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The chief cause of poverty in science is imaginary wealth.
The chief aim of science however is not to open the door
to infinite wisdom, but to put some limit to infinite error.

Bertholt Brecht, Galileo

A mamma e papà
e in memoria di Gabriella

ABSTRACT

Chronic lymphocytic leukaemia (CLL) is a malignant lymphoproliferative disorder which typically affects elderly people. It is the commonest leukemia in the Western adults, accounting for 25-30% of all leukemias and for 10% of all hematological neoplasms. Although new modalities such as combination therapy with fludarabine, cyclophosphamide (CTX) and the anti-CD20 antibody rituximab have greatly improved clinical outcome in a fraction of patients, CLL is largely considered incurable and there is a continuous need to develop new treatment strategies. Anti-cancer active specific immunotherapy aims at activating the patient´s immune system to recognize and eliminate the tumor. A number of clinical observations as well as several preclinical studies indicate that CLL is responsive to immune effector functions.

In the first part of this thesis, we investigated the ability of a promiscuous HLA class II epitope, hTERT (611–626) (GV1001) to elicit antileukemic immune responses *in vitro*. We demonstrated that CLL patients with hTERT-expressing leukemic cells have naturally occurring hTERT-specific T cells that proliferate and can be expanded *in vitro* and used to lyse autologous CLL cells. We therefore identified telomerase as a vaccine candidate in CLL. We then analyzed hTERT mRNA splicing patterns in CLL by a newly designed quantitative PCR assay and showed that the expression of the functional transcript of hTERT (hTERT-FL) is independent from disease phase in IgHV mutated but not in unmutated patients. This finding highlights the necessity of focusing on this transcript when analyzing hTERT expression and encourages further studies to assess whether hTERT-FL could generate novel epitopes that may serve as immunotherapy targets.

In the second part of the thesis, we studied safety, immune and clinical effects of vaccination with autologous DC loaded with apoptotic CLL cells (Apo-DC) in CLL patients in a phase I clinical trial. Using a combination of leukapheresis and affinity-based technologies (CliniMACS®) for monocyte enrichment, we were able to produce a sufficient amount of DC vaccine that met accepted and established quality criteria. Sixteen patients were accrued stepwise in three different cohorts receiving Apo-DC alone, Apo-DC + granulocyte-macrophage-colony-stimulating-factor (GM-CSF), or Apo-DC + GM-CSF + low-dose CTX. Vaccination was well tolerated and increased leukemia-specific immunity in 10/15 (66%) of the patients (2/5, 3/5 and 5/5 in the three cohorts, respectively). No significant difference in time-to progression (TTP) between immune-responders and non-immune responders was observed. An additional patient was immunized repeatedly for a long period of time and achieved a complete response in blood and a nodular partial response in bone marrow. $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T-cells (T_{regs}) measured in one year follow-up period were significantly lower in immune-responders vs non-responders ($p<0.0001$). In this study, we demonstrated that vaccination with Apo-DC is a feasible approach that can generate immune responses and potentially clinical responses and that combination with GM-CSF and low-dose CTX functions as an immunological adjuvant in this setting.

In conclusion, the studies presented in this thesis suggest that immunotherapy is a promising approach in CLL and promote further investigation to better define the vaccination strategy and combination with immune enhancing/modulating drugs which holds the greatest potential to generate immune responses and clinical benefit in CLL patients.

LIST OF PUBLICATIONS

I. Kokhaei P*, **Palma M***, Hansson L, Österborg A, Mellstedt H, Choudhury A. Telomerase (hTERT 611-626) serves as tumor antigen in B-cell chronic lymphocytic leukemia and generates spontaneously antileukemic, cytotoxic T cells. *Experimental Hematology*, 2007, 35, 297-304.

II. **Palma M**, Kokhaei P, Hansson L, Hojjat-Farsangi M, Choudhury A, Österborg A, Mellstedt H. Expression of human Telomerase Reverse Transcriptase splice variants in chronic lymphocytic leukemia. *(Manuscript)*.

III. Adamson L, **Palma M**, Choudhury A, Eriksson I, Näsman-Glaser B, Hansson M, Hansson L, Kokhaei P, Österborg A, Mellstedt H. Generation of a dendritic cell-based vaccine in chronic lymphocytic leukemia using CliniMACS platform for large-scale production. *Scandinavian Journal of Immunology*, 2009, 69, 529-536.

