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mRNA expression of tumor associated antigens in patients with chronic lymphocytic leukemia

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To my wife Agnieszka

and

my daughter Justyna

Content

Abbreviations

A	Adenosine
AML	Acute myeloid leukemia
AMV	Avian myeloblastosis virus
APC	Antigen presenting cell
Apo-DC	Dendritic cells pulsed with apoptotic bodies
BAGE	B melanoma antigen
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCG	Bacillus of Calmette and Guérin
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B cell receptor
bp	Base pairs
C	Cytidine
°C	Temperature in Celcius degree
CA9/G250	Carboanhydrase 9
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
CEA	Carcinoembryonic antigen
CML	Chronic myeloid leukemia
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
DLI	Donor lymphocyte infusion

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DW	Distilled Water
EBV	Epstein-Barr-Virus
EDTA	Ethylenediamine tetraacetic acid
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence-activated cell sorting
G	Guanosine
g	Gram (weight)
GEP	Gene expression profiling
GM-CSF	Granulocyte monocyte colony stimulating factor
GvHD	Graft versus host disease
Gy	Gray
HCV	Hepatitis C virus
HER	Human epidermal growth factor receptor
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
HSJ2	Heat shock protein
hTERT	Human telomerase catalytic subunit
HV	Healthy volunteer
IFNγ	Interferon gamma
Ig	Immunoglobulin

IgVH	Variable part of the heavy chain of Ig
IL	Interleukin
IMP	Influenza matrix protein
kb	Kilo base
kDa	Kilo Dalton
mAb	Monoclonal antibody
MAZ	Myc-associated zinc-finger protein
$MgCl_2$	Magnesium chloride
µg	Microgram (weight)
MHC	Major histocompatibility complex
µl	Microliter
MLPC	Mixed lymphocyte peptide culture
µM	Micromolar
ml	Milliliter
min	Minute
mM	Millimolar
MM	Multiple Myeloma
MoDC	Monocyte-derived DC
MPP11/MIDA	M-phase phosphoprotein 11
MUC/EMA	Epithelial membrane antigen
NBT	Nitroblue tetrazolium
NK	Natural killer
NY-Ren60	Renal cell cancer antigen

OFAiLRP	Oncofetal antigen immature laminin-receptor protein
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PINCH	Particularly interesting new Cys-His protein
PRAME	Preferentially expressed antigen in melanoma
PSA	Prostate specific antigen
PWM	Pokeweed Mitogen
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RHAMM	Receptor for hyaluronic acid mediated motility
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SEREX	Serological analysis of recombinant cDNA expression libraries
T	Thymidine
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TBP	TATA-Box binding protein
TAE	Tris acetate EDTA buffer

Taq	Thermophilus aquaticus
TCR	T cell receptor
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
WT1	Wilms tumor gene 1
ZAP-70	Zeta chain associated protein 70

1 Introduction

1.1 B-cell chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in Western countries and USA (Dighiero et al. 1996). The disease is characterized by the accumulation of monoclonal B lymphocyte originated leukemic cells which are phenotypically CD5+/CD23+/CD19+ and express surface immunoglubulin at low level. Accumulation of malignant cells in the blood, bone marrow, lymph nodes and spleen is progressive and considered to be caused by the dysregulation of programmed cell death (apoptosis) rather then increased proliferation (Kitada et al. 1998). The disease affects mainly eldery individuals and is mostly characterized by a long natural history (Dighiero et al. 1996, Keating et al. 2003). Recently, two subtypes of the disease could be defined according to the molecular markers CD38, expression of zeta chain associated protein 70 (ZAP-70) or the IgVH mutational status, all of these factors associated with a variable clinical course (Crespo et al. 2003, Rassenti et al. 2004). A cohort of patients with slowly progressive disease is characterized by low expression of CD38, low expression of the ZAP-70 molecule and mutational IgVH status. In contrast, patients who experience a progressive disease show high expression levels of CD38 and ZAP-70, as well as an unmutated status of IgVH. Some genetic abberations are also of prognostic relevance: 11q deletion and 17p deletion and to some extent trisomy 12 have been classified as unfavorable

aberrations, in contrast the13q deletion as favorable (Kröber et al. 2002).

1.1.1 Classification of B-CLL

In European countries and the U.S., two classification systems for B-CLL patients are currently in use, i.e. the Rai and the Binet classification. In this paper, the clinical stage of disease was assessed according to the Rai classification (Rai et al. 1975).

Table 1. The classification of B-CLL patients according to Rai (1975).

Rai classification:	Details of classification:
stage 0	blood lymphocytosis and more than 30% lymphocytes in bone marrow
stage 1	like in stage 0 + enlarged lymph nodes
stage 2	stage 1 + spleen and liver enlargement
stage 3	stage 2 + anemia (with hemoglobin of less than 11 g per deciliter)
stage 4	stage 3 + low platelet count (less than 100,000 per microliter)

Blood parameters (lymphocytosis, platelet count and hemoglobin level) and enlargement of spleen and liver divide patients with B-CLL into 5 distinct stages. Patients with less advanced disease are classified in stage 0 and most advanced cases are cassifed in stage 4 state.

According to Rai classification of B-CLL patients the U.S. National Cancer Institute could be devided into three groups (Table 2).

Table 2. Groups of patients according to the U.S. National Cancer Institute.

Low-risk group	Rai Stage 0. Patients are expected to have, on the average life expectancy in excess of 14 years
Intermediate-risk group	Rai Stage 1 and Stage 2 together. The median life expectancy is around 8 years.
High-risk group	Rai Stage 3 (anemia, defined as hemoglobin of less than 11) and Stage 4 (thrombocytopenia, platelets < 100 G/L).

Clinically B-CLL patients could be classified into three risk groups (according to U.S. National Cancer Institute). Low-risk group represents patients with early disease and a life expectancy of more then 14 years, in contrast to high-risk patients who are in most advanced stages of disease.

1.1.2 Treatment of CLL

In patients at early stages (0 and 1 - according to Rai classification) of B-CLL a “watch and wait” strategy is generally suggested, as no advantage of early initiated treatment has been observed (Dighiero 1997).

However, some patients with progressive disease and unfavourable prognostic factors must undergo chemotherapy earlier. The decision when to initiate treatment is not simple. Many new treatment regimens according to prognostic factors are under current clinical evaluation. For many years, chlorambucil was the first line treatment. However new strategies with fludarabine, cyclophosphamide and monoclonal antibodies (rituximab (anti-CD20) and alemtuzumab (anti-CD52)) have entered clinical treatment protocols (Byrd et al. 2004). Recently, strategies with immune vaccines become another therapeutic option for B-CLL patients (Hus et al. 2005).

1.2 Tumor associated antigens (TAAs)

The anti-leukemic effect obtained by graft versus leukemia (GvL) reaction after allogeneic stem cell transplantation or after by donor lymphocyte infusion (DLI) suggests the existence of immunogenic antigens in leukemias (Marks et al. 2002, Ritgen et al. 2004). Tumor associated antigens (TAAs) are molecules preferentially expressed by tumor cells, but not in normal tissues. These antigens are proteins that could serve as distinctive molecular markers of disease as well as possible specific targets for immunotherapies (Jäger et al. 2003). Monoclonal antibodies directed against these structures and presensitized T cell clones could act selectively against tumor cells.

1.2.1 Types of TAAs

There are several types of tumor associated antigens (TAAs) (Table 3): Cancer/germline antigens of the BAGE or the MAGE gene families (Boel et al. 1995, Liu et al. 2004). These antigens are considered to be good candidate antigens to future immunotherapies because of their expression on tumor cells and on normal germ cells but not in other normal somatic tissues. Other types of TAAs are expressed in normal tissues but overexpressed in tumor cells, e.g. HER2/neu. The use of these TAAs as targets is not unproblematic because of the possible danger of eliciting autoimmune reactions (Stockinger 1999). There are suggestions that a two log difference in the expression of TAAs on normal cells versus tumor cells is required for safe immunotherapy. Mutated proteins constitute further target structures for immunotherapies. For example the bcr-abl mutation, characteristically observed in chronic myeloid leukemia, results from the t(9;22) translocation, the so called the “Philadelphia chromosome”. This gene rearrangement results in the production of a novel oncoprotein, BCR/ABL, a constitutively active tyrosine kinase (Bocchia et al. 2005).

Antigens connected with differentiation represent another type of TAAs. The prostate specific antigen (PSA), i.e. the product of the *KLK3* gene is an important tumor marker used in the diagnosis and monitoring of prostate cancer (Fong et al. 2003). Elevated PSA levels were also reported in some breast and gynecologic cancers (Kucera et al. 1997).

Viruses are important co-factors in the etiology of some malignancies. Viral genomes are detected in tumor cells and code viral antigens. In principle, these

antigens are attractive targets for immunotherapeutic attacks because T cells directed against these structures should not be effectively removed from the T cell repertoire by central tolerance-inducing mechanisms. The success of therapy directed at Epstein-Barr-Virus (EBV) or cytomegalovirus (CMV) antigens in transplanted patients suggests that this type of response might be of clinical relevance (Rooney et al. 2001)

1.2.2 Serological analysis of recombinant cDNA expression libraries (SEREX)

SEREX is a powerful method to search for new immunogenic antigens. It is based on a detectable humoral immune response against tumor cells in patients with malignancies (Chen 2000, Greiner et al. 2000). Briefly, the clones from a cDNA expression library that are reactive with patients sera are selected. DNA is isolated and positive clones are sequenced. To identify antigens, gene databases are screened for homology. In the next step of allogeneic serology, the frequency of IgG antibodies against these SEREX antigens in patients and in HVs are collected.Thereafter, the gene expression of SEREX antigens is screened by RT-PCR and Western Blot. The SEREX methodology is schematically depicted in Figure 1.

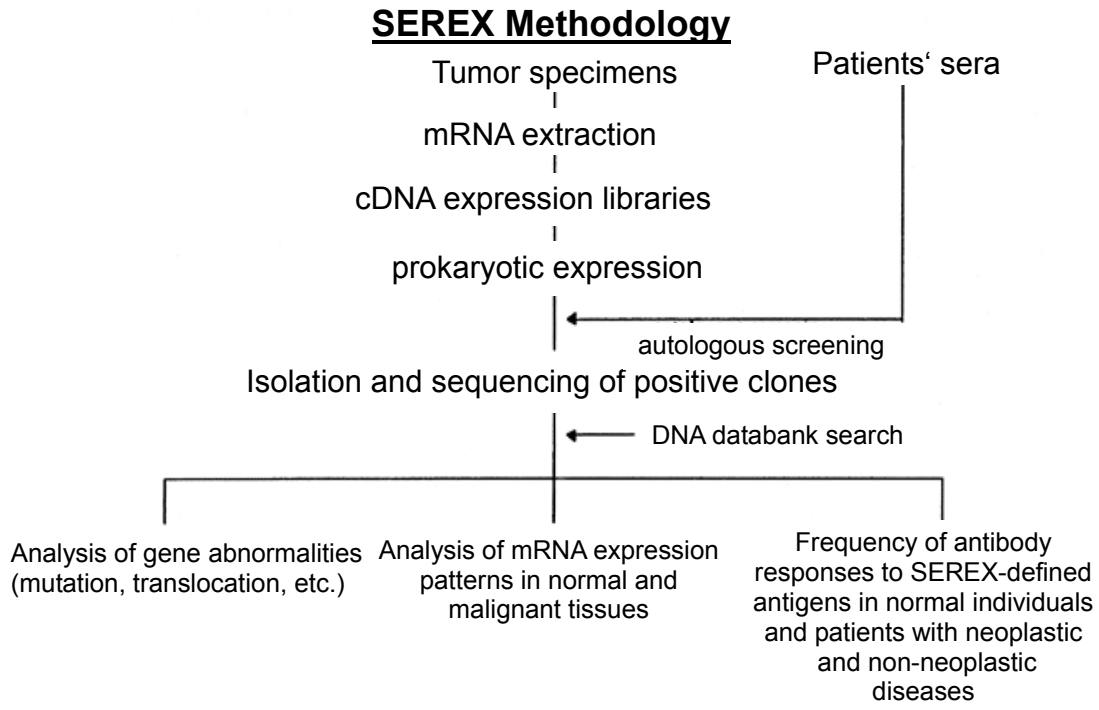
Table 3. Types of tumor associated antigens

after Benoit Van den Eynde and Pierre van der Bruggen (van den Eynde et al. 1997)

Cancer-Testis Cancer/Germline Antigens	Differentiational Antigens	Mutated Antigens	Overexpressed Antigens	Viral Antigens
MAGE	Tyrosinase	MUC1	MAZ	HPV
BAGE	PSA	p53	PRAME	EBV
NY-ESO 1		Bcr-abl	Survivin	HCV
SSX2			Proteinase 3	
			NewRen60	
			WT1	
			G250	
			Survivin	
			hTERT	
			PINCH	
			HSJ2	
			Her/Neu	
			CEA	

According to the expression pattern in healthy vs. malignant tissues, tumor associated antigens could be classified into five groups: Cancer-Testis (which could be deteceted only in healthy testis and in tumors), Differentiational (normally expressed only during differentiation), mutated (mutated forms of naturally appearing proteines), overexpressed (expressed also in healthy tissues but overexpressed by tumors) and viral antigens (whose expression is associated with viral infection). Examples of antigens for each group are shown in Table 3.

