation by analyzing three surface proteins that are highly expressed in the activated state. Non stimulated cells (A) are opposed to LPS (B), PGN (C) and HRV (D) stimulation for 48 hours. Detection of surface receptors occurred with conjugated CD1a, CD40 and HLA-DR antibodies (antibody names in respective boxes). One representative experiment of two independent experiments is shown.

The binding capacity of labeled antibodies on the surface of non- stimulated DCs for all three activation marker is marginal compared to the strongly increased fluorescence intensity for LPS or PGN stimulated APCs (Figure 20). Figure 21 demonstrates that none of the TfR antibodies compromise the intensity of the activation process from LPS stimulated DCs. Similar data was

obtained for the stimulation via PGN. However, activation of DCs via human rhinovirus (R-DC) resulted in clearly reduced expression of all three activation markers compared to the other stimuli (Figure 20 D). Given that DCs are not activated in the presence of HRV and based on the fact that stimulated as well as non- stimulated DCs are not influenced by the treatment with any of the tested CD71 mAbs, we were not surprised to note as well no effects of antibody treatment simultaneously to HRV stimulation.

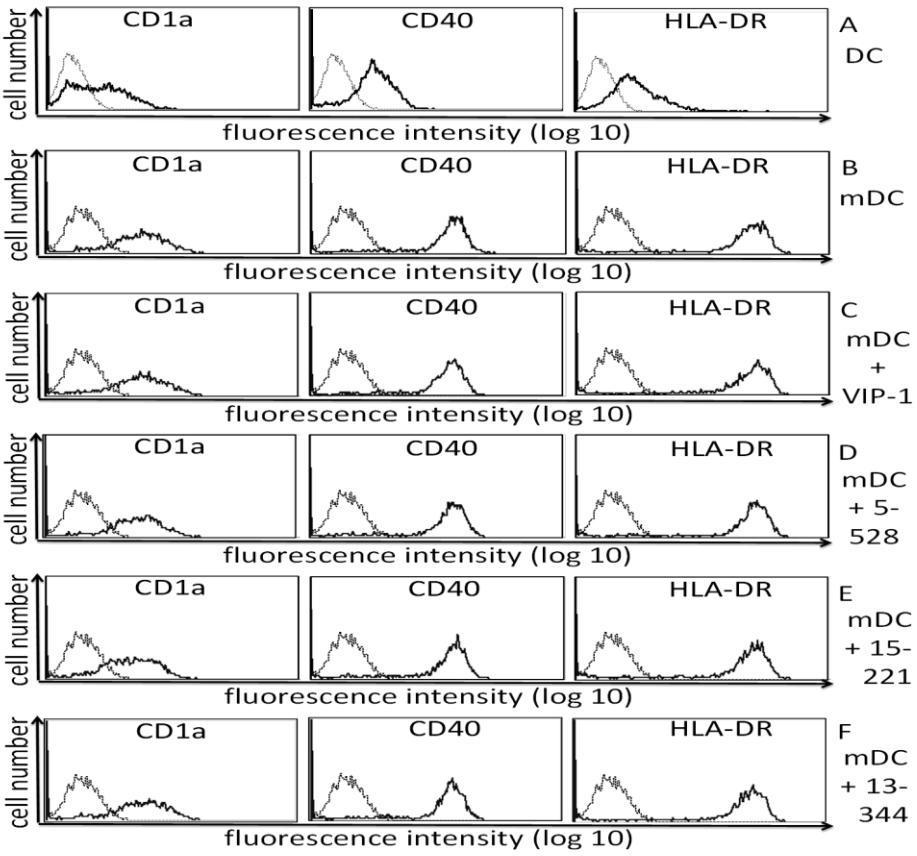

Figure 21: Activation profile of DCs treated with LPS for 48 hours alone (A) or in combination with CD71 mAbs (C-F). Activation of DCs happened also including PGN or HRV (data not shown) for stimulation. None of the tested Tfr binding antibodies reduce the upregulation of activation surface molecules. One representative experiment of four independent experiments is shown.

4.5.2. Treatment of stimulated DCs with CD71 mAbs has down modulating effects on the surface TfR expression

After observing that stimulation and simultaneously incubation of mAbs did not lead to alterations on the DC activation behavior, it was of interest if TfR surface expression is influenced during activation plus simultaneously CD71 antibody

treatment. Therefore dendritic cells were mAb treated and stimulated with LPS, PGN and HRV as described before and afterwards marked with conjugated CD71 mAbs.

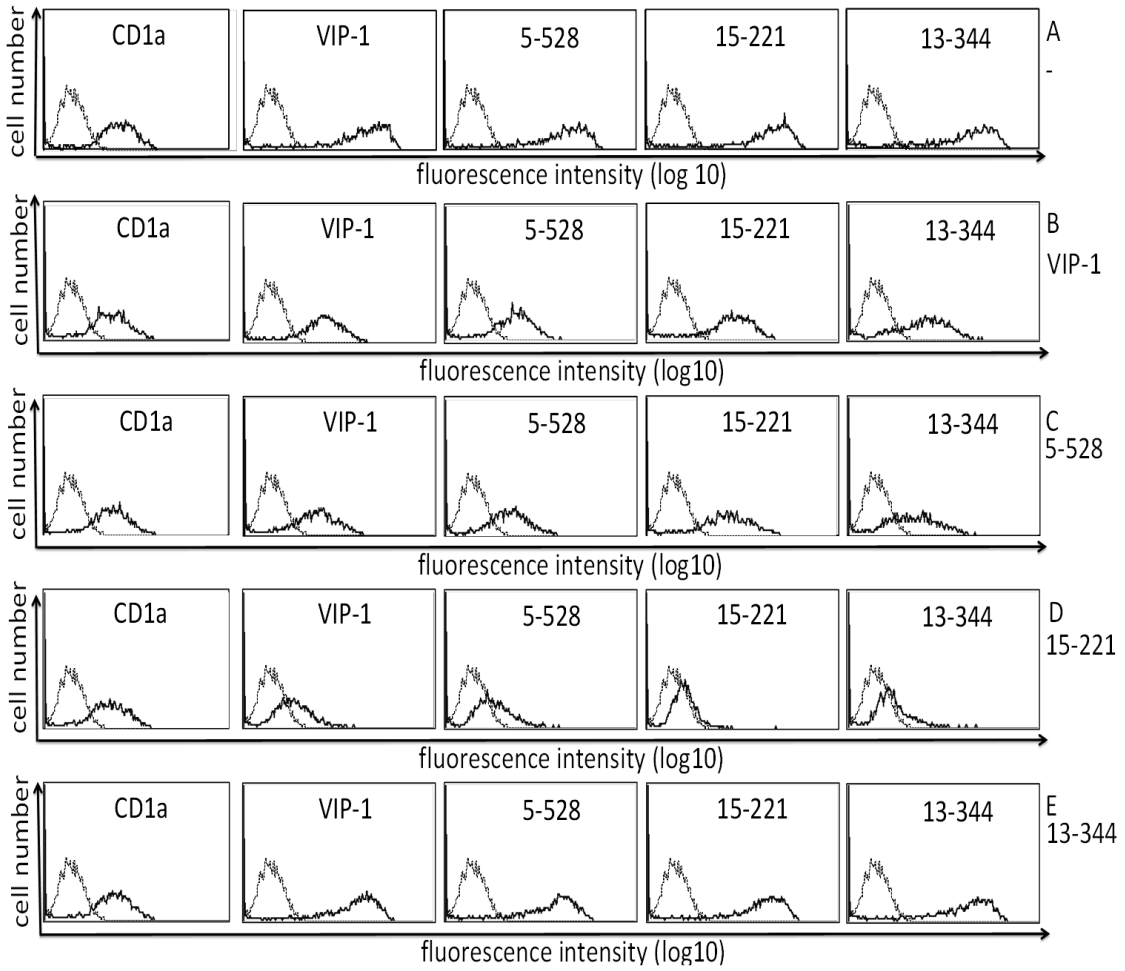

Figure 22: Characterization of the TfR surface expression on 48 hours PGN stimulated DCs combined to respectively one of the mAbs (clone names specified on the right side). All of the tested CD71 mAbs cause down regulatory effects on the surface expression of TfR proteins. The strongest inhibitory effect is detectable for treatment with mAb 15-221 (D), whereas mAb 13-344 (E) addition to cultivating DCs has moderate consequences. One representative experiment of three independent experiments is shown.