IV. **Palma M**, Hansson L, Näsman-Glaser B, Eriksson, Adamson L, Widén K, Horváth R, Kokhaei P, Vertuani S, Choudhury A, Österborg A, Mellstedt H. Vaccination with dendritic cells loaded with apoptotic bodies (Apo-DC) of autologous leukemic cells in chronic lymphocytic leukemia. *(Manuscript)*.

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B. Kokhaei P, Adamson L, **Palma M**, Österborg A, Pisa P, Choudhury A, Mellstedt H. Generation of DC-based vaccine for therapy of B-CLL patients. Comparison of two methods for enriching monocytic precursors. *Cytotherapy* (2006), 8 (4): 318-326.

C. **Palma M**, Adamson L, Hansson L, Kokhaei P, Rezvany R, Mellstedt H, Österborg A, Choudhury A. Development of a dendritic cell-based vaccine for chronic lymphocytic leukemia. *Cancer Immunology and Immunotherapy* (2008), 57:1705–1710.

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AFP	Alpha-fetoprotein
Ag	Antigen
AIHA	Autoimmune hemolytic anemia
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ATM	Ataxia telangiectasia-mutated
BAFF	B-cell activation factor of the TNF family
BCG	Bacillus Calmette-Guerin
BCR	B-cell receptor
BM	Bone marrow
CAP	Cyclophosphamide, adriamycine, prednisone
CBC	Complete blood count
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
CFC	Cytokine Flow-cytometry
CLL	Chronic lymphocytic leukemia
CD40L	CD40 ligand
CDR	Complementarity-determining region
CFAR	Fludarabine/cyclophosphamide/rituximab/alemtuzumab
cFLIP	cellular FLICE-inhibitory protein
CHOP	Cyclophosphamide, adriamycine, vincrinstine, prednisone
CMV	Cytomegalovirus
CR	Complete response
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTX	Cyclophosphamide
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
EBV	Epstein Barr virus
ECOG	Eastern Cooperative Oncology Group
ELISPOT	Enzyme-linked Immunospot
EMEA	European Medical Agency
EORTC	European Organization for Research and Treatment of Cancer
FA	Fludarabine/alemtuzumab
FADD	Fas-associated death domain
FC	Fludarabine/cyclophosphamide
FCA	Fludarabine/cyclophosphamide/alemtuzumab
FCO	Fludarabine/cyclophosphamide/oblimersen
FCR	Fludarabine/cyclophosphamide/rituximab
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
FMNL1	Formin-related protein in leukocytes 1
FR	Fludarabine/rituximab

GC	Germinal center
GITR	Glucocorticoid-induced TNF receptor family-related gene
GM-CSF	Granulocyte-macrophage-colony-stimulating-factor
GMP	Good manufacturing practice
GVL	Graft-versus-leukemia
HDAC	Histone deacetylase
HER-2	Human Epidermal growth factor Receptor 2
HLA	Human leukocyte antigen
HPV	Human papillomavirus
hTERT	Human telomerase reverse transcriptase
ICAM	Intracellular adhesion molecule
Id	Immunoglobulin idiotype
IDO	Indoleamine 2,3-dyoxigenase
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IgHV	Ig heavy chain V
IL	Interleukin
IMC	Immature myeloid cell
iSBTc	International Society for Biological Therapy of cancer
IWCLL	International Workshop on chronic lymphocytic leukemia
LDT	Lymphocyte doubling time
LFA	Leukocyte function–associated antigen
LMP	Low-molecular-mass protein
LN	Lymph nodes
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBL	Monoclonal B lymphocytosis
M-CSF	Macrophage colony stimulating factor
MDM2	Murine double minute 2 oncoprotein
MDSC	Myeloid-derived suppressor cell
MIF	Macrophage migration inhibitory factor
MZ	Marginal zone
NF-kB	Nuclear factor-κB
NK cell	Natural killer cell
NKR	NK cell receptor
NLCs	Nurse-like cells
NKT cell	Natural killer T cell
NO	Nitric oxide
OFA-iLRP	Oncofetal antigen immature laminin receptor protein
OR	Overall response
OS	Overall survival
PB	Peripheral blood
PR	Partial response
PC	Proliferation center
PD	Progressive disease
pDC	Plasmocytoid DC