Figure1. Serological expression cloning (SEREX) approach for the definition of human tumor antigens. (after Yao-Tseng Chen (Chen 2000))



A cDNA expression library was created on the basis of mRNA extracted from tumor samples. Thereafter, the cDNA was cloned into a prokaryotic organism (*E. coli*), plotted onto nirtocellulose membranes and incubated with patients' sera. Reactive clones were selected. The DNA was isolated and positive clones were sequenced. To identify the respective gene, databases on the web were screened for homology. To further characterize such SEREX-defined antigens, the frequency of IgG antibodies against this particular antigen in patients and in healty volunteers were compared. Subsequently, the tissue expression pattern and gene abnormalities were analyzed.

1.3 Immunotherapy

Tumor immunotherapy represents an anti-cancer approach to manipulate the patient's immune system to recognize and fight selectively against tumor cells. Immunotherapies are designed to induce or enhance the immune response against unique neoplasmatic targets, so-called tumor associated antigens (TAAs). The prevailing techniques of tumor immunotherapy can be divided into two groups called non-specific and antigen-specific therapies (Roitt et al. 2000).

1.3.1 Non-specific immunotherapy

For many years, it was known that the administration of attenuated forms of a mycobacterial strain called Bacille Calmette-Guérin (BCG) had anticancer effects. The exact mechanism by which BCG elicits anti-cancer immune responses in certain patients was not determined but some scientists speculate that BCG probably activates both macrophages and lymphocytes (Prescott et al. 2000).
The use of cytokines in immunotherapy can also lead to the destruction of tumor cells. For example, administration of IL-2 can increase the clinical outcome in renal cell carcinoma and melanoma patiens (Eklund et al. 2004).

1.3.2 Specific immunotherapy

A restricted recognition of tumor cells by the immune system is required for specific immunotherapy (Jäger et al. 2003). This goal might be achieved by the administration of monoclonal antibodies directed against tumor cells or by

infusion of T cell clones that recognize neoplasmatic target (the so-called adoptive immunotherapy or adoptive T cell transfer).

1.3.2.1 Antibody therapy

In current clinical practise for the treatment of B-CLL patients is rituximab, a monoclonal antibody directed against CD20-molecules expressed on B-cells which are mainly tumor cells in B-CLL (Robak 2004). Another drug broadly used by oncologists is Herceptin (Trastuzumab), an antibody against the human epidermal growth factor receptor 2 (HER2), a cell-surface tyrosine kinase receptor overexpressed by 25% to 30% of breast cancers (Jones et al. 2004).

1.3.2.2 Adoptive immunotherapy

Adoptive T-cell therapy is an attempt to harness and amplify the tumor-eradicating capacity of a patient's own T cells and then re-transfuse these effectors to the patient in such a state that they effectively eliminate residual tumor cells (Golab et al. 2002). Unfortunately, the cytotoxic effect of the transfused lymphocytes is rather short, due to rather limited half life time of cytotoxic T lymphocytes (CTL).

1.3.2.3 Cancer vaccination

Hitherto, cancer vaccination strategies rely on the injection of known immunogenic tumor antigens or antigens presenting cells (APCs) coincubated with specific antigen *ex vivo*. Either adopted T cells transfered into the patient or

T cells stimulated *in vivo* are supposed to induce and/or enhance immune responses against the tumor cells. The success of vaccine strategies depends on two main components of the immunoresponse: the antigen presenting part and the effector part, i.e. lymphocytes capable to kill tumor cells in an antigen-specific manner. Dendritic cells (DCs) are the most potent antigen presenting cells which can present antigens delivered by direct infusion (Hart 1997).

A relatively ancient but still useful form of anti-cancer vaccination strategy is to extract whole tumor cells, lysate or irradiate them, and inject the crude extract into the patient (Kokhaei et al. 2004). To enhance the antitumor effect, such material could be transferred into the patient in the presence of an adjuvant such as BCG. There is evidence that the BCG supplement creates a suitable environment within the patient so that the antigens associated with the tumor cells can be properly presented to the immune system for the subsequent generation of tumor specific T cells to induce an anti-tumor effect (Prescott et al. 2000). Other possible adjuvants are cytokines, i.e. the granulocyte monocyte colony stimulating factor (GM-CSF). Adjuvants, cytokines and co-stimulators are considered to improve the anti-tumor effect by creating an optimal *in vivo* environment for the presentation of tumor-associated antigens to the immune system. The main advantage of vaccination with tumor cells extract is the fact that this approach circumvents the need for definition of a particular tumor associated antigen (TAA).

However, TAAs have been defined for many tumors. Antigens need to be cleaved into peptides and processed in the endoplasmatic reticulum before they

are presented to the immune system as MHC-peptide complexes. Such immunogenic epitope peptides presented *via* MHC molecules represent potential targets for immunotherapies.

Another option is the delivery of *ex vivo* antigen experienced DCs. Antigen presenting cells could be pulsed with tumor-associated protein or RNA that codes for a tumor-associated protein or immunogenic fragments (peptides) isolated from a tumor antigen. DCs pulsed with tumor-associated peptide or lysate were shown to be effective in the treatment of metastatic melanoma (Mosca et al. 2003).

The main advantage of a peptide-based vaccine is that it allow an exact monitoring of T cell responses to this particular antigen by ELISPOT assays, flow cytometry analysis of intracellular IFNγ or tetramer staining (Nagorsen et al. 2004, Greiner et al. 2005). The potential of peptide/proteine vaccination as an anti-cancer therapy might be enhanced by establishing the exact peptide dose, by the use of adjuvants, by synergistic cytokine combinations, and by optimizing the methods of delivery.

1.3.2. Dendritic cells (DCs)

DCs represent not more than 1% of PBMC in healthy volunteers (Hart 1997). DCs may be obtained from blood or generated *in vitro* from precursor cells. For DC based antitumor therapeutical strategies it is crucial to obtain large amounts of DCs. *In vitro* generation of DCs from precursor cells has been favoured as a source of cells as by this approach greater numbers of DCs can be produced.

DCs could be generated from CD34+ bone marrow stem cells. Another source for the generation of DCs represent monocytes. After incubation with IL-4 and GM-CSF, monocytes develop into immature DC-like cells. To induce maturation of such monocyte-derived DCs (MoDCs) at least TNF-α is required, if not a cocktail of cytokines such as TNF-α, IL-1β, IL-6 and PGE2 (Jonuleit et al. 1997). Such manipulated monocytes are able to induce and maintain immune responses (Garderet et al. 2001).

1.4 Immunology of CLL

Generating specific immune responses by cytotoxic T lymphocytes (CTLs) against CLL cells might be cumbersome, as CLL patients suffer from immunosuppression increasing with the progress of the disease (Goldman 2000, Tsiodras et al. 2000). T cell function as well as antigen presentation by DCs were reported to be impaired in CLL (Scrivener et al. 2003, Orsini et al. 2003). However, CTL responses against modified immunoglobulin derived peptides and tumor-derived RNA could be generated in CLL patients (Harig et al. 2001, Müller et al. 2004), thus indicating the potential of immune reactions in these patients against various undefined, as well as defined antigens.

1.5 Immunotherapies for patients with B-CLL

Despite new therapeutical strategies resulting in a better outcome of CLL patients, this particular disease still remains uncurable, with the exception of allogeneic stem cell transplantation. There is a fervent need to develop novel strategies to

fight effectively against this neoplasia. Immunotherapy targeting specifically tumor cells might represent a novel strategy of treatment. Recently, studies pursuing different approaches have been published: Strategies with whole tumor lysates, irradiated cells or total mRNA, for which one is not obliged to define TAAs, or with pulsed DCs to induce antitumor response are under current clinical investigation (Hus et al. 2005, Kokhaei et al. 2004).

Goddard et al. (Goddard et al. 2003) in his comprehensive work on DCs pulsed with tumor lysates demonstrated HLA class II-restricted proliferative and cytotoxic T-cell responses to B-CLL. Also DCs pulsed with tumor-derived RNA could generate cytotoxic T lymphocytes (CTLs) in CLL patients (Müller et al. 2004). In another study, three strategies for the delivery of antigens to DCs, namely apoptotic bodies (Apo-DCs), tumor lysates, and tumor RNA were compared. In all six CLL patients, Apo-DCs induced higher HLA-restricted, T cell responses than DC pulsed with tumor lysate or RNA (Kokhaei et al. 2004).

To our knowledge, no result from leukemia specific vaccination using TAAs in B-CLL patients has been published yet.

1.6 TAAs in B-CLL

In the literature, only few tumor associated antigens have been defined for B-CLL (Giannopoulos 2005). The following paragraphs summarize data for these TAAs.

1.6.1 B cell receptor and immunoglobulins

The B cell receptor (BCR) and immunoglobulin (Ig) are clonal markers containing tumor specific epitopes, which can act as target for a T cell mediated immune

response in B-cell malignancies (Trojan et al. 2000). However, CTLs responses generated against naive Ig-derived peptides were rather weak (Harig et al. 2001). Harig et al. (Harig et al. 2001) cite as a main reason for this phenomena the low peptide-binding affinity of Ig-derived peptides to the major histocompatibility complex (MHC) class I molecules, resulting in a low immunogenicity. Vuillier et al. (Vuillier et al. 2003) also investigated the B cell specific idiotype as a vaccine target in CLL *in vitro*. After long time of restimulation (5 rounds), only in a small number of cases very limited CTL responses were detected. To enhance the affinity to MHC class I molecules, Harig et al. (Harig et al. 2001) modified Ig-derived peptides, but CTL responses against this heteroclitic Ig-derived peptides could be generated only in a few patients.

1.6.2 Survivin

Based on the transfection of DCs with total RNA extracted from tumor cells, Müller et al. induced specific CTL responses *in vitro* in the blood from B-CLL patients. Interestingly, some of these *in vitro* generated T cells recognized CLL lymphocytes that expressed the TAA designated survivin (Müller et al. 2004). Reichardt and Brossard raised the question whether survivin might be an interesting candidate antigen for CLL targeting immunotherapies (Reichardt et al. 2005). Survivin is a member of the apoptosis inhibitors gene family, which also plays a critical role in the regulation of the cell cycle and mitosis (Caldas et al. 2005). Survivin shows a tumor restricted expression in a variety of human malignancies (Gordan et al. 2002). However, the detection of the expression of survivin in B-CLL seems not to be trivial. Survivin is overexpressed in bone

marrow (BM) cells (Nakagawa et al. 2004) and lymph nodes from patients with B-CLL (Graziero et al. 2001), but is not expressed on non-stimulated CLL cells (Zeis et al. 2003, Giannopoulos et al. 2005). Graziero et al. (Graziero et al. 2001) detected the expression of this antigen in CLL cells only after stimulation with CD40L. The ligation of B cell CD40 by its ligand CD154 expressed on activated T cells has been demonstrated to play an important role in T cell mediated B cell activation (van Kooten et al. 2000). A direct contact of B cells with activated T cells takes place in lymph nodes, bone marrow and spleen (van Kooten et al. 2000).