Pre-incubation of CD71 mAbs result in a clear reduction in the surface TfR emergence. The strongest down regulatory effect was detectable for DC treatment with mAb 15-221 (Figure 22 D) followed by mAbs 5-528 (Figure 22 C) and VIP-1. (Figure 22 B) mAb 13-344 (Figure 22 E) on the other hand shows only a limited effect on the surface arrival of TfR proteins. The lower count of surface receptors for the respective antibody therapies was obtained in an

identical order for LPS stimulation. However, DC incubation of HRV combined to CD71 mAbs showed almost no effects given that HRV stimulation alone lead to only a weak detection of CD71 surface molecules. The reduced expression of TfR proteins on surfaces of stimulated cells lead to the assumption that incubation of antibodies directed against the TfR results in the accumulation of the receptor proteins in the cell interior, maybe through the inhibition of the recycling mechanism back to the cell surface. The theory of receptor protein accumulation in the cell interior is confirmed by the in vitro study of the TfR internalization process through the conjugation of mAb 13-344 on the DC surface [82].TfR antibodies do not alter the cytokine secretion of dendritic cells

After 48 hours of respective CD71 antibody plus LPS, PGN or HRV14 stimuli incubation, the cytokine dispersion of the DC supernatant was determined via LUMINEX. Based on the activation of DCs, the total cytokine production is increased for stimulation with the bacterial components LPS and PGN. But still only slight effects induced by TfR mAbs were noted for any of the secreted cytokines. Minimally reduced cytokine data were observed for IL-8, IL-12p40 and IL-12p70 for the treatment with mAb 15-221. On the other hand a slightly increased IL-1β expression was observable when DCs were incubated in combination with mAb 13-344 (Figure 23). These cytokine variances are only in a small range and it seems to be unlikely that these aberrations affect the behavior of any cells. Nearly no cytokine secretion was detectable for HRV stimulated DCs (unpublished results).

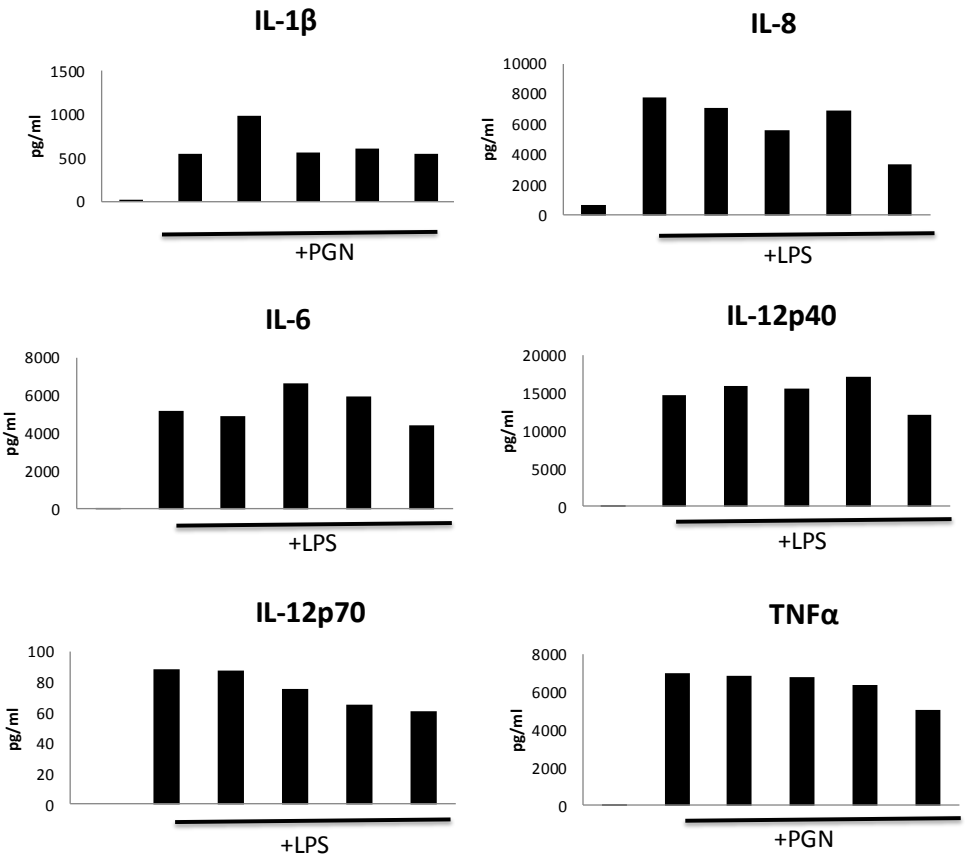

Figure 23: The combination of stimuli plus CD71 mAbs were incubated to DCs for 48 hours and analyzed via LUMINEX. Respective stimuli are denoted under the CD71 mAbs. Results of one representative experiment of three independent experiments are shown.

4.6. T cell proliferation mediated by activated plus CD71 mAb pretreated DCs is not affected

For the activation process of DCs as well as for T cells it is probable that iron has important functions. Furthermore it is evident that this trace element is required for the function of the DNA synthesis via ribonucleotide reductase during the proliferation of each cell. Inhibition of TfRs leads consequently to a non-functional T cell increasement. However, the previously collected data showed no effects of CD71 mAb treatment on the activation process of DCs. To test the implication of antibody incubation on the activation process of T cells mediated by alloreactive APCs, DCs were again mixed with respective antibodies and simultaneously activated via LPS, PGN or HRV14. For the initiation of the mixed leukocyte reaction (MLR), T cells were added 48 hours

after DC treatment and incubated for 5 days. Stimulation of DCs implicated in the rise of methyl-3H-thymidine integration into newly formed T cells from 10 000cpm up to 50 000cpm, whereupon the highest values are attained for PGN stimulation (Figure 24). Equally to the previous mentioned data of the DC activation by using different stimuli and compared to the other stimuli we detected also a strongly limited T cell proliferation by DC stimulation with HRV14. Concerning the effects of antibody treatment, we observed only minimal fluctuations in the thymidine incorporation between the different mAbs. The degree of the alloreactive reaction among non-treated DCs and those incubated with one of the four mAbs is equally low. This data confirms prior microscope observations where neither proliferation enhancement nor inhibition between the various antibodies has been noted. In summary none of the tested CD71 antibodies combined to different stimuli during the pre-incubation process with DCs shows any implications on the T cell proliferation during the MLR.