PDGF	Platelet-derived growth factor
PFS	Progression-free survival
PGE_2	Prostaglandin E_2
PS	Performance status
qPCR	quantitative real-time PCR
RAG	Recombinase activating gene
RECIST	Response Evaluation Criteria in Solid Tumors
RHAMM	Receptor for hyaluronic acid-mediated motility
RIC	Reduced-intensity conditioning
RT-PCR	Reverse transcriptase polymerase chain reaction
SHM	Somatic hypermutation
SDF-1	Stromal cell-derived factor-1
SI	Stimulation index
SLL	Small lymphocytic lymphoma
SCT	Stem cell transplantation
SD	Stable disease
SEREX	Serological identification by recombinant expression cloning
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TGF	Tumor growth factor
TD	T cell-dependent
Th1	T helper 1
Th2	T helper 2
TI	T cell-independent
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T-PLL	T-cell prolymphocytic leukemia
TTP	Time to progression
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	regulatory T cell
VEGF	Vascular endothelial growth factor
WBC	White blood cells
ZAP70	Tyrosine kinase zeta-associated protein 70

CONTENTS

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is a malignant lymphoproliferative disorder of mature B lymphocytes. It is characterized by the progressive accumulation of mature-looking, functionally incompetent, long-lived B lymphocytes in blood, bone marrow, lymph nodes, spleen, liver or other lymphoid tissues [1].

CLL typically affects elderly people and is characterized by an extremely heterogeneous clinical course, with some patients living for decades without requiring treatment whereas others progress rapidly despite therapy. In up to 10% of the cases, CLL transforms into a high-grade non-Hodgkin lymphoma [2].

Over the past decade there have been major advances in understanding the pathogenesis of the disease and in the treatment. Molecular patterns have been identified that define patient subgroups with different prognosis or predictive of response to therapy and new treatment strategies have been designed which improved both response rates and duration of responses. Nevertheless, the disease is still considered incurable even though improvement in overall survival has been recently demonstrated following new active treatment regimens [3]. There is a continuous need to develop new treatment strategies, in particular in certain disease settings, such as asymptomatic high-risk disease, or as maintenance therapy, or for patients not eligible for up-front aggressive chemo-immunotherapy [4].

1.2 EPIDEMIOLOGY

CLL is the commonest leukemia in the Western adults, accounting for 25-30% of all leukemias and for 10% of all hematological neoplasms [5]. Based on registry data, the age-adjusted incidence rate in the United States was found to be 3.9/100000 per year. The incidence rates in men are nearly twice as high as in women. Compared to the Whites, the disease is rarer among Blacks and much rarer in Asian/Pacific Islanders (75% and 23% that of Whites, respectively) [6]. There is substantial geographical variation in CLL incidence with higher rates reported in Northern America and Europe [5]. In Sweden the incidence is 4.7/100000 persons per year [7].

The increased incidence of CLL reported from the 1950s onwards is primarily due to increased detection of early stage disease. Indeed, from the 1950s to the 1990s, the incidence of disease doubled from 2.6 to 5.4 per 100000 persons/year [8] and the median survival after diagnosis increased from 3 years to 7 years [9], while the mortality rate remained relatively stable [10]. Where access to automated hematology analyzer technology is widespread and relatively inexpensive, the incidence of CLL has indeed stabilized.

CLL predominantly affects elderly people, with a median age at diagnosis of 72 years [6], which may have important clinical consequences for tolerability of more intensive treatment regimens [11]. Almost 70% of newly diagnosed CLL patients are older than 65; less than 2%, younger than 45; 9.1%, between 45 and 54; 19.3%, between 55 and 64;

26.5%, between 65 and 74; 30.0%, between 75 and 84; and 13.2%, have more than 85 years of age [4].

1.3 ETIOLOGY

The etiology of CLL is still unknown. The possible causal relationship with environmental and occupational exposures, such as pesticides [12], magnetic fields [13], farming and animal breeding [14] and viruses [15, 16], has been investigated, but the associations found with these exposures were rather weak.

On the other hand, there is strong and consistent evidence that a genetic component contributes to the etiology. A family history of CLL or other lymphoid malignancies is indeed one of the strongest risk factors for the development of the disease. First-degree relatives of CLL patients are at significantly higher risk for developing the disease (relative risk = 7.5) [17]. Several familial clusters of