CD40 signaling of CLL cells increases the susceptibility of immune recognition, but on the other side CD40 stimulation promotes the survival and the cycle cell arrest of CLL cells (Banchereau et al. 1994). Following CD40 activation, the antigen presenting capacity of CLL cells was increased and these cells became able to present TAAs and to induce proliferation of T cells with a specificity for leukemic cells (Buhmann et al. 1999, Krackhardt et al. 2002a). Therefore, not the entire population of malignant cells in the peripheral blood (PB) might be reached when survivin is targeted by an immunotherapy for patients with B-CLL. However Schmidt et al. (Schmidt et al. 2003) demonstrated the efficient lysis of the autologous CLL cells due to the induced CTL response against survivin. Specific T cell reactivity against survivin-derived HLA-B35 restricted epitopes was also found in the PB from patients with B-CLL, multiple myeloma (MM) and melanoma (Reker et al. 2004). Vaccines against survivin might target a proliferation compartment located in pseudofollicules of lymph nodes in CLL patients

(Granziero et al. 2001) and might be an effective component of polyvalent vaccines. Moreover, it could be an effective tool to eliminate CLL cells from the circulation presuming that activation also takes place in the spleen (Caligaris-Cappio 1996).

1.6.3 Fibromodulin

A very powerful tool to identify new TAAs in B-CLL is gene expression profiling (GEP). Based on GEP data (Klein et al. 2001), fibromodulin was recently proposed to be a novel TAA in B-CLL. CTL responses were detected against four different HLA-A2 binding fibromodulin-derived peptides (Mayr et al. 2005). However the expression of fibromodulin is not strictly tumor restricted. Fibromodulin is also expressed on normal connective tissue, binds collagen fibers (Hedbom et al. 1993) and is involved in wound repair (Hakkinen et al. 1996). Therefore, vaccination with fibromodulin protein or peptide might induce auto-immune reactions.

1.6.4 Adipophilin

Recently, using the very sophisticated combination of DNA chip analysis and isolation of peptides from the tumor's cell surface, Weinschenk et al. (Weinschenk et al. 2002) identified a peptide derived from the adipophilin protein capable of inducing CTLs in a braod variety of malignancies. Adiopophilin, adipose differentiation-related protein is expressed only marginally on healthy tissue (Schmidt et al. 2004). Schmidt et al. (Schmidt et al. 2004) generated CTL capable to lyse primary CLL cells which expressed adipophilin while normal B

cells were spared, indicating that this TAA might be suitable for the development of CLL specific immunotherapy.

1.6.5 Human telomerase catalytic subunit (hTERT)

Nair et al. (Nair et al. 2000) identified the telomerase catalytic subunit (hTERT) as a potentially important target for anti-cancer immunotherapeutic strategies in a broad variety of malignancies. More than 85% of human cancers exhibit strong telomerase activity when compared to the low expression in normal adult tissues (Nair et al. 2000, Vonderheide et al. 1999). However, the expression of hTERT in B-CLL patients is not so straightforward to be interpreted. Tchirkov et al. (Tchirkov et al. 2004) noted an increased hTERT expression in advanced stages of B-CLL and - in opposition to others (Verstovsek et al. 2004) - proposed hTERT to be a prognostic marker in B-CLL. Moreover, the telomerase activity of PBMN from B-CLL patients was comparable to this observed in healthy volunteers (Damle et al. 2004).

1.6.6 Oncofetal antigen-immature laminin receptor protein (OFAiLRP)

T cells isolated from PB of B-CLL patients were demonstrated *in vitro* to be reactive against DCs pulsed with peptide derived from oncofetal antigen-immature laminin receptor proteine (OFAiLRP) (Siegel et al. 2003). The RT-PCR results suggest that OFAiLRP might not be the best target for immunotherapy because of its strong expression on PBMCs from healthy volunteers (Giannopoulos et al. 2004) and renal tissue (Su et al. 2003), thus indicating the

potential danger of auto-immune reactions. However, the posttranslational changes were not considered in these studies as the difference between the 32-44kDa and the 67kDa form of LRP is not detectable on the mRNA level. The mature form of LRP (67kDa) appears to be the dimerized from of iLRP (32-44kDa) with noncovalent binding to the galactose-binding protein galactein-3 (Rohrer et al. 1999) which is downregulated during tumorigenesis, resulting in the overexpression of the monomeric form of LRP (Castronovo et al. 1996). No CTL responses from cells stimulated by DCs transfected with OFAiLRP RNA against BM and CD34+ from healthy individuals were observed (Siegel et al. 2003).

1.6.7 Antigens identified by SEREX

SEREX has been used as another powerful method to detect TAAs in a variety of malignancies. Despite hypogammaglobulinemia and autoimmune phenomena currently observed in patients with B-CLL, Krackhardt et al. (Krackhardt et al. 2002b) were able to identify 14 novel antigens in B-CLL (KW-1 to KW-14) by SEREX. The antigen KW-2 revealed a cancer/testis antigen-like expression profile. A splice variant of KW-4 showed a tumor restricted expression pattern and KW-13 is highly expressed in CLL patients. Peptide-specific CTL responses against 12 peptides derived from KW-2 and KW-13 were demonstrated. But, thus generated CTLs were not able to lyse naive CLL cells.

1.7 Rationale of the study

The anti-leukemic effect obtained by graft vs. leukemia (GvL) reaction or by donor lymphocyte infusion (DLI) suggests the existence of immunogenic antigens in leukemias (Marks et al. 2002, Ritgen et al. 2004). Identification of TAAs as target structures might open the way to mono- or polyvalent vaccines against leukemia including DC vaccination as well as immunotherapies using specific antibodies. A tumor-restricted expression would be favourable for future immunotherapies because of the absence of the problems related to immune tolerance and autoimmunity (Stockinger 1999).

Here, we investigated on the following leukemia/tumor associated antigens (LAAs)/TAAs known from literature and from our own SEREX study on myeloid leukemias (Greiner et al. 2002): the receptor for hyaluronic acid mediated motility (RHAMM/CD168) and the M-phase phosphoprotein 11 (MPP11), the preferentially expressed antigen in melanoma (PRAME), the Wilms tumor gene 1 (WT1), the carboanhydrase 9 (CA9/G250), the particularly interesting new Cys-His protein (PINCH), the heat shock protein (HSJ2) and the myc-associated zinc-finger protein (MAZ), the proteinase 3, the renal cell cancer antigen (NY-Ren60) and human telomerase catalytic subunit (hTERT) as TAAs which are shared in different types of cancers and leukemias (Vonderheide et al. 1999, Schmidt et al. 2003).

Recently survivin, fibromodulin and the OFAiLRP have been characterized as TAAs in B-CLL with the potential to elicit specific anti-tumor response (Schmidt et al. 2004, Mayr et al. 2005, Siegel et al. 2003). In this study, the mRNA expression

of all of the above mentioned TAAs/LAAs was assessed by conventional RT-PCR assays in PBMCs from B-CLL patients. Moreover, because of the exquisite RHAMM/CD168 expression pattern, quantitative RT-PCR and mixed lymphocyte peptide cultures followed by ELISPOT assays were employed to assess the expression level and the immunogenicity of this novel leukemic antigen in patients with B-CLL.

1.8 The aim of this study

- The purpose of this study was to define the expression pattern of TAAs/LAAs in PBMCs isolated from B-CLL patients.
- We compared the expression pattern of particular TAAs in B-CLL patients to healthy volunteers.
- The expression level and the immunogenicity of new defined leukemic antigens in B-CLL were assessed using mixed lymphocyte peptide cultures (MLPCs) followed by enzyme-linked immunosorbent spot (ELISPOT) assays.

2 Materials and Methods

2.1 Cell samples

Peripheral blood samples were taken from 20 healthy volunteers at the German Red Cross Blood Center, Ulm (Germany) and from 30 B-CLL patients treated in the Department of Hematooncology at the Medical University of Lublin (Poland) according to clinical CLL study protocols approved by the local ethics committee. Informed consent was obtained from all patients and healthy volunteer blood donors with respect to the use of their blood for scientific purposes. The clinical characteristics of the patients in this study is summarized in Table 4.

2.1.1 Culture of cell lines

The human cell lines were cultured in a standard medium consisting of RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 10 % (v/v) AB serum (German Red Cross Blood Center, Ulm, Germany) , 2 mM L-glutamine (Biochrom AG, Berlin, Germany), 100 units/ml penicillin (Initrogen Gibco, Grand Island, USA) and 100 units/ml streptomycin (Invitrogen Gibco). The cell lines T2 and the K562 were obtained from the „Deutsche Sammlung von Mikroorganismen und Zellkulturen“ (www.dsmz.de,Braunschweig, Germany).

T2 cells are TAP-deficient hybridoma cells resulting from the fusion of a lymphoblastic B-cell line with lymphoblastic T-cell line (ATCC-CRL-1992). T2 cells were used in ELISPOT assays for the HLA-A*0201 peptide epitopes. The T2 cell line was selected as a target because it expresses only the HLA-A*0201 allele,

which was the restriction element for the peptides used in this analysis. K562 is a cell line established from the pleural effusion of a 53-year-old woman with chronic myeloid leukemia (CML) in the state of blast crisis (DSMZ No ACC 10).

2.1.2 Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll – Biocoll Separation Solution (Biochrom AG, Berlin, Germany) density gradient centrifugation. The viability of obtained PBMCs was always >95%, as determined by trypan blue staining (Trypan Blue Solution 0.4%, Sigma-Aldrich, Munich, Germany). The viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany) and stored for RNA preparation at – 192 °C in liquid nitrogen.

2.1.3 mRNA preparation and reverse transcription

For the isolation of mRNA from PBMCs cells, the μMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions. Briefly, after cell lysis, RNA was incubated with 50 μl Oligo (dT) Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) then lysed by flow in the magnetic field through the MACS column Type μ (Miltenyi Biotec). After washing isolated mRNA was realeased from column by pre-heated Elution Buffer (Miltenyi Biotec). The quality and quantity of the isolated mRNA was assessed using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). Fifty ng of mRNA was reverse transcribed into 20 μl of cDNA using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics,

Mannheim, Germany). For each conventionel RT-PCR and for each reaction in quantitative qRT-PCR 1 µl of the cDNA preparation was used.

Table 4. Basic characteristics of 30 CLL patients.

#	Patient Sex/Age	Rai Stage	WBC (G/L) / Lymphocytes (%)	Doubling time (months)	Hb (g/L)	Peripheral lymph nodes (cm)	ZAP-70 expression (% on $CD5^+/19^+$ cells)	$CD38^+/19^+$ (%)
1	M/50	1	45 /86	>12	144	1.5	12.70	2.64
2	M/58	3	25.7 /58	n.a.	99	2.5	n.a.	n.a.
3	M/69	1	35 /75	3	154	2	27.26	10.21
4	M/53	0	61.1 /90.3	12	150	n.e.	n.a.	n.a.
5	M/74	0	79 /76	n.a.	139	n.e.	n.a.	n.a.
6	M/67	0	26.3 /76	>18	145	n.e.	n.a.	n.a.
7	M/71	2	187 /93	1	120	2	4.87	28.04
8	M/65	3	118 /86	4	107	1.5	6.71	0.19
9	M/58	2	288 /67	2	108	n.e.	28.37	21.82
10	M/63	4	88 /88	1	156	1.5	38.09	2.33
11	M/71	3	260 /88	0.5	102	4	26.40	38.99
12	F/51	0	26.5 /71.4	20	142	n.e.	15.88	26.67
13	M/72	1	66.5 /83.3	21	126	n.e.	22.70	6.26
14	M/74	3	83.3 /75	n.a.	140	2.5	7.78	8.43
15	M/52	2	96.1 /87	n.a.	149	3-4	49.05	29.93
16	F/53	1	68.1 /84.8	11	115	1.5	2.63	12.27
17	F57/	1	42.6 /86	17	124	1.5	3.06	2.76
18	M/53	4	295 /79	1	94	2	29.15	38.07
19	F/73	1	39.7 /85.5	>38	139	1.5	19.10	21.46
20	M/72	0	14.4 /62.1	>38	138	n.e.	23.90	18.57
21	M/78	0	16.6 /63.3	> 54	133	n.e.	39.90	30.74
22	M/72	0	26.6 /75	15	126	n.e.	32.55	45.12
23	M/71	0	24.9 /75.2	>26	145	n.e.	2.0	2.11
24	M/50	0	16.4 /75.8	21	135	n.e.	33.64	17.42
25	M/57	2	93.6 /95	4	169	n.e.	60.07	25.34
26	F/58	1	49.6 /76	6	119	n.e.	n.a.	n.a.
27	F/58	0	49.5 /71	58	137	n.e.	n.a.	n.a.
28	M/68	0	245 /64	n.a.	119	n.e.	n.a.	n.a.
29	M/68	4	17.6 /94	n.a.	97	3	47.52	9.41
30	F/57	2	75 /85	20	140	n.e.	4.62	1.85

Characteristics of 30 B-CLL patients included in the study. The table give information on age/sex, stage of disease (according to the Rai classification), white blood cells counts (WBC) (in G/L), lymphocytes counts (as % of WBC), doubling time (in months), hemoglobin (Hb) (in g/L), diameter of peripheral lymph nodes (in cm), the expression of ZAP-70 (as % on CD5+/19+ cells) and coexpression of CD38+/19+ (in %). n.a. = not analyzed, n.e. = not enlarged.