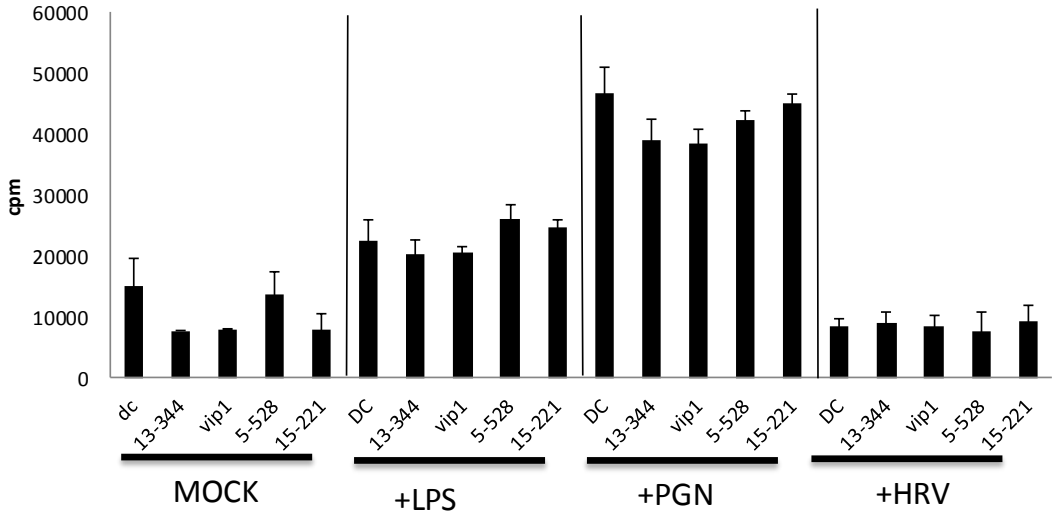

Figure 24: Monitoring of the T cell proliferation mediated by two days CD71 antibody pre-treated DCs. Various antibodies and stimuli were combined and mixed to DCs cultivated in 1640 RPMI medium including 10% FCS. 48 hours later the mixed leukocyte reaction was initiated by $5*10^5$ preprocessed DCs mixed with 10^5 T cells. For the determination of the T cell proliferation, methyl-3H- thymidine was added 5 days later. Integration of methyl-3H-thymidine into the newly formed DNA was detected via scintillation counter. Results of one representative experiment of two independent experiments are shown. Mean ± SD of triplicate measurements is represented.

4.6.1. MAb 15-221 or VIP-1 directly added at the beginning of the mixed leukocyte reaction efficiently inhibit T cell proliferation

In a second mixed leukocyte approach, DCs were activated in the same way as described above but unlike to the first assay, antibodies directed against TfR proteins were added simultaneously with the T cells at the beginning of the MLR. Similarly to the first approach we observed the strongest T cell proliferation induction via DC stimulation with PGN followed by LPS activation. Addition of TfR antibodies at the same time point as T cells resulted in a clear inhibition of T lymphocyte proliferation in MLRs incubated with antibodies VIP-1 or 15-221. Contrarily a slightly enhanced T cell expansion is observable for the treatment with mAb 13-344. Both inhibition and enhanced T cell proliferation are independent of the stimuli variant. These data are opposite to the result of the first MLR and raise the question of which part of the IS is influenced by the addition of CD71 mAbs directly at the beginning of the MLR. Given that we found no influence of antibody treatment on the DC activation process, we assumed rather a correlation with a nonfunctional T cell activation and out of it resulting proliferation process.

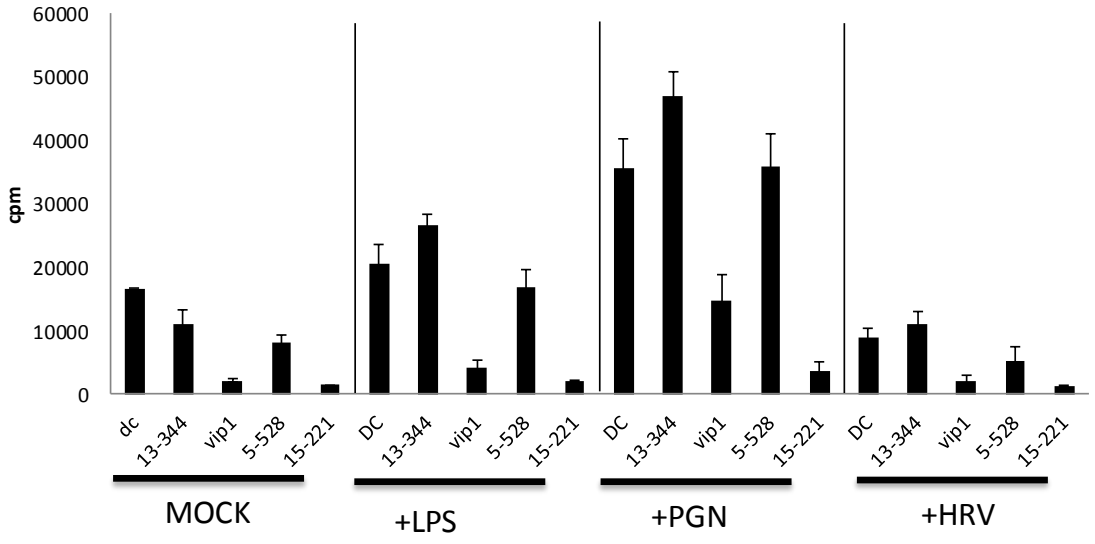

Figure 25: Previously activated DCs were combined to T cells of a different donor to set up a mixed leukocyte reaction (MLR). Stimulated DCs were rendered in a 96 well-plate and TfR antibodies were subjoined simultaneously with T cells in 96 well plates. Methyl-3H-thymidine was added after five days of incubation and the degree of cell incorporation analyzed.

Results of one representative experiment of two independent experiments are shown. Mean ± SD of triplicate measurements is represented.

4.7. In vitro stimulation of T cells in the presence of CD71 mAbs influences cytokine production and proliferation

The observation of the strongly reduced T cell multiplication while mAb VIP-1 and 15-221 treatment at the outset of the MLR is contrary to the other so far obtained results. Indeed all of our tested antibodies showed the capability to bind to human cell surfaces but none of them possess inhibitory functions concerning the proliferation of human B and T cell lymphoma. Moreover the incubation of DCs with TfR antibodies in combination with diverse stimuli manifested in the same way no influences in the maturing process into professional APCs. These facts indicate that mAb VIP-1 as well as 15-221 block a supposed iron dependent intermediate step during the T cell activation process. To test this assumption we stimulated T cells via plate bound CD3 (OKT3) antibodies alone or in combination with CD28 or CD63 co-stimulatory antibodies. Additional soluble CD71 mAbs were added before the cytokine level in the supernatant and the intensity of T cell increasement were determined 48 hours after initiation of the stimulation process. A clear reduction of the cytokine secretion was detectable for IL-10, IL-13, IL-17 plus IL-22, when T cells were treated with mAb VIP-1 or 15-221. In most of the data we observed a stronger reduction in the cytokine production of mAb 15-221 incubated T cells than for the treatment with mAb VIP-1 (Figure 23). Equally considerably lower cytokine levels were perceived for the IL-2 secretion after the incubation with mAb 15-221.

IFNγ is produced by various cells of the innate immunity (mainly APCs and macrophages) and its detection by type II receptors lead to an increased differentiation of T_H4^+ cells into the T_H1 subgroup and controls the activation of cells mainly of the innate immunity (e.g. NK cells) [58]. However, the main producer cells of IFNγ are T_H1 cells, a subcategory of the T helper lymphocytes [23, 58]. Indeed the analysis of the T cell supernatant resulted in strongly increased levels of IFNγ levels but contrary to nearly all the other cytokines we detected barely any reduction for the T cell treatment with any of the tested CD71 mAbs. A second apparently not affected cytokine expression concerns

the volume of the IL-4 production. Beside mast cells, the highest part of IL-4 is established via the T helper lymphocyte derived T_H2 cells [53]. As expected, in vitro stimulation of T cells showed a high secretion of IL-4, but none of the antibodies demonstrated a clearly noticeable effect on the cytokine production process. The variance of activation stimuli between CD3 plus CD28 or CD3 plus CD63 also did not change the properties of CD71 mAb treatment on the T cell cytokine secretion pattern.