2.2 Reverse transcriptase polymerase chain reaction (RT-PCR) of TAA

ß-actin was used as house-keeping gene. The expression of the genes of interest was assessed by RT-PCR and classified into one of the two groups: positive: detectable mRNA expression of the antigen or negative: no detectable mRNA expression of the gene of interest. The mRNA isolated from K562 cells tested positive for all genes and therefore served as a positive control. Distilled water (DW) was used as a negative control. False positive results as a consequence of genomic DNA contamination were excluded by the design of primers located in different exons. The sequence of the primers for RT-PCR, temperatures of denaturation, annealing and elongation and the cycle numbers are shown in Table 5. All primers were synthetized by MWG-Biotech AG, Ebersberg, Germany. Products of two bands appeared as a result of RT-PCR and were observed in all positive for RHAMM/RHAMM^{-exon4} B-CLL cases. Figure 2 shows schematic map of RHAMM/CD168 with sites of primers used in this study.

2.3 Agarose gel electrophoresis

Dried agarose (Seakem LE; Cambrex Bio Science Rockland, Rockland, ME, USA) was dissolved in the 100 ml 1 x TAE buffer consisting of 40 mM Tris (Roth, Karlsruhe, Germany), 20 mM glacial acetic acid (Roth) (97 %) and 1 mM EDTA (Roth) by heating. The gel was casted into a mold which was fitted with a well-forming comb. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus (Horizon 11-14, Horizontal Gel

Elektrophoresis System, Gibco Life Technologies, Gaithersburg, USA). After amplification, the PCR products with a DNA-ladder marker (peqLab Biotechnologie GmbH, Erlangen, Germany) were loaded into the samples wells to aid in fragment size determination. PCR fragments were separated by size in the agarose gel with Molecular Weight Marker VI (Roche Diagnostics). Electrophoresis was performed at 100 V for 40 min with the gene power supply (Power Pack P25, Biometra, Göttingen, Germany) at room temperature. Then the gel was dyed in the presence of 50 ml 1 x TAE buffer containing 2.5 µM ethidium bromide (10 mg/ml; Pharmacia Biotech, Uppsala, Sweden) for 30 min. The DNA bands were visualized by ultraviolet light (Transilluminator, UVP Inc., Upland, CA, USA) and photographed using a video camera (MWG Biotech AG).

2.4 Quantitative "real time" reverse transcriptase polymerase chain reaction (qRT-PCR) for RHAMM

For the quantitative measurement of the mRNA expression of $RHAMM^{FL}$ quantitative real-time RT-PCR was performed using the Light Cycler SYBR Green I technology according to the manufacturer's protocol (Roche Diagnostics) (Greiner et al. 2000). The TATA-Box binding protein (TBP) was used as a house-keeping gene. An initial denaturation step at 95°C for 10 min was followed by 40 cycles of 10 s at 95°C, 15 s at 62°C, 20 s at 72°C for the TBP. For the gene RHAMM/CD168 an initial denaturation step at 95°C for 10 min was followed by 45 cycles of 10 s at 95°C, 10 s at 66°C, 12 s at 72°C. 0.1 µl of cDNA was used per qRT-PCR.

Table 5a. Primer sequences and PCR conditions for TAAs/LAAs in PBMCs of B-CLL patients and HVs. (part 1)

Antigen Name	Forward Primer	Reverse Primer	
	Accesion Number	T_m = Melting Temperature	Number of Cycles
BAGE	5' TGG CTC GTC TCA CTC TGG 3'	5' CCT CCT ATT GCT CCT GTT G 3'	
	NM_001187.1	T_m =64°C	35 Cycles
G 250 (CA9)	5' ACT GCT GCT TCT GAT GCC TGT 3'	5' AGT TCT GGG AGC GGC GGG A 3'	
	NM_0011216.1	T_m =68°C	35 Cycles
HTERT	5' CCT CTG TGC TGG GCC TGG ACG ATA 3'	5' ACG GCT GGA GGT CTG TCA AGG TAG	
	NM_003219.1	T_m =68°C	35 Cycles
MAZ	5' CCT TCC GCG ACG TCT ACC ACC TGA 3'	5' CTA CTG CTG CCG CTG CCG CTG 3'	
	NM_002383.1	T_m =68°C	32 Cycles
MIDA	5' AAG ATC ATT ATG CAG TTC TTG GAC 3'	5' CCA ATA ACA TCT TTG GCA GTT CT 3'	
	X_98260	T_m =60°C	35 Cycles
NY-ESO-1	5' ATG GAT GCT GCA GAT GCG G 3'	5' GGC TTA GCG CCT CTG CCC TG 3'	
	NM_139250	T_m =60°C	35 Cycles
NY-REN60	5' GAA TCG CCC CAG CCT CTT TG 3'	5' ACT CTG CGC ATC CAC TTT CTT CAG	
	NM_032582.2	T_m =58°C	30 Cycles
PINCH	5' GCC AAC TGC GGG AAG GAG 3'	5' GGA AGC AAA CAT CAC CAA ATA 3'	
	NM_004987.2	T_m =56°C	32 Cycles
PRAME	5' GTC CTG AGG CCA GCC TAA GT 3'	5' GGA GAG GAG GAG TCT ACG CA 3'	
	NM_006115	T_m =64°C	35 Cycles
Proteinase 3	5' ACC TCA GTC CAG CTG CCA 3'	5' GAA AGT GCA AAT GTT ATG 3'	
	NM_002777.2	T_m =52°C	35 Cycles
RHAMM	5' GGA AGC AAG GCT AAA TGC TG 3'	5' ACC TGC AGC TTC ATC TCC AT 3'	
	NM_012484.1	T_m =66°C	35 Cycles
$RHAMM^{FL}/RHAMM^{-exon4}$	5' GGC CGT CAA CAT GTC CTT TCC TA 3'	5' TTG GGC TAT TTT CCC TTG AGA CTC 3'	
	NM_012484.1	T_m =68°C	35 Cycles
Fibromodulin	5' CAA CAC CTT CAA TTC CAG CA 3'	5' ACC TGC AGC TGG GAG AAG T 3'	
	NM_002023.2	T_m =55°C	35 Cycles
Syntaxin	5' CAG TGG GCA AAG CGA GGT GTT 3'	5' ACT GTG ACG CCA ATG ATG ACT GCT	
	NM_004604.2	T_m =58°C	30 Cycles
WT1	5' ATG AGG ATC CCA TGG GCC AGC A 3'	5' CCT GGG ACA CTG AAC GGT CCC CGA	
	NM_000378.2	T_m =64°C	35 Cycles
HSJ	5' AGG AGC AGT AGA GTG CTG TC 3'	5' GAC AGC ACT CTA CTG CTC CT 3'	
	NM_008298.1	T_m =56°C	35 Cycles

Table 5b. Primer sequences and PCR conditions for TAAs/LAAs in PBMCs of B-CLL patients and HVs. (part 2)

Antigen Name	Forward Primer		Reverse Primer	
	Accesion Number	T_m = Melting Temperature		Number of Cycles
OFAiLRP	5' ATG TCC GGA GCC CTT GAT GTC CTG CAA ATG 3'		5' TTA AGA CCA GTC AGT GGT TGC TCC TAC CC 3'	
	J_03799	T_m =68°C		35 Cycles
Survivin	5' CGA CCC CAT AGA GGA ACA TAA A 3'		5' GGA ATA AAC CCT GGA AGT GGT G 3'	
	AF077350	T_m =59°C		30 Cycles
β-actin	5' GCA TCG TGA TGG ACT CCG 3'		5' GCT GGA AGG TGG ACA GCG A 3'	
	XM_037235	T_m =68°C		24 Cycles
TBP	5'CAC GAA CCA CGG CAC TGA TT 3'		5'TTT TCT TGC TGC CAG TCT GGA C 3'	
	NM_003194.2	T_m =60°C		40 Cycles

Table 5 (a and b) displays the conditions of reverse transcriptase polymerase chain reactions (RT-PCRs) for tumor/leukemia associated antigens (TAAs/LAAs). Tumor antigen name, forward primer sequence, reverse promer sequence, accession number (from BLAST database), melting temperature and number of cycles are described for each gene assessed in this study.

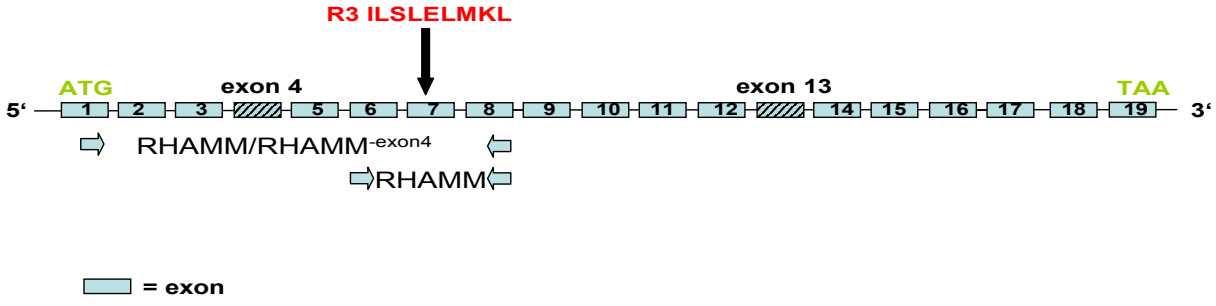
To quantify the mRNA expression of RHAMM/CD168, a conventional PCR for RHAMM/CD168 from K562 was performed and the amount of product cDNA was measured by photometry using an Eppendorf BioPhotometer (Eppendorf). A serial dilution of cDNA was subjected to PCR to obtain standard curves. The amount obtained by quantitative measurement of mRNA in attogram (ag) for RHAMM/CD168 and TBP was calculated into copy numbers and normalized to the house keeping gene TBP, of which hitherto no retro-pseudogenes are known. Quantitative measurement of mRNA by real-time RT-PCR for RHAMM/CD168 was performed for cohort of 24 other patients with B-CLL in early stages 0-2 according to the Rai classification.

2.5 Peptides

RHAMM/CD168-derived peptide sequences with HLA-A*0201-binding motifs were predicted using two different computer algorithms based on known binding affinities (Rammensee et al. 1999). Candidate peptides were also checked for their cleavage pattern by a prediction algorithm for proteasomal cleavage (Nussbaum et al. 2001).

RHAMM peptide R3 (pos. 165-173: ILSLELMKL; see also Figure 2) was chosen because it targets the most immunogenic RHAMM/CD168 peptide in AML, CML and HV (Greiner et al. 2005). The influenza matrix protein (IMP) derived peptide (GILGFVFTL) served as a positive control (Scheibenbogen et al. 2002). Peptides were synthesized by Thermo Electron Corporation (Ulm, Germany) to a minimum of 95% purity as measured by high performance liquid chromatography.

Figure 2. A schematic map of RHAMM/CD168



The RHAMM/CD168 sites of primers and the location of the T cell epitope peptide R3 used in this study are indicated. The pair of primers RHAMM/RHAMM^{-exon4} was used for conventional RT-PCR, the pair of primers for RHAMM in “real time” RT-PCR experiments, R3 peptide was used as a highly immunogenic epitope of RHAMM/CD168 in mixed lymphocyte peptide cultures and ELISPOT assays. Boxes indicate exons, splice variants –exon4 and –exon13 as desribed in the literature are marked.