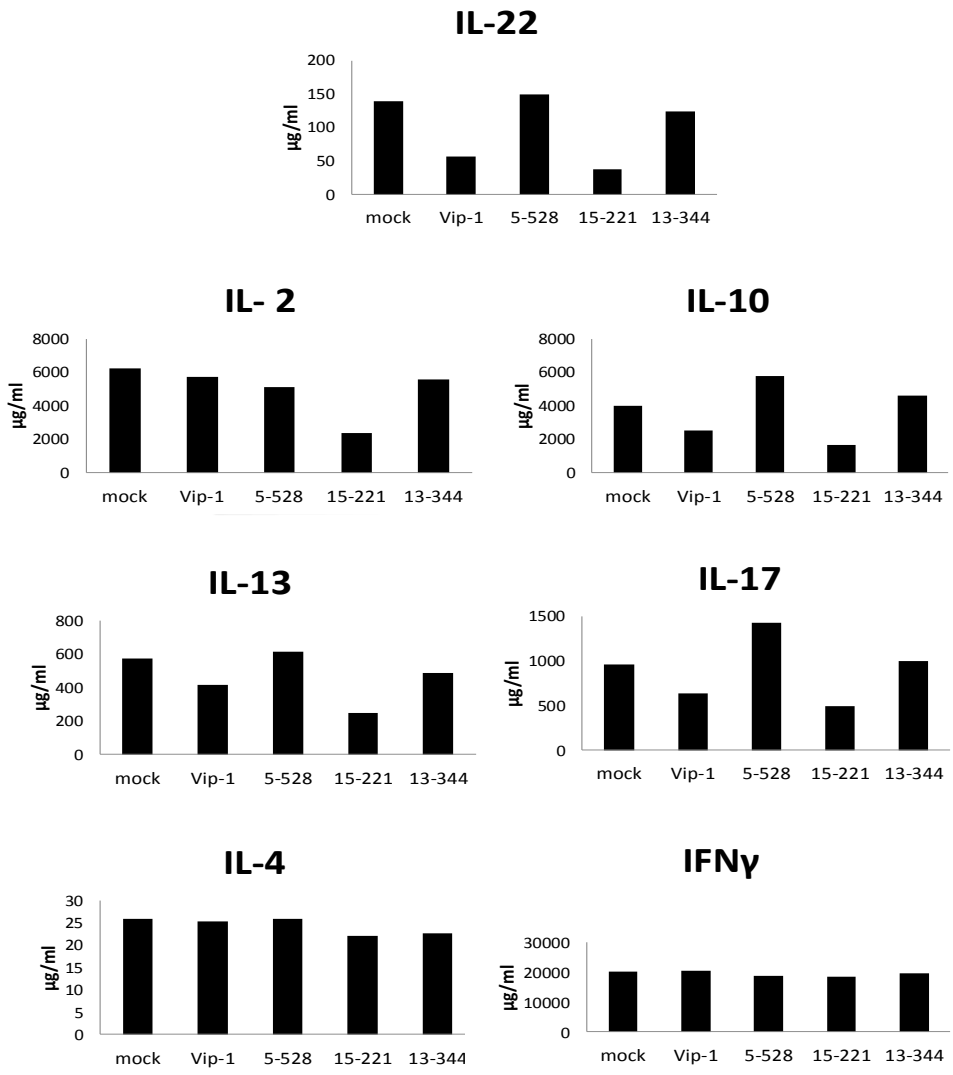

Figure 26: T cell stimulatory CD3 (OKT3) immunoglobulins alone or in combination with CD28 co-stimulatory antibodies were fixed on the bottom of 96- well plates and incubated with 10^5 T cells plus soluble CD71 mAbs for 48 hours. T lymphocytes treated with mAb VIP-1 or 15-221 exhibit a clear reduction in the secretion of IL-10, IL-13, IL-17 and IL-22, whereas the level of IL-2 cytokines is mainly reduced by treatment with mAb 15-221. Contrary none of

the tested antibodies influence the IL-4 and IFNγ cytokine secretion. Similar results were obtained for the combination of OKT3 plus CD63 antibodies. Results of one representative experiment of two independent experiments are shown.

The evaluation of the T cell proliferation accords with the results obtained from the cytokine analysis. For all three stimuli combinations T cell propagation is potently reduced for the respective addition of one of the two mAbs VIP-1 or 15-221. The highest T cell augmentation was detectable for the antibody combination OKT3 plus CD28. In all assays we detected a higher decrease for the treatment with antibody 15-221 than for mAb VIP-1.

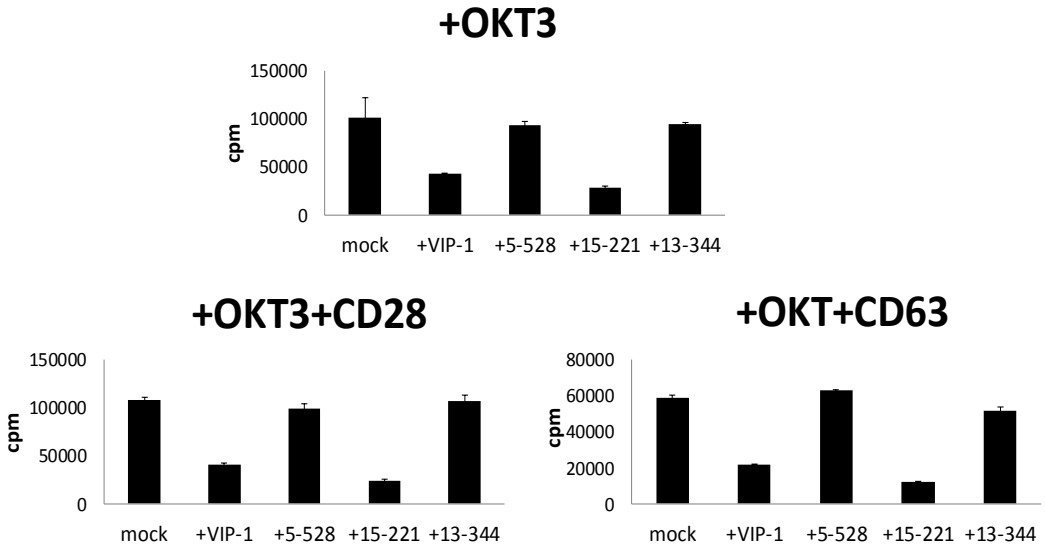

Figure 27: In vitro T cell proliferation assay mediated by plate bound CD3 antibodies alone or joined with either CD28 or CD63 antibodies. T cell proliferation was stimulated by plate bound antibodies directed against CD3 chains of the TCR alone or in combination with antibodies targeted on co-stimulatory ligands. The T cell augmentation was analyzed after 48 hours incubation and detected via incorporation of methyl-3H-thymidin into newly formed DNA 18 hours after addition to the proliferation assays. Results of one representative experiment of two independent experiments are shown. Mean ± SD of triplicate measurements is represented.

5. DISCUSSION

Iron is essential functions for the activity of diverse enzymes, whereas abnormal regulation implicates harmful effects through low or high cellular concentrations. The integration process of iron into the cell interior depends on the endosomal absorption of iron loaded Tf proteins bound to TfRs on cell surface. This study was constructed on four murine mAbs that recognize epitopes of the human TfR (CD71). In the first part of the study we confirmed that all mAbs are specific for CD71. Binding studies on fast dividing B and T cell lymphoma showed the binding of mAbs VIP-1, 15-221 and 5-528 on the surface of Daudi, Raji, K562 and also on Jurkat cell. Antibody 13-344 bound to murine AKR and BW cell lines as well as to above mentioned human cell lines. MAb VIP-1 was already known to recognize specifically human CD71 proteins [78- 80] and was therefore used as a positive control. The murine BW cell line expressing the human surface TfR is similarly recognized by all four tested antibodies and exhibit similar fluorescence intensity for staining. This was further confirmed by silver stain analysis, where isolates precipitated with CD71 mAb coated beads showed a band at molecular weight similar to that of CD71 monomers. The upregulation of TfRs on the surface of pre-stimulated DCs have been already reported [24, 27] and was also further confirmed in this study. Previously characterizations of mAb 13-344 in our lab showed further a strong conjugation to pre-stimulated DCs [82]. Addition of CD71 mAbs –VIP-1 or 15-221- directly to a MLR resulted in a strong inhibited T cell proliferation. These results were surprising, because co-cultivation of monocytes with mAb VIP-1, 15-221, 13-344 or 5-528 showed influences neither on the differentiation of DCs nor on the production of cytokines. Hence we were interested if inhibitory effects are via DCs by delaying activation signals or probably by down-regulating of costimulatory surface molecules. Another possibility could be also its effect via T cell activation. Similarly, in an in vitro stimulation assay we were able to demonstrate an inhibitory effect of both antibodies VIP-1 and 15-221 on the T cell proliferation.

Probably the most prominent CD71 mAb described in the literature, 42/6 represses efficiently Tf attachment to its receptor on many human cell types

[70-73]. Similarly to other CD71 mAbs, the mechanism for the inhibition of this IgA isotype is not completely clear. One theory acts on the assumption that mAb 42/6 causes sterical changes in the extracellular part of the receptor [73], whereas other opinions are based on a competitive occupation of nearby areas of Tf binding sites and thus inhibiting the attachment of iron loaded Tf on the receptor [70]. Multivalent mAbs mainly directed against murine CD71 determinants are supposed to cross-link TfRs on cell surfaces. It has been also shown that monomeric antibodies of the IgG isotype enhance the degradation of surface TfRs on murine tumor cells [49, 72]. Concerning the formation of the IS, numerous mAbs directed against the TfR on the surface of T cells are described [31]. Antibody FG 2/12 binds proximate to the TF binding site and seems to reduce the binding areas for DC through sterical retardation of Lck and CD3ε conglomeration. Addition of mAb FG 2/12 seems therefore to inhibit signal transduction prior IS formation [31]. Another CD71 mAb, FG 1/5, has minimal effects on NK cell mediated cell lysis. The context between NK cell mediated cell killing has been detected with mAb 2/12 [87]. The CD71 mAbs tested in our study are IgG immunoglobulins similarly to those mentioned before. Cultivation of human or murine lymphomas in combination with respective CD71 mAbs did not alter the cell multiplication. The unaffected cell expansion after treatment with mAbs VIP-1, 15-221, 5-528 or 13-344 indicates that respective antibodies do not cover the stalk region or the lateral receptor domains of Tf attachment sites and therefore do not compete with Tf for binding areas. None of the four CD71 mAbs prevent the internalization process of iron loaded Tf in monocyte derived DCs and probably do therefore not compete for the iron dependent differentiation process. However, for some cell lines alternative iron uptake mechanisms can be observed. For the exclusion of the iron absorption by unknown alternative ways, we performed proliferation assays in FCS free medium in parallel to the cell augmentation in standard medium. Beside nutrients, FCS contains dissolved iron components and probably also calf Tf proteins similar to that of human. Cells incubated in the medium lacking FCS did not grow and most of them died within two days. Nevertheless it is hard to conclude that cells died exclusively based on the Tf deletion or iron restriction, because FCS contains other nutrients as well as cell growth factors.

A more detailed view on the antibody binding sites showed that mAb VIP-1 and 5-528 recognize identical epitopes. The even more attenuated fluorescence signal of VIP-1 antibodies for mAb 5-528 pretreated KM-H2 cells, changes to a less attenuated signal when KM-H2 cells are preincubated with mAb VIP-1 and stained with mAb 5-528. This demonstrates that mAb 5-528 covers a larger part of mAb common shared sequence area than antibody VIP-1. The mutual inhibition is maybe of interest for the preparation of an antibody binding map from the whole extracellular domain of human TfR proteins, but definitely for the analysis of TfR surface expression via conjugated immunglobulins.

In 2002, sequencing of the murine genome identified a tremendous sequence similarity with the human genome. TfR proteins isolated from mice and separated via SDS- PAGE indicated a molecular weight of 200 kDa (100 kDa for each monomer), that differ only in 10 kDa from human non-reduced receptor proteins [47]. The structural homology between human and mice TfR proteins may permit the detection of evolutionary conserved sequence regions in both species and probably account for the crossreaction of mAb 13-344 on human and murine CD71 proteins. Exactly as mammals, insects express ferritin and Tf proteins. The structure of Tf proteins for a few insect species exhibit also similarities with that of comparable peptides in mammalians [46], but in insects there is no evidence either for the existence of iron regulatory proteins, such as Hepcidin or ferroportin-1, or for the iron absorption mechanism via Tf cell surface receptors [48]. However the iron uptake system in arthropods is regulated, it is not surprising that none of our tested anti- CD71 mAb detected any proteins on the surface of cells from the moth type *Spodoptera litura*.

In the course of maturation from monocytes to DCs, more and more TfR proteins appear on the cell surface [24].The strong binding of conjugated CD71 antibodies after 48 hours of continuous DCs activation is in accordance with the already described strong expression of TfR on stimulated DCs. It is surprising that non proliferating cell types possess cell surface receptors in a similar fashion compared to rapidly growing lymphoma cells. The further development of lymphoid and myeloid progenitors differs in many details, starting from participating cytokines to the organ where maturation occurs. Especially for lymphocytes it is reported that iron plays important roles in the maturation

process and in the transduction of environmental signals [22, 31, 62]. Iron seems to be equally associated with the development and activation process of DCs and would demonstrate a common feature between both lineages.

DC stimulation via PGN or LPS and synchronous treatment with mAbs VIP-1, 15-221, 5-528 or 13-344 revealed no deviation in the strong expression of molecules associated with the activation process. Based on probably the important effect of TfRs during DC development plus from the results of unaffected lymphoma proliferation assays lead to suggestion that none of the tested antibodies bind on sequence areas essential for the TfR/Tf/iron complex internalization activity. Apart from the iron integration, TfRs are suspected to influence the signal transduction in lymphocytes via tyrosine phosphorylation of B- or T cell receptor associated ITAM motifs. Since DCs are non- proliferating cells, it could be speculated, that similar events account for the upregulation of TfRs on DC surfaces. Such events may influence the conversion into professional APCs or the migration to secondary lymphoid organs (e.g.: upregulation of surface proteins). Concerning the morphological changes in CD71 mAb incubated cells, no deviations were noted compared to non- treated DCs. In spite of this the appearance of TfRs increases in later developmental steps from monocytes toward DCs and suggests the requirement of iron for the formation process. The almost unchanged cytokine profile of mature DCs stimulated with PGN or LPS alone as well as in the combination to antibody treatment agree with the unaffected pattern of activation marker and confirms that none of the tested antibodies affect the maturation from DCs to APCs.

Surface stainings of stimulated DCs by associates of our lab in 2004 showed a clear reduction of the fluorescence signal after a time period between 30 and 120 minutes when samples remained at room temperature [82]. Fluorescence microscopic analysis verified the internalization and accumulation of applied antibodies 13-344 in the cell interior (Figure 28).

In a similar way we observed a clear reduction of TfR appearance on DC surfaces after the incubation with stimuli and CD71 mAbs for two days. It is likely that the decreasing fluorescence signal for TfR proteins results from the same mechanism. However the receptor mediated integration activity into endosomes has been observed independently to the attachment of Tf to its

receptor and CD71 recycle back to the cell surface within a short time period [6, 13, 14]. According to that conclusion we would not expect to observe down modulatory effects of TfR surface proteins based on the treatment with mAbs that show no effects on DC differentiation.

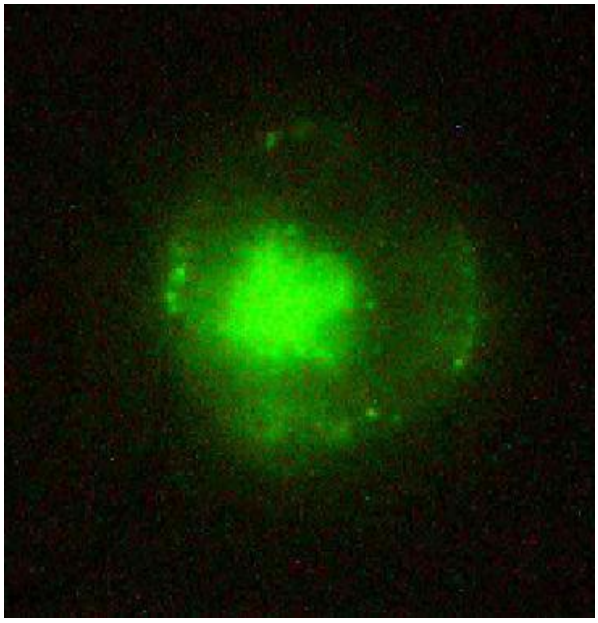

Figure 28: Accumulation of fluorescence labeled CD71 mAb 13-344 in the cell interior after incubation at room temperature for 120 minutes. Picture of the receptor mediated uptake adapted from [82].