2.6 Mixed lymphocyte peptide culture (MLPC)

PBMC from B-CLL patients were separated by Ficoll and subsequently selected by CD8 Magnetic Beads (Miltenyi Biotec) through a MACS column (Miltenyi Biotec). CD8 negative antigen presenting cells (APCs) were irradiated with 30 Gy and pulsed with a R3 peptide or a IMP at a concentration of 20 µg/ml for 2 h. After co-incubation with CD8 positive T lymphocytes overnight, the MLPC was supplemented with 10 U/ml IL-2 (recombinant human Interleukin-2, Sigma-Aldrich, Munich, Germany) and 20 ng/ml IL-7 (recombinant human Interleukin-7, Strathmann Biotec AG, Hamburg, Germany) on day +1. On day +7, the medium was changed and new irradiated and peptide-pulsed CD8 negative APCs were added as one week before. Again, the MLPC was supplemented with IL-2 and IL-7. After a total of 16 days of culture, T cells were harvested and evaluated for their specific cytotoxicity in a standard ELISPOT assay against T2 cells pulsed with the RHAMM/CD168 peptide, with the positive control IMP peptide, with unspecific positive control Pokeweed Mitogen (PWM) (Sigma-Aldrich) or without peptide, as described by another group earlier (Scheibenbogen et al. 2002).

2.7 Enzyme-linked immunosorbent spot (ELISPOT) assay for the detection of interferon gamma (IFN-γ) secretion

IFN-γ ELISPOT assays were performed according to the manufacturer's protocol (Mabtech, Hamburg, Germany) and as described earlier in detail (Greiner et al. 2002). Briefly, 96-well nitrocellulose plates (Millipore, Schwalbach, Germany) were coated with INF-γ mAbs (Mabtech) and incubated overnight at 4°C. After

washing with PBS (Invitrogen Gibco, Grand Island, USA), plates were blocked with 10% human AB serum (German Red Cross Blood Bank, Ulm) for 2 h at 37°C. 1 x 10^4 presensitized CD8+ T-lymphocytes and 4 x 10^4 target cells (peptide pulsed T2 cells) were added to each well. As a positive control, not connected with antigen recognition, 1 µg/ml Pokeweed Mitogen (PWM) (Sigma-Aldrich) was used. After incubation in RMPI 1640 medium (Biochrom AG) overnight, plates were washed with PBS. 0.2 µg/ml IFN-γ mAbs (Mabtech, Hamburg, Germany) were added to each well, plates were incubated for 2 h at room temperature. Cells were washed with 1 µg/ml streptavidin-alkaline phosphatase (Mabtech) for 2 h. Plates were washed and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate p-toluidine / nitroblue tetrazolium, Sigma-Aldrich) was used for colorization according to the manufacturer's instructions.

Plates were dried up at room temperature and thereafter evaluated by the use of an ELISPOT reader consisting of a video camera and a computer system with pattern recognition software (CTL, Reutlingen, Germany).

2.8 Granzyme B ELISPOT assay

The granzyme B ELISPOT assay was performed as described elsewhere (Scheibenbogen et al. 2002) to determine specific lysis of RHAMM/CD168 (peptide) positive target cells according to the manufacturer's instructions (BD, San Diego, USA).

2.9 Cell culture with CD40L

Based upon data from literature, a period of the three days was chosen as stimulation time period (Graziero et al. 2001, Gricks et al. 2004). On day 1, PBMCs from CLL patients were separated by Ficoll and subsequently selected by CD19 Magnetic Beads (Miltenyi Biotec) through a MACS column (Miltenyi Biotec). CD19 positive cell were cultured in RPMI1640 medium (Biochrom AG) supplemented with 0.5 µg/ml soluble, human CD40L (sCD40L) + 2 µg/ml enhancer for ligands (Alexis, San Diego, USA) and cultured for three days.

2. 10 Tetramer staining

CD8+ T cells (1×10^6), stimulated with irradiated CD8 negative antigen presenting cells in the presence of R3 peptide or an irrelevant peptide MAGE3-derived peptide (FLWGPRALV), were stained with the tetramer HLA-A2/R3*PE (Ludwig Institute for Cancer Research, Lausanne, Switzerland) at a concentration of 5 µg/ml and anti-CCR7 pure rat antibody (BD, Heidelberg, Germany) in the dark and incubated for 40 min at room temperature. Thereafter, anti-CD8*PerCP (BD, Heidelberg, Germany), anti-CD45RA*FITC (BD, Heidelberg, Germany) and secondary antibody goat anti-rat IgG*APC (Caltag Laboratories, Burlingame, USA) were added at 4ºC for 20 min in the dark. After washing twice, stained cells were analyzed by flow cytometry.

2.11 Software used and statistical analysis

To compare RHAMM/CD168 expression by several groups of patients, the alternative t-test was used. Results lower than 0.01 were considered to be significant. Statistical analyses were performed using STATISTICA 5.1 (StatSoft, StatSoft Polska, Poland).

3 Results

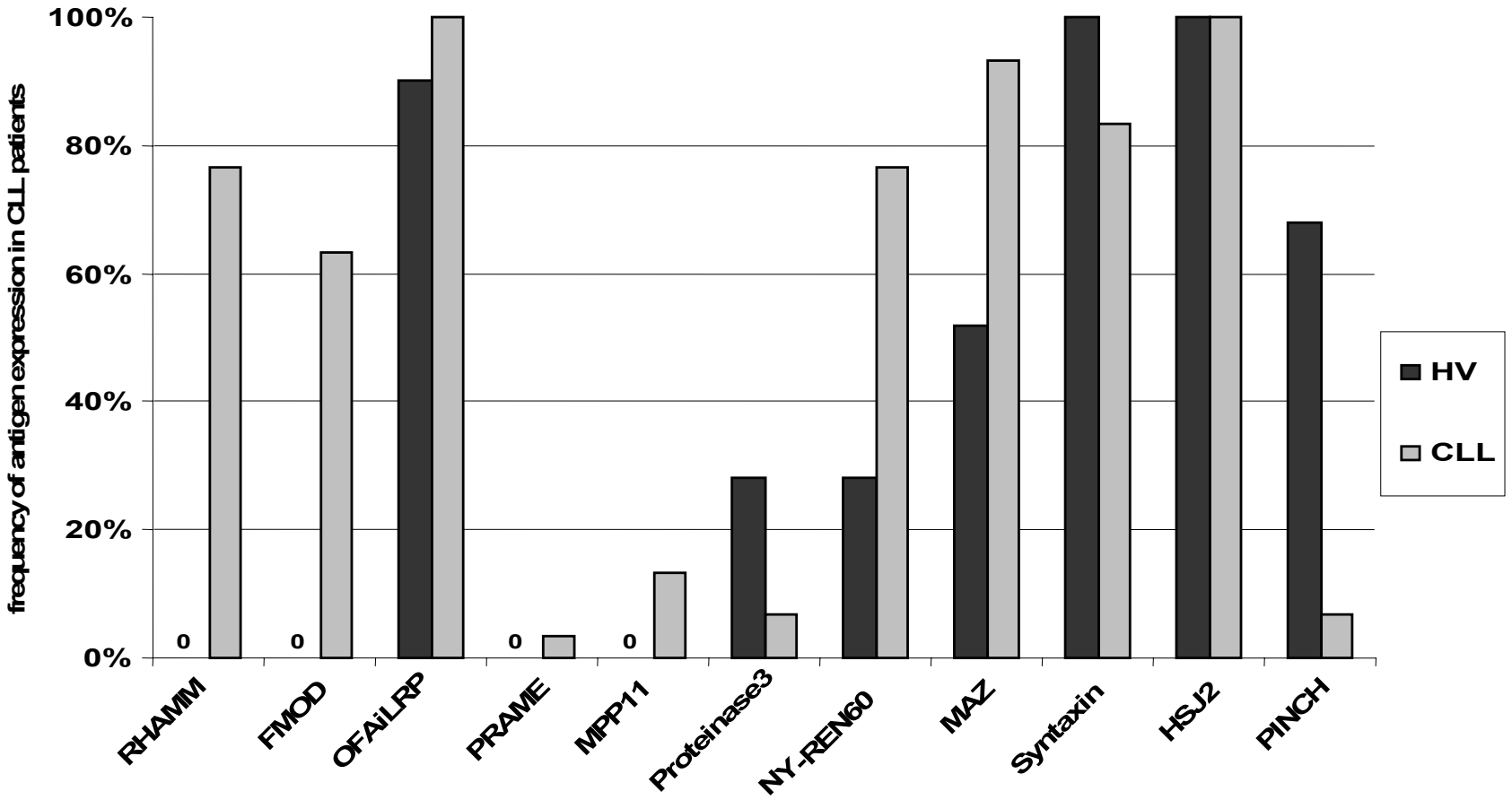
3.1 Differential mRNA expression of TAAs in CLL patients versus healthy volunteers

The mRNA expression of different immunogenic antigens was evaluated in PBMCs from 30 B-CLL patients. Several antigens with reported expression in other leukemia types (AML, CML and the cell line K562) showed no mRNA expression in naive B-CLL samples: **WT-1**, **MAGE A1, BAGE, G250** and **h-TERT.** A frequency of <20% in patients with CLL was noted for the mRNA expression of **MIDA, PINCH, PRAME** and **proteinase 3**. **MIDA** was detected in 13% of CLL patients, **PINCH** in 7%, **PRAME** in 3% and **proteinase 3** in 7% of these patients. More then half of the examined CLL patients showed a positive mRNA expression for **RHAMM/RHAMM^{-exon4}** (77%), **fibromodulin** (63%), **NY-Ren 60** (77%) and **syntaxin** (83%). The highest frequencies of mRNA expression in CLL patients were detected for **HSJ2** (100%), **MAZ** (93%) and **OFAiLRP** (100%). Figure 3 gives a comparison of the mRNA expression frequencies of T/LAA in B-CLL patients versus healthy volunteers.

3.2 Survivin expression before and after stimulation with sCD40L

No mRNA expression of survivin in PBMCs was detected in all naive B-CLL samples. Survivin is also not expressed in PBMN form HV (Figure 4).

Figure 3. Expression of selected TAAs in B-CLL patients versus healthy volunteers.



Expression of selected TAAs in B-CLL patients versus healthy volunteers (HV). The frequency of antigen expression in 30 B-CLL patients and 20 healthy volunteers (HV) was assessed by conventional RT-PCR as described in the Material and Methods section.

Figure 4. The example of expression of survivin in PBMCs from patients with B-CLL (A) and from healthy volunteers (B).

Representative results for survivin expression in PBMCs from 9 patients with B-CLL (CLL1-9) are shown in panel A, and results from 9 HV (HV1-9) are depicted in panel B. Panel C displays the expression of β-actin in B-CLL patients. Results were obtained by RT-PCR as described in details in the Material and Methods Section. The cell line K562 was used as positive control and distilled water (DW) as negative control. "M" indicates Molecular Weight Marker VI (Roche).

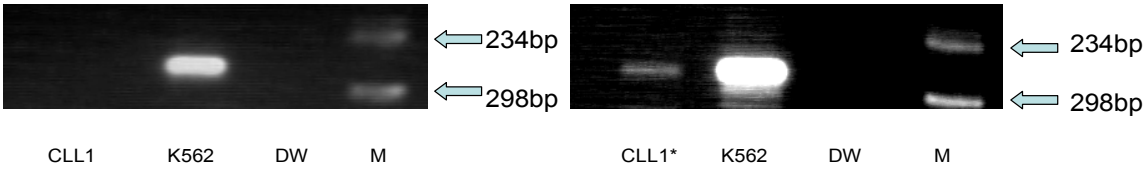
Survivin expression was only detectable on CD19 positive cells after 4 days of stimulation with sCD40L (Figure 5).

3.3 RHAMM/CD168 mRNA expression in different stages of CLL by conventional RT-PCR

RHAMM/RHAMM^{-exon4} is more frequently expressed in advanced stages of disease according to Rai (3 and 4) then in early stages (0-2) according to Rai

(100% vs. 61%, p=0.014) (Figure 6).