On the other hand, activated DCs produce TfRs in large quantities and cross linkage of antibody as well as receptor molecules within the cytoplasm may prevent the recirculation to the cell surface. It is reported that TfRs possess important functions in the establishment and maintenance of IS [31]. The reduced expression of surface CD71 molecules on stimulated DCs after mAb treatment implicated no effects in regard to the activation process of T cells. Hence it is likely that after formation of the IS only a limited number of receptors are required for the maintenance of the contact sites. The results of MLRs where T cells were added to CD71 mAb pretreated DCs are in strong contrast to the second T cell proliferation assay, where antibodies were added simultaneously with T cells to pre-activated DCs. The strong repressive effect on the proliferation at mAb VIP-1 or 15-221 treated T cells were also clearly remarkable via microscopy. Based on the fact that neither VIP-1 nor 15-221 reduced the activation intensity of DCs and antibody incubation to DCs for two

days also showed no influence on the activation of T cells, we assumed a direct influence on the later cell type.

Both the high proliferation of antibody treated Jurkat T- cell leukemia cell line as well as the T cell activation by mAb pretreated DCs showed us that the cell multiplication process itself seems not to be affected. For that reason we assumed that both antibodies interrupt with activities required for the T cell activation. It is to notice that leukemia cells, such as Jurkat cells, are immortal and strongly uncontrolled concerning the cell cycle modulation and the completion of effector functions. Identically to tissue cells of healthy donors the synthesis of new DNA is dependent on iron molecules for the usage of metalloproteins, but possible consequences on the effector function are not observable.

In vitro stimulation of T cells via CD3 antibodies alone or in combination with antibodies directed against costimulatory ligands resulted in a strong T cell proliferation also in the presence of CD71 mAb 5-528 and 13-344, but not for the treatment with mAb VIP-1 or 15-221. The clear growth repression probably caused by strongly reduced activation signal transduction has also been observed in the cytokine secretion of IL-2, IL-10, IL-13, IL-17 and IL-22. The assumption of reduced activation signal based on CD71 mAbs is in conflict with the high secretion of IFNγ that comes along with the activation of T cells and fulfills important in vivo challenges in the activation of macrophages and NK cells as well as in the upregulation of MHC peptides on cell surfaces [58, 59]. IFNγ proteins are decoded by the JAK-STAT translation pathway after detection of various cytokines and are secreted into the cytoplasm. The unchanged level of IFNγ in the supernatant is surprising when referred to the enormous growth reduction in mAbs VIP-1 and 15-221 treated T cells. Beside genes for cytokines, the JAK-STAT signaling mechanism include many regulatory proteins that also encode for IFNγ proteins [59]. This positive feed-back loop may explain increasing levels of IFNγ. IFNγ itself influences the protein assembly further through the $CD4^+$ precursor T cells differentiation into the T_H1 subgroup, which belongs again to the main IFNγ producers. However both regulatory mechanisms do not explain the unchanged cytokine quantity between augmented and inhibited cell growth. Moreover cytokines IL-2, IL-10,

IL-13 and IL-22 are equally produced by the participation of JAK-STAT signaling pathways, but contrary to IFNγ we detected clearly lower levels upon mAb VIP-1 or 15-221 incubation.

In summary, the presented data demonstrates the binding of four monoclonal antibodies to epitopes of the human TfR. Antibody 13-344 possesses additional binding reactivity to similar surface proteins on murine lymphocytes. None of the antibodies influence either the high proliferative capacity of lymphoma cells or suppress DC activation. However, immunosuppressive functions were detected for mAbs VIP-1 and 15-221 related to the T cell, increase the probability of preventing the establishment of signaling pathways important for activation processes. Both antibodies are suitable to limit T cell proliferation efficiently without affecting DC activation and are therefore a potential starting point in the fight against T cell lymphoma. Contrarily mAbs 13-344 and 5-528 show no effect on the activation or proliferation of all tested immune cells and can be used as a promising diagnostic tool for the detection of cell activation and proliferation. .

6. ABBREVIATIONS

abbr.	meaning	abbr.	meaning
APC	Antigen-presenting cell	MLR	Mixed Leukocyte Reaction
Asp	Aspartic acid	mRNA	messenger RNA
ATP	Adenosine Tri-Phosphate	MTf	MelanoTransferrin
cSMAC	central SupraMolecular Activation Cluster	NADPH	Nicotinamide Adenine Dinucleotide Phosphate
pSMAC	peripheral SupraMolecular Activation Cluster	NFκB	Nuclear Factor κB
DC	Dendritic Cell	NK cells	Natural Killer cells
DMT1	Divalent Metal Transporter 1	OH	Hydroxyl
ETC	Electron Transport Chain	PAMP(s)	Pattern associated molecular pattern(s)
Fe II / Fe III	Iron oxidation state II / III	PGN	PeptidoGlycaN
HFE	gene coding for human hemochromatosis protein	PRR(s)	Pattern Recognition Receptor(s)
HLA	Human Leukocyte Antigen	PTK	Protein Tyrosine Kinase
HRV	Human Rhino Virus	ROI	Reactive Oxygen intermediate
ICAM	IntraCellular Adhesion Molecule	TCR	T Cell Receptor
Ig	Immunoglobulin	Tf	Transferrin
IL	InterLeukin	TfR	Transferrin Receptor
iNOS	Inducible Nitric Oxid Synthase	TGF	Transforming Growth Factor
IRE(s)	Iron Response Element(s)	Thr	Threonine
IRP(s)	Iron Regulatory Protein	TNF	Tumor Necrosis Factor
ITAM	Immunoreceptor Tyrosine-based activation motif	TRAF	TNF Receptor Associated Factor
kDa	kilo Dalton	UTR	UnTranslated Region
LFA-1	Lymphocyte Function-associated Antigen	β2m	beta-2 microglobulin
LPS	LipoPolySaccharid		
mAb	monoclonal Antibody		
MHC	Major Histocomatibility Complex		

7. REFERENCES

[1] Papanikolaou, G., Pantopoulos, K., 2005. Iron metabolism and toxicity. Toxicol. Appl. Pharmacol.: 202 (2): 199-211

[2] Weiss, G., 2004. Modification of iron regulation by the inflammatory response. Best. Pract. Res. Clin. Haematol.: 18 (2): 183-201

[3] Cook, J.D., Skikne, B.S., Baynes, R.D., 1993. Serum transferrin receptor. Annu. Rev. Med.: 44: 63-74

[4] Testa, U., Kühn, L., Petrini, M., et al., 1991. Differential regulation of iron regulatory element-binding protein(s) in cell extracts of activated lymphocytes versus monocytes-macrophages. J. Biol. Chem.: 266 (21): 13925-13930

[5] Brinkmann, M., Teuffel, R., Laham, N., et al., 2007. Expression of iron transport proteins divalent metal transporter-1, Ferroportin-1, HFE and transferrin receptor-1 in human monocyte- derived dendritc cells. Cell Biochem. Funct.: 25 (3): 287-296

[6] Daniels, T.R., Delgado, T., Rodriguez, J.A., et al., 2006. The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin. Immunol.: 121 (2): 144-158

[7] Qian, Z.M., Li, H., Sun, H., Ho, K., 2002. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol. Rev.: 54 (4): 561-587

[8] Rahmanto, Y. S., Dunn, L.L., Richardson, D.R., 2007. The melanoma tumor antigen, melanotransferrin (p97): a 25-year hallmark- from iron metabolism to tumorigenesis. Oncogene: 26 (42): 6113-6124

[9] Aisen, P. 2004. Transferrin receptor 1. Int. J. Biochem. Cell Biol.: 36 (11): 2137-2143

[10] Wang, J., Pantopoulos, K., 2011. Regulation of cellular iron metabolism. Biochem. J.: 434 (Pt 3): 365-381

[11] Pantopoulos, K., Hentze, M.W., 1998. Activation of iron regulatory protein-1 by oxidative stress in vitro. Proc. Natl. Acad. Sci. USA: 95 (18): 10559-10563

[12] Itoyama, K., Kawahira, Y., Murata, M., et al., 1999. Fluctuations of some characteristics in the common cutworm, *Spodoptera litura* (Lepidotera: Nuctoidae) reared under different diets. Appl. Entomol. Zool.: 34 (3): 315-321

[13] Trowbridge, I.S., Collawn, J.F., Hopkins, C.R., 1993. Signal- dependent membrane protein trafficking in the endocytotic pathway. Annu. Rev. Cell Biol.: 9: 129-161

[14] Kurten, R.C., 2003. Sorting motifs in receptor trafficking. Adv. Drug Delivery Rev.: 55 (11): 1405-1419

[15] Weiss, G., 2002. Iron and immunity: a double-edged sword. Eur. J. Clin. Invest.: 32 (s1): 70-78

[16] Walker, B.L., Tiong, J.W.C., Jefferies, W.A., 2001. Iron metabolism in mammalian cells. Int. Rev. Cytol.: 211: 241-278

[17] Lambert, L.A., Mitchell,S.L., 2007. Molecular evolution of the transferrin receptor/glutamate carboxypeptidase II family. J. Mol. Evol.: 64 (1): 113-128

[18] Moura, I.C., Centelles, M.N., Arcos-Fajardo.M., et al., 2001. Identification of the transferrin receptor as a novel immunoglobulin (Ig)A1 receptor and its enhanced expression on mesangial cells in IgA nephropathy. J. Exp. Med.: 194 (4): 417-425

[19] Manger, B., Weiss, A., Hardy, K.J., et al., 1986. A transferrin receptor antibody represents one signal for the induction of IL 2 production by a human T cell line. J. Immunol. 136 (2): 532-538

[20] Neckers, L.M., Cossman, J., 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc. Natl. Acad. Sci. USA: 80 (11): 3494-3498

[21] Bayer, A.L., Baliga, P., Woodward, J.E., et al., 1998. Transferrin receptor in T cell activation and transplantation. J. Leukocyte Biol.: 64 (1): 19-24

[22] Salmerón, A., Borroto, A., Fresno, M., et al., 1995. Transferrin receptor induces tyrosine phosyphorylation in T cells and is physically associated with the TCR zeta chain. J. Immunol.: 154 (4): 1675-1683

[23] Abbas, A.K., Lichtman, A.H., Pillai, S. (2007). Celllar and molecular immunology. 6th edition. Philadelphia. Elsevier

[24] Pasquier, B., Leppelletier, Y., Baude, C., et al., 2004. Differential expression and function of IgA receptors (CD89 and CD71) during maturation of dendritic cells. J. Leukoc. Biol.: 76 (6): 1134-1141

[25] Ferlazzo, G., Wesa, A., Wei, W.Z., et al., 1999. Dendritic cells generated either from CD34+ progenitor cells or from monocytes differ in their abillity to activate antigen-specific CD8+ T cells. J. Immunol.: 163 (7): 3597-3604

[26] Fagarasan, S., Honjo, T., 2003. Intestinal IgA synthesis: Regulation of front-line body defences. Nat. Rev. Immunol.: 3 (1): 63-72

[27] Testa, U., Petrini, M., Quaranta, M.T., et al., 1989. Iron up- modulates the expression of transferrin receptors during monocyte-macrophage maturation. J. Biol. Chem.: 264 (22): 13181-13187

[28] Bourgeade, M.F., Silbermann, F., Kühn, L., 1992. Post-transcritpional regulation of transferrin receptor mRNA by IFNγ. Nucleic Acids. Res.: 20 (12): 2997-3003

[29] Byrd, T.F., Horwitz, M.A., 1993. Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes. Coordinate upregulation by iron transferrin and downregulation by interferon gamma. J. Clin. Invest.: 91 (3): 969-976

[30] Dustin, M.L., Chakraborty A.K., Shaw A.S., 2010. Understanding the structure and function of the immunological synapse. Cold Spring Harb Perspect Biol.; 2 (10):a002311.

[31] Batista, A., Millán, J., Mittelbrunn, M., et al., 2004. Recruitment of transferrin receptor to immunological synapse in response to TCR engagement. J. Immunol.: 172 (11): 6709-6714

[32] Alarcón B, Mestre D, Martínez-Martín N., 2011. The immunological synapse: a cause or consequence of T-cell receptor triggering? Immunology; 133(4):420-5.

[33] Lee, K.H., Holdorf, A.D., Dustin, M.L., et al., 2002. T cell receptor signaling precedes immunological synapse formation. Science: 295 (5559): 1539-1542

[34] Cua, D.J., Tato, C.M., 2010. Innate IL-17-producing cells: the sentinels of the immune system. Nat. Rev. Immunol.: 10 (7): 479-489

[35] Romagnani, S., 1997. The Th1/Th2 paradigm. Immunol. Today: 18 (6): 263-266

[36] Ozaki, K., Leonard, W.J., 2002. Cytokine and cytokine receptor pleiotropy and redundancy. J. Biol. Chem.: 277 (33): 29355-29358

[37] Taniguchi, T.1988. Regulation of cytokine gene expression. Annu. Rev. Immunol.: 6: 439-464

[38] Ihle, J.N., Kerr, I.M., 1995. Jaks and Stats in signaling by the cytokine receptor superfamily.Trends. Genet.: 11 (2): 69-74

[39] Goetz, F.W., Planas, J.V., MacKenzie, S., 2004. Tumor necrosis factors. Dev. Comp. Immunol.: 28 (5): 487-497

[40] Beutler, B., Cerami, A., 1988. The history, properties, and biological effects of cachectin. Biochemistry: 27 (20): 7575-7582

[41] Kishimoto, T., 1989. The biology of interleukin-6. blood:74 (1): 1-10

[42] Siegal, F.P., Kadowaki, N., Shodell, M., et al., 1999. The nature of the principal type 1 interferon-producing cells in human. Science: 284 (5421): 1835-1837

[43] Zdanov, A., Schalk-Hihi, C., Gustchina, A., et al., 1995. crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon γ. Structure: 3 (6): 591-601

[44] Trinchieri, G., 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol: 3 (2): 133-146

[45] Hamza, T., Barnett, J.B., Li, B., 2010. Interleukin 12 a key immunoregulatory cytokine in infection applications. Int. J. Mol. Sci.: 11 (3): 789-806

[46] Locke, M., Nichol, H., 1992. Iron economy in insects: Transport, metabolism, and storage. Annu. Rev. Entomol.: 37: 195-215

[47] Van Agthoven, A., Goridis, C., Naquet, P., et al., 1984. Structural charateristics of the mouse transferrin receptor. Eur. J. Biochem.: 140 (2): 433-440

[48] Pham, D.Q., Winzerling, J.J., 2010. Insect ferritins: Typical or atypical? Biochim. Biophys. Acta.: 1800 (8): 824-833

[49] White, S., Taetle, R., Seligman, P.A., et al., 1990. Combinations of anti- transferrin receptor monoclonal antibodies inhibit human tumor cell growth in vitro and in vivo: evidence for synergistic antiproliferative effects. Cancer Res.: 50 (19): 6295-6301

[50] Liao, W., Lin, J. X., Leonard, W. J., 2001. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr. Opin Immunol. 23 (5): 598-604