Figure 5. Increased expression of survivin after 3-days stimulation of B-CLL cells with sCD40L



The induced expression of survivin after 3 days of stimulation of CD19 positive cells from a B-CLL patient (CLL1) with sCD40L. Results were obtained by RT-PCR for survivin. The cell line K562 served as a positive control. Distilled water (DW) was used as negative control.

3.4 Expression analysis of RHAMM by real-time RT-PCR

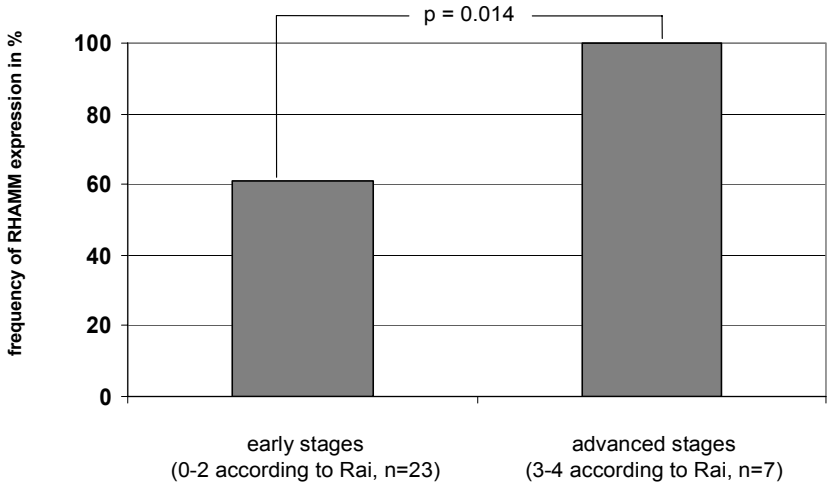
Quantitative measurement of mRNA by real-time RT-PCR for RHAMM was performed for 24 samples from CLL patients. The results from all patients were transformed into copy numbers as described earlier (Li et al. 2005). Thereafter, the percentage of TBP was calculated (Table 6).

Table 6. Results from real time RT-PCR of the housekeeping gene TBP and RHAMM/CD168 in 24 CLL patients as assessed by LightCycler technology.

Patient No.	Rai stage	RHAMM/CD168 (copy numbers)	TBP (copy numbers)	Ratio $RHAMM^{FL}$/TBP (%)
1	1	229	25,268	0.90
2	1	555	36,260	1.53
3	1	324	42,809	0.75
4	0	243	35,816	0.67
5	0	224	61,188	0.36
6	1	199	36,694	0.54
7	1	212	40,216	0.52
8	1	149	41,312	0.36
9	0	358	49,752	0.72
10	1	592	81,436	0.72
11	1	988	51,766	1.90
12	1	301	32,562	0.92
13	1	186	54,845	0.33
14	1	125	73,698	0.17
15	0	434	48,285	0.89
16	0	146	77,902	0.18
17	0	850	58,274	1.45
18	0	1,238	151,033	0.81
19	0	388	33,440	1.16
20	1	244	27,768	0.88
21	1	1,106	50,330	2.19
22	0	175	32,469	0.53
23	0	18	5,830	0.31
24	1	362	25,186	1.43

24 B-CLL patients at early stages of the disease (stage 0 and 1 according to the Rai classification) were examined by quantitative RT-PCR. Results obtained by LightCycler "real time" RT-PCR for RHAMM/CD168 and for TATA-binding protein (TBP) were transformed into copy numbers, and the relative ratio RHAMM/TBP was calculated.

Figure 6. Stage of disease and RHAMM/RHAMM^{-exon4} expression in B-CLL patients.



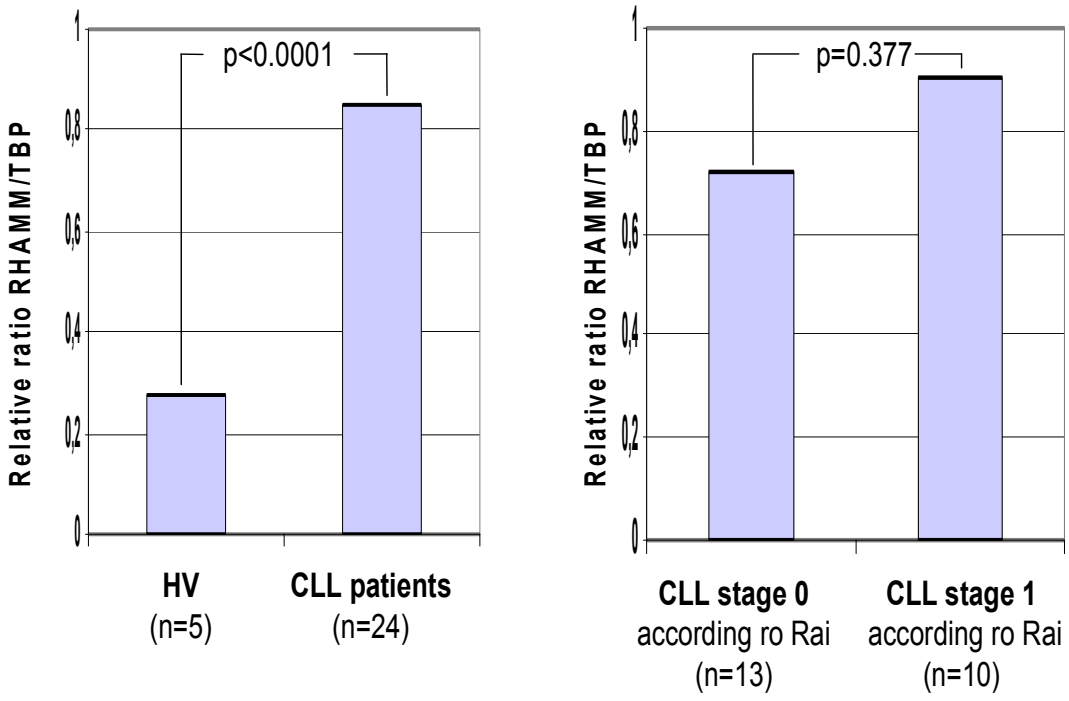
Correlation of the stage of disease and RHAMM/RHAMM^{-exon4} expression. The frequency of RHAMM/CD168 expression in B-CLL patients in early (0-2) and advanced (3-4) stages of the disease according to the Rai classification. A conventional RT-PCR was performed as described in the Material and Methods section. n indicates the number of patients.

3.5 The mRNA expression of RHAMM/CD168 is significantly higher in B-CLL patients than in HVs

The mean difference of the ratio RHAMM/TBP between CLL patients and the healthy volunteers group was 0.57. Two-tailed p value of the alternative t-test was extremely significant ($p<0.0001$).

The tendency (p=0.38) to higher relative expression of RHAMM between CLL patients in Rai stage 0 and 1 was noted (Fig. 7).

Figure 7. The relative ratio of RHAMM/TBP in B-CLL patients as assessed by LightCycler real-time RT-PCR.



The relative ratio of RHAMM/TBP in early stage B-CLL patients and healthy volunteers (HVs) as assessed by LightCycler real-time RT-PCR. Quantitative results obtained by LightCycler "real time" RT-PCR for RHAMM/CD168 and for the TATA-binding protein (TBP) were transformed into copy numbers, and the relative ratio RHAMM/TBP was calculated. Panel A displays the difference of RHAMM/CD168 quantity in 24 patients with early stages of B-CLL versus 5 healthy volunteers (HVs). Panel B shows the level of RHAMM/CD168 in B-CLL patients with stage 0 and 1 of the disease according to the Rai classification.

3.6 The relative ratio RHAMM/TBP differs not significantly in ZAP-70 positive and negative B-CLL patients

The ZAP-70 status of CLL patients was evaluated by cytometric analysis. The cut-off level for ZAP positiveness was set at 20% as other groups (Crespo et al. 2003). In ZAP-70 positive CLL patients the mean relative RHAMM level (average = 0.9271, SD = 0.5158, range 0.36 to 1.91, n=9) did not differ significantly (p=0.23) the ZAP-70 negative group (average = 0.6728, SD = 0.3695, range 0.34 to 1.53, n=12).

3.7 Synopsis of ELISPOT analysis for interferon gamma (INFγ) and Granzyme B

In ELISPOT assays for the release of interferon gamma (INFγ) and Granzyme B no or only sight (<10 spots/40 000 CD8+ cells) CTL responses even against influenza matrix protein (IMP) were observed after 8 days of culture. After 16 days of culture, CTL responses against RHAMM peptide and IMP could be observed (Fig. 8a, b).

Figure 8 (a and b) displays specific CTL responses against RHAMM/CD168 in one selected B-CLL patient after 16 days of MLPC (as assessed by ELISPOT assay for IFN-γ (Fig. 8a) and Granzyme B (Fig. 8b), numbers indicate the mean value of spots in triplicate per 40,000 CD8+ cells. After 16 days of culture, T cells were harvested and evaluated for their specific cytotoxicity in production of IFN-γ (Fig. 8a) and Granzyme B (Fig. 8b) in a standard ELISPOT assay against T2 cells pulsed with the RHAMM/CD168 R3 peptide, with the influenza matrix protein (IMP) peptide as a peptide-specific control, with the lectin pokeweed mitogen (PWM) as positive control or without peptide as a negative control. Results were evaluated by the use of an ELISPOT reader consisting of a video camera and a computer system with pattern recognition software.

Figure 8a. Specific CTL responses against RHAMM/CD168 in one selected B-CLL patient after 16 days of MLPC (as assessed by ELISPOT assay for IFNγ, numbers indicate spots per 40,000 CD8+ cells).

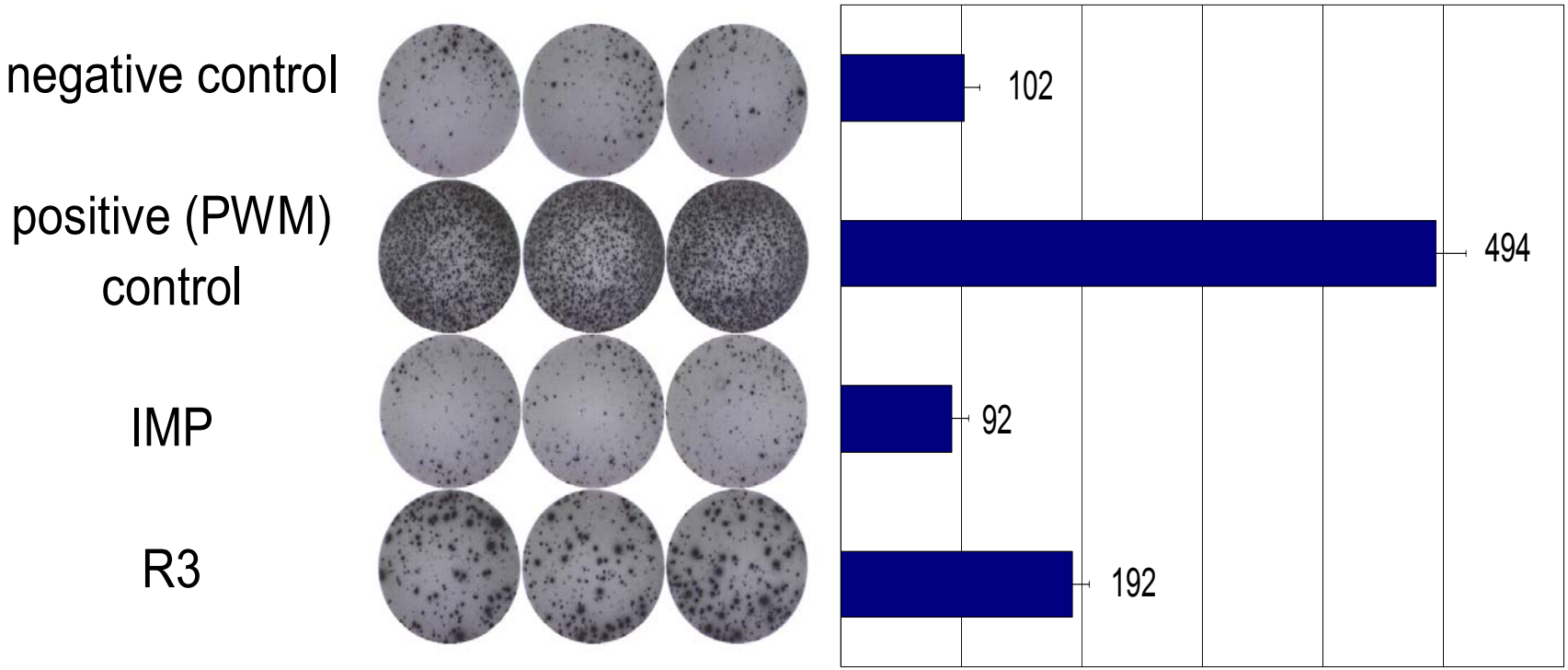


Figure 8b. Specific CTL responses against RHAMM/CD168 in one selected B-CLL patient after 16 days of MLPC (as assessed by ELISPOT assay for Granzyme B, numbers indicate spots per 40,000 CD8+ cells).

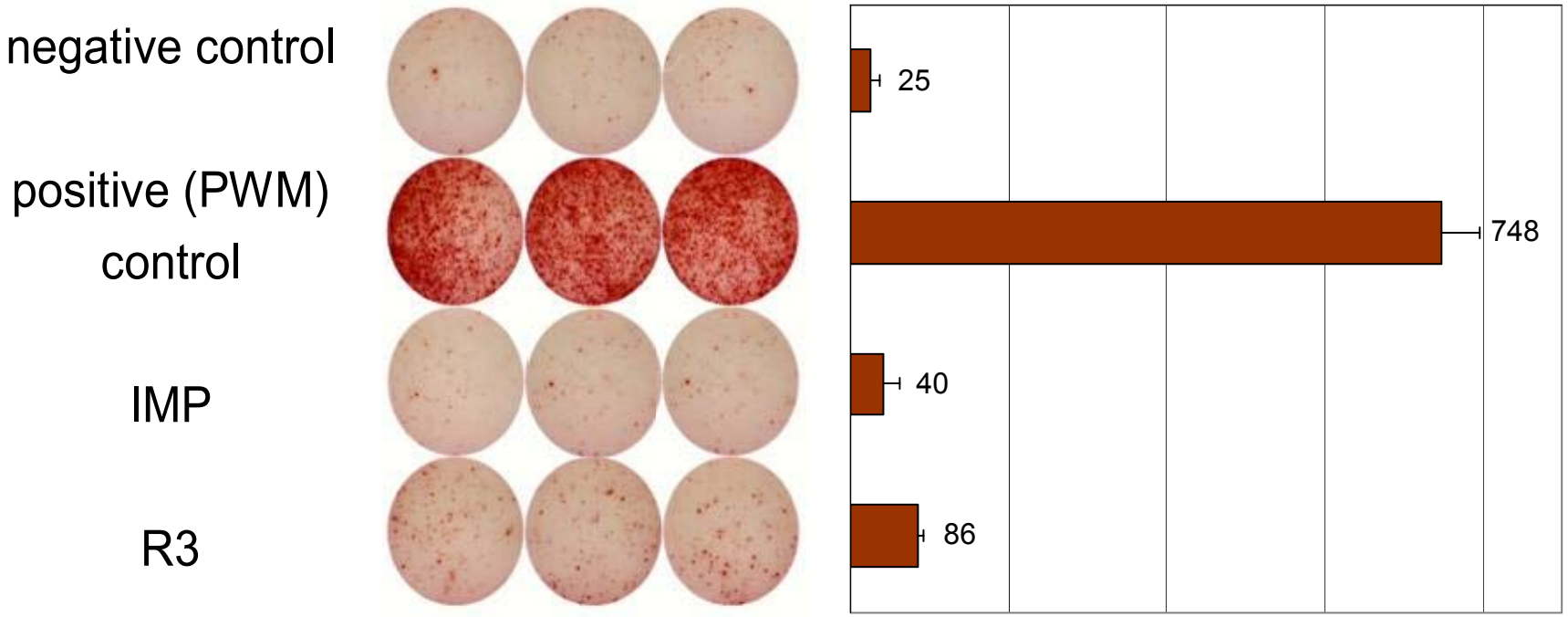
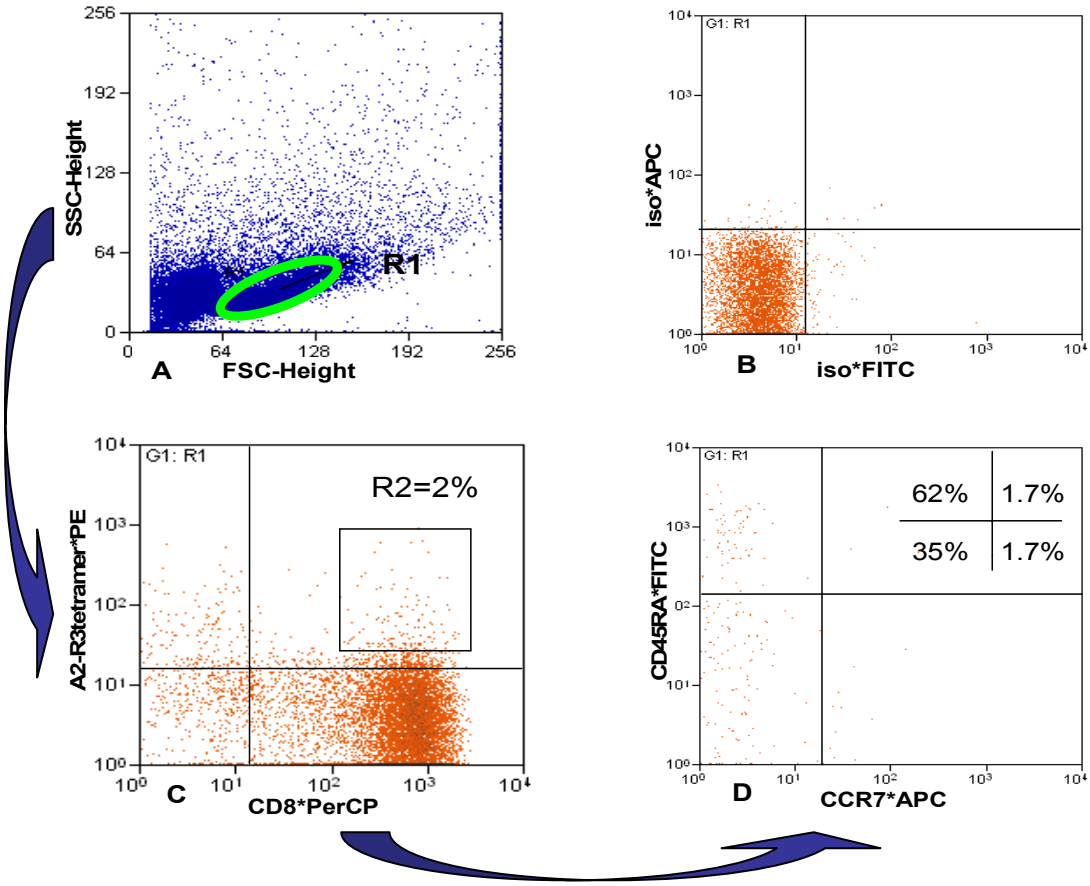


Figure 9. Four color-staining of R3-specific T lymphocytes.



CD8+ lymphocytes from an CLL patient were subjected to two rounds of stimulation with autologous CD8- APCs in the presence of the R3 peptide (Figure 9c) as described in the Material and Methods section. The figure 9 displays representative results obtained in such presensitized T lymphocytes using four-color staining of CD8 and HLA-A2/R3 tetramer peptide. The lymphocytes (gated in R1 of Figure 9a) were gated as double positive for CD8+ and HLA-A2/R3 tetramer+ (gate R2 of Figures 9c) and further characterized by counterstaining for CCR7 and CD45RA (Figure 9d). The majority of the T cells revealed to be CD8+HLA-A2/R3-teramer+CCR7-CD45RA^{high} effector T cells (as compared with iso-control Figure 9b).

3.8 Tetramer staining of CD8+ T lymphocytes specifically recognizing peptide R3 in the context of HLA-A2

CD8+ lymphocytes from three other CLL patients were subjected to two rounds of stimulation with autologous CD8- APCs in the presence of the R3 peptide or in the presence of an irrelevant MAGE3-derived peptide. FACS analysis with four-color revealed a frequency of CD8+ T cells specifically recognizing the R3 peptide ranging

from 0.2 to 2.2% in the presence of the R3 peptide (Figure 9c) in contrast with 0.01 to 0.3% in the presence of the irrelevant peptide MAGE3. CD8+HLA-A2/R3-tetramer+ T lymphocytes elicited by stimulation through the R3 peptide were gated (R2 gate) and further characterized as predominantly (62%) CCR7-CD45RA^{high} effector T cells (Figure 9d).

4 Discussion

Vaccination strategies for patients with B-CLL using whole tumor lysates (Hus et al. 2005), irradiated cells or total RNA to induce anti-tumor immune responses are under current clinical investigation (Kokhaei et al. 2004). These immunotherapeutical approaches circumvent the definition of specific antigens. However, to reach a high concentration of T cell epitope peptide and to induce thereby a clinically relevant T cell response, the definition of immunogenic TAAs and their T cell epitopes in B-CLL is highly desirable. Moreover only vaccination with antigen peptides might allow a straightforward assessment of T cell responses to these particular peptides (Nagorsen et al. 2004).

In this study, we found several expression patterns of TAAs in B-CLL patients vs. healthy donors (HVs), with some TAAs showing an exquisite expression in leukemic blasts but not normal cells (Fig. 3). A tumor-restricted expression would be favourable for future immunotherapies because of the absence of danger to induce auto-immune reactions by vaccination of patients with these TAAs.

High frequencies of such tumor-restricted antigens as **RHAMM/CD168** and **fibromodulin** (60-80%) and lower (<20%) expression frequencies **PRAME** and **MPP11** were noted. Intrestingly, no expression of **survivin** and **hTERT** was observed.

Hitherto, **survivin**, **OFAiLRP** and recently **fibromodulin** have been characterized as TAAs in B-CLL which can elicit an anti-tumor responses (Schmidt et al. 2004, Mayr et al. 2005, Siegel et al. 2003).

Survivin is expressed in CD40L stimulated CLL cells on the mRNA (Fig. 5) and on the protein level (Granziero et al. 2001), but not in non-stimulated CLL cells on the protein level (Zeis et al. 2003). This might be the reason why we were not able to detect mRNA signal for survivin in the PBMCs of B-CLL patients in this study who were mainly early stage patients.

T cells isolated from PBMCs of B-CLL patients were demonstrated *in vitro* to be reactive against **OFAiLRP** peptide pulsed DCs (Siegel et al. 2003). The mRNA expression results obtained in the present study suggest that OFAiLRP might not be the best target for immunotherapies because of its strong expression in PBMCs from healthy donors indicating the potential danger of auto-immune reactions. In this work, we did not consider the post-translational changes and the difference between the monomeric immature and the dimerized mature form of LRP, which can not be differentiated on the mRNA level.

Our results indicate that fibromodulin and RHAMM/CD168 are the most interesting possible targets for immunotherapy in B-CLL because of their tumor-restricted and high frequent expression.

The most important finding of this study was the definition of **RHAMM/CD168**, a new promising TAA candidate in B-CLL patients, which is expressed in tumor cells but neither in PBMN cells nor in CD34+ cells from healthy donors (Greiner et al. 2003, Fig. 3). An increased CTL response against a RHAMM/CD168-derived peptide was observed after vaccinations with DCs pulsed with CLL cell lysate (Hus et al. 2005). RHAMM/CD168 was first described as a molecule required for cell motility and cell cycle progression through the G2 and M phase

(Turley et al. 1993). RHAMM/CD168 plays an essential role in the organization and maintenance of the mitotic spindle apparatus and strongly activates extracellular-regulated kinase (erk1) (Zhang et al. 1998, Assmann et al. 1999). RHAMM/CD168 was found to be overexpressed in multiple myeloma (MM) and breast cancer (Craine et al. 1999, Assmann et al. 1998). High RHAMM^{-exon4}/RHAMM ratios in the bone marrow from MM patients correlate with poor survival, independent of standard prognostic factors (Maxwell et al. 2004). The expression of RHAMM/CD168 on the mRNA level and a weak expression of this antigen on the protein level were noted (Turley et al. 1993, Till et al. 1999).