[51] Malek, T.R., 2003. The main function of IL-2 is to promote the development of T regulatory cells. J. Leukoc. Biol.: 74 (6): 961-965

[52] Scott, M.J., Godshall, C.J., Cheadle, W.G., 2002. Jaks, stats, cytokines, and sepsis. Clin Diagn Lab Immunol.: 9 (6): 1153-1159

[53] Nelms, K., Keegan, A.D., Zamorano, J., et al., 1999. The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol.: 17: 701-738

[54] Wynn, T.A., 2003. IL-13 Effector functions. Annu.Rev.Immunol.: 21: 425-456

[55] Kolls, J.K., Lindén, A., 2004. Interleukin-17 family members and inflammation. Immunity: 21 (4): 467-476

[56] Kawaguchi, M., Adachi, M., Oda, N., et al., 2004. IL-17 cytokine family. J Allergy Clin Immunol: 114 (6): 1265-1273

[57] Jones, B.C., Logsdon, N.J., Walter, M.R., 2008. Structure of IL-22 bound to ist high affinity IL-22R1 chain. Structure: 16 (9): 1333-1344

[58] Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-γ: an overview of signals, mechanisms and functions. J. Leukoc. Biol.: 75 (2): 163-189

[59] Shuai, K., Liu, B., 2003. Regulation of jak-stat signalling in the immune system. Nat. rev. Immunol.: 3 (11): 900-911

[60] Li, M.O., Wan, Y.Y., Sanjabi, S., et al., 2006. Transforming growth factor- β regulation of immune responses. Annu. Rev. Immunol.: 24: 99-146

[61] Letterio, J.J., Roberts, A.B., 1998. Regulation of immune respones by TGF-β. Annu. Rev. Immunol.: 16: 137-161

[62] Neckers, L.M., Yenokida, G., James, S.P., 1984. The role of the transferrin receptor in human B lymphocyte activation. J. Immun.: 133 (5): 2437-2441

[63] Ned, R.M., Swat, W., Andrews, N.C., 2003. Transferrin receptor 1 is differentially required in lymphocyte development. Blood: 102 (10): 3711-3718

[64] Seligman, P.A., Kovar, J., Schleicher, R.B., Gelfand, E.W., 1991. transferrin- independent iron uptake supports B lymphocyte growth. Blood: 78 (6): 1526-1531

[65] Brown, J.P., Hewick, R.M., Hellström, K.M., et al., 1982. Human melanoma-associated antigen p97 is structurally and functionally related to transferrin. Nature: 296 (5853): 171-173

[66] Thorstensen, K., Romsol, I., 1990. The role of transferrin in the mechanism of cellular iron uptake. Biochem. J.: 271 (1): 1-9

[67] Chua, E., Clague, J.E., Sharma, A.K., et al., 1999. Serum transferrin receptor assay in iron deficiency anaemia and anaemia of chronic disease in the elderly. Q. J. Med.: 92 (10): 587-594

[68] Kaplan, J., Jordan, I., Sturrock, A., 1991. Regulation of the transferrin-independent iron transport system in cultured cells. J. Biol. Chem.: 266 (5): 2997-3004

[69] Trowbridge, I.S., Domingo, D.L., 1981. Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumour cells. Nature 294 (5837): 171-173

[70] Trowbridge, I.S., Lopez, F., 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhhibits human tumor cell growth in vitro. Proc. Natl. Acad. Sci. USA: 79 (4): 1175-1179

[71] Mendelson, J., Trowbridge, I., Castagnola, J., 1983. Inhibition of human lymphocyte proliferation by monoclonal antibody to transferrin receptor. Blood: 62 (4): 821-826

[72] Lesley, J.F., Schulte, R.J., 1985. Inhibition of cell growth by monolonal anti-transferrin receptor antibodies. Mol. Cell. Biol.: 5 (8): 1814-1821

[73] Taetlee, R., Castagnola, J., Mendelsohn, J., 1986. Mechanisms of growth inhibition by anti-transferrin receptor monoclonal antibodies. Cancer Res.: 46 (4): 1759-1763

[74] Knapp, W., Dörken, B., Rieber, R.E., et al., 1989. CD antigens 1989. IJC: 44 (1): 190-191

[75] Besancon, F., Bourgeade, M.F., Testa, U., 1985. Inhibition of transferrin receptor expression by interferon-α in human lymphoblastoid cells and mitogen-induced lymphocytes. J. Biol. Chem.: 260 (24): 13074-13080

[76] Karpova, M.B., Schoumans, J., Ernberg, I., et al., 2005. Raji revisited: cytogenetics of the original Burkitt`s lymphoma cell line. Leukemia: 19 (1): 159-161

[77] Abraham, R.T., Weiss, A., 2004. Jurkat T cells and development of the T- cell receptor signalling paradigm. Nat. Rev. Immunol.: 4 (4): 301-308

[78] McMichael, A J. (1987). Leucocyte Typing III. white Cell Differentiation Antigens. Oxford University Press.

[79] Knapp, W. (1989). Fourth international workshop and conference on human leucocyte differentiation antigens. Vienna. Oxford University Press.

[80] Barclay, A.N., Birkeland, M.L. (1993).The Leukocyte Antigen FactsBook. London. Academic press limited.

[81] Nichol, H., Law, J.H., Winzerling, J.J., 2002. Iron metabolism in insects. Annu. Rev. Entomol.: 47: 535-559

[82] Seemann C., 2004. Molekulare und funnktionelle charakterisierung des "13-344-antigens" auf dendritischen Zellen". Diploma thesis, Akademie für den medizinisch-technischen Laboratoriumsdienst am AKH der Stadt Wien.

[83] Cella, M., Engering, A., Pinet, V., et al., 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature: 388 (6644): 782-787

[84] Leitner J, Grabmeier-Pfistershammer K, Steinberger P., 2010. Receptors and ligands implicated in human T cell costimulatory processes. Immunol. Lett.: 128 (2): 89-97.

[85] Melián, A., Beckman, E.M., Porcelli, S.A., et al., 1996. Antigen presentation by CD1 and MHC-encoded class I-like molecules. Curr. Opin. Immunol.: 8 (1): 82-88

[86] Hermandez, J.B., Newton, R.H., Walsh, C.M., 2010. Life and death in the thymus-cell death signaling during T cell development. Curr. Opin. Cell Biol.: 22 (6): 865-871

[87] Lopez Guerrero, J.A., Redondo, J.M., Alarcón, B., et al., 1989. Different functional domains on the transferrin receptor molecule defined by monoclonal antibodies. Immunology: 66 (2): 252-257

[88] Santtila, S., Savinainen, K., Hurme, M., 1998. Presence of the IL-1RA Allele 2 (IL1RN*2) is associated with enhanced IL-1β production in vitro. Scand. J. Immunol.: 47 (3): 195-198

[89] Kirchberger, S., Majdic, O., Blüml, S., et al., 2008. The cytoplasmic tail of CD45 is released from activated phagocytes and can act as an inhibitory messenger for T cells. Blood: 112 (4): 1240-1248

8. CURRICULUM VITAE

Personal data

Name:	Andreas Staudigl
Date of birth:	June, 1st, 1984
Place of birth:	Korneuburg, Austria
Nationality:	Austria
Residence:	A-2103 Langenzersdorf
E-mail:	staudigl.andreas@gmail.com

Education

1991-1995	elementary school, VS Langenzersdorf
1995-2003	Bundesgymnasium und Bundesrealgymnasium “Bertha von Suttner”, 1210 Vienna
2003-2005	Bundesgymnasium, Bundesrealgymnasium und Wirtschaftskundliches Bundesrealgymnasiums für Berufstätige, Henriettenplatz 6, 1150 Vienna
January 2005	Matura passed
Since Otober 2006	studies in Genetics and Microbiology,
	University of Vienna, Austria
July 2011- May 2012	diploma thesis at the Institute of Immunology, Medical University of Vienna

Spoken languages:

German (native language)

English (fluently)

French (basic knowledge)