Our group defined RHAMM/CD168 as a novel immunogenic antigen in patients with acute myeloid leukemia (Greiner et al. 2005).

Here, we show that RHAMM/CD168 is expressed in all stages of the examined B-CLL patients with the tendency of upregulation of RHAMM/RHAMM^{-exon4} expression in higher B-CLL stages (Fig. 6 and Fig. 7b). In colon cancer and CML, high RHAMM/CD168 expression is associated with metastases and higher tumor stages (Greiner et al. 2003, Yamada et al. 1999). We focused in this study on patients with early stages of B-CLL, presuming that these patients might potentially profit from immunotherapy.

Besides the status of IgVH mutation (Kröber et al. 2002), the zeta chain associated protein 70 (ZAP-70) was found to be a strong prognostic factor in B-CLL patients (Crespo et al. 2003). In the present study, RHAMM/CD168 was expressed in both ZAP-70 positive (+) and negative (-) B-CLL patients.

As demonstrated by other groups (Scrivener et al. 2003), a severe impairment of T cell function could be noted in patients with B-CLL. In several B-CLL patients evaluated in this work, no T cell reactivity to an unspecific stimulus like a lectin PWM was observed in a mixed lymphocyte reaction after presensitization, indicating that the T cell compartment in these B-CLL patients was impaired. The impaired T cell reactivity in B-CLL patients might be explained by the influence of CD19+/CD5+ leukemic cells on the T cell function as well as on the DC population (Orsini et al. 2004, Scrivener et al. 2003) and might be overcome by specific adjuvants such as IL-12, IL-15 or sCD40L (Goddard et al. 2003b, von Bergwelt-Baildon et al. 2004). After two weeks of presensitization we could observe CD8+ T cells reactive to PWM, IMP peptide or RHAMM/CD168 peptide in other B-CLL patients (Fig. 8a and Fig. 8b). The lysis of tumor cells by activated IFN-γ producing T lymphocytes through Granzyme B plays a major role in tumor rejection (Russell et al. 2002). In our experiments, we could induce IFN-γ and Granzyme B secreting CD8+ T cells recognizing targets pulsed with R3 peptide *in vitro.*

Summarizing the results of our immunological analysis, we demonstrated high expression frequencies of the leukemia-specific antigen RHAMM/CD168 in patients with B-CLL with no respect to stage or ZAP-70 status. In a subgroup of CLL patients, we detected CD8+ T cells isolated from PBMCs reactive to R3, a newly characterized T cell epitope peptide derived from RHAMM/CD168.

4.1 Conclusions

We conclude that RHAMM/CD168 might be an interesting target antigen for clinical peptide vaccination in patients with B-CLL, especially in early stages of the disease when the T-cell function is still preserved and the tumor load rather limited. RHAMM/CD168 is expressed on both ZAP-70 (+) and ZAP-70 (-) CLL patients rendering both subgroups of CLL patients eligible for future RHAMM targeted immunotherapies.

5 Summary

Specific immunotherapy might be a novel option for targeted therapy of patients with B-cell chronic lymphocytic leukemia (B-CLL), a disease characterized by a prolonged natural course. To define potential target antigens for immunotherapies in B-CLL, we screened the mRNA expression of eleven tumor/leukemia associated antigens (TAA/LAAs) from the literature: fibromodulin, survivin, oncofetal antigen-immature laminin receptor protein (OFAiLRP), B-melanoma antigen (BAGE), the carboanhydrase 9 (CA9/G250), melanoma antigen (MAGE1), the preferentially expressed antigen in melanoma (PRAME), proteinase, Syntaxin, human telomerase catalytic subunit (hTERT) and the Wilms tumor gene 1 (WT1), as well as six TAA/LAAs defined previously by serological analysis of recombinant cDNA expression libraries (SEREX) of myeloid leukemias patients by our group: the receptor for hyaluronic acid mediated motility (RHAMM/CD168), the M-phase phosphoprotein 11 (MPP11), the particularly interesting new Cys-His protein (PINCH), the heat shock protein (HSJ2), the myc-associated zinc-finger protein (MAZ) and by others: the renal cell cancer antigen NY-Ren60. Peripheral blood mononuclear cells (PBMCs) from 30 B-CLL patients and 20 healthy volunteers (HVs) were evaluated by conventional and quantitative RT-PCR. To evaluate the immunogenicity of the newly defined TAA RHAMM/CD168 in CLL, mixed lymphocyte peptide cultures (MLPC) and enzyme-linked immunosorbent spot (ELISPOT) assays were performed. No expression of WT-1, h-TERT, BAGE, G250, MAGE1 and survivin was observed in B-CLL patients or HVs. Low (2-20%) expression frequencies of MPP11, PINCH, PRAME and proteinase were detected. mRNA of RHAMM/CD168, fibromodulin, syntaxin and NY-Ren60 was expressed in 55-90% and of HSJ2, MAZ and OFA-iLRP in 90-100% of the patients. Only RHAMM/CD168, fibromodulin, PRAME and MPP11

showed expression in B-CLL patients but not in HVs, all others antigens showed expression frequencies of 28-100% in HVs. RHAMM mRNA expression was significantly higher in B-CLL patients than in HVs.

No significant difference of RHAMM/CD168 expression was observed between zeta chain associated protein 70 (ZAP-70) positive and negative patients. Specific cytotoxic T lymphocyte (CTL) responses in B-CLL patients against RHAMM/CD168 could be detected *in vitro*. In MLPC, RHAMM/CD168 specific responses by CD8+HLA-A2/R3tetramer+CCR7-CD45RA^{high} effector T cells were detected.

We conclude that RHAMM/CD168 might be an interesting target for future immunotherapies in both ZAP-70 (+) and ZAP-70 (-) CLL patients.

5.1 Zusammenfassung

Eine spezifische Immuntherapie für Patienten mit Chronischer Lymphatischer Leukämie vom B-Zell-Typ (B-CLL) könnte eine neue therapeutische Option für diese Tumorentität darstellen. Die Definition von immunogenen Antigenen ist dazu eine wichtige Voraussetzung.

In mononukleären Zellen des peripheren Blutes von 30 B-CLL Patienten und 20 Probanden untersuchten wir die Expression von folgenden Antigenen aus der Literatur und aus unserem serologischen Screening von recombinanten cDNA Expressions Bibliothek (SEREX) Studien bei Patienten mit Akuter myeloischer Leukämie (AML) und Chronischer myeloischer Leukämie (CML): Fibromodulin, Survivin, Oncofetal antigen-immature laminin receptor protein (OFAiLRP), B-melanoma antigen (BAGE), Carboanhydrase 9 (CA9/G250), melanoma antigen (MAGE1), preferentially expressed antigen in melanoma (PRAME), Proteinase, Syntaxin, Human telomerase catalytic subunit (hTERT), Wilms tumor gene 1 (WT1), Receptor for hyaluronic acid mediated motility (RHAMM/CD168), M-phase phosphoprotein 11 (MPP11), particularly interesting new Cys-His protein (PINCH), heat shock protein (HSJ2), Myc-associated zinc-finger protein (MAZ) und renal cell cancer antigen NY-Ren60.

Es wurde keine Expression für WT-1, h-TERT, BAGE, G250, MAGE1 und Survivin bei B-CLL-Patienten und Probanden detektiert. Eine geringe (2-20%) Häufigkeit der Expression für MPP11, PINCH, PRAME und Proteinase wurde festgestellt. mRNA für RHAMM/CD168, Fibromodulin, Syntaxin und NY-Ren60 wurde bei 55-90% der Patienten mit B-CLL exprimiert und mRNA für HSJ2, MAZ and OFA-iLRP bei 90-100%. Nur für RHAMM/CD168, Fibromodulin, PRAME und MPP11 konnten wir eine Tumor-restringierte Expression zeigen. Die höchste Häufigkeit der Expression wurde

für RHAMM/CD168 und Fibromodulin bei Patienten mit B-CLL beobachtet.

Für die Expression von RHAMM/CD168 wurde kein Unterschied bei ZAP-70 positiven und negativen Patienten mit B-CLL beobachtet.

RHAMM/CD168 ist in verschiedenen soliden Tumoren und bei Patienten mit AML und CML überexprimiert. Aufgrund der serologischen Daten für AML- und CML-Patienten und aufgrund seines Tumor-spezifischen Expressionsmusters ist das Antigen RHAMM/CD168 für eine spezifische Immuntherapie von Leukämien geeignet.

Wir wiesen in Enzyme-linked immunosorbent spot (ELISPOT)-Assays bei immunkompetenten B-CLL-Patienten RHAMM/CD168-R3-Epitop-Peptid-spezifische CTLs nach, die sowohl Interferon-gamma als auch Granzym B sezernierten, also ein zytotoxisches Potential besaßen. In der durchflusszytometrischen Analyse konnten diese R3-spezifischen T-Zellen als CD8+HLA-A2/R3tetramer+CCR7-CD45RA^{high} Effektor-T-Zellen charakterisiert werden.

Zusammenfassend konnten wir zeigen, dass RHAMM/CD168 und Fibromodulin in B-CLL häufig exprimiert sind und dass funktionelle T-Zellantworten gegen das RHAMM/CD168 HLA-A2-restringierte Peptid R3 *in vitro* bei Patienten mit B-CLL evozierbar sind.

RHAMM/CD168 könnte daher ein interessantes Target für zukünftige Immunotherapie-Strategien bei Patienten mit B-CLL darstellen.

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7 Publications

1. Dmoszyńska A, Giannopoulos K. Rola wysokodawkowej chemioterapii i autotransplantacji krwiotwórczych komórek macierzystych w szpiczaku plazmocytowym – 18 lat doświadczeń. (High dose chemotherapy and autologous hemopoietic stem cell transplantation in multiple myeloma – 18 years of experience). Acta Haematologica Polonica 35: 343 – 349 (2004)

2. Giannopoulos K, Greiner J, Roliński J, Dmoszyńska A, Hus I, Döhner H and Schmitt M. The mRNA expression of tumor associated antigens (TAAs) in CLL patients. Onkologie. 27: 1-230 (Abstract) (2004)

3. Dmoszyńska A, Giannopoulos K. Nowotwory hematologiczne wieku starszego. (Hematological malignancies in older patients). Postępy Biologii Komórki 32: 115-124 (2005)

4. Greiner J, Li L, Ringhoffer M, Barth TFE, Giannopoulos K, Guillaume P, Ritter G, Wiesneth M, Döhner H, Schmitt M. Identification and characterization of epitopes of the receptor for hyaluronic acid mediated motility (RHAMM/CD168) recognized by CD8 positive T cells of HLA-A2 positive patients with acute myeloid leukemia. Blood Apr 12 [Epub ahead of print] (2005)

5. Gianopoulos K, Karaś P, Tabarkiewicz J, Roliński J, Dmoszyńska A. The assessment of dendritic cells subsets and lymphocyte subpopulations after taking a Finnish sauna. Pol. J. Environmental Studies 14: 109-113 (2005)

6. Giannopoulos K. Immunotherapeutical targets for B-cell chronic lymphocytic leukemia. Acta haematologica Polonica 36: 76-82 (2005)

7. Giannopoulos K, Dmoszyńska A, Roliński J, Hus I, Schmitt M. The expression of survivin in patients with B-cell chronic lymphocytic leukemia (B-CLL). Acta haematologica Polonica 36: 83-88 (2005)

8. Hus I, Roliński J, Tabarkiewicz J, Wojas K, Bojarska-Junak A, Greiner J, Giannopoulos K, Dmoszyńska A, Schmitt M. Allogeneic dendritic cells pulsed with tumor lysates or apoptotic bodies as immunotherapy for patients with early stage B-cell chronic lymphocytic leukemia (B-CLL). Leukemia (in press) (2005)

papers submitted

9. Giannopoulos K, Li L, Bojarska-Junak A, Roliński J, Dmoszyńska A, Hus I, Greiner J, Renner C, Döhner H and Schmitt M. Expression of RHAMM/CD168 and other tumor associated antigens in patients with B-cell chronic lymphocytic leukemia.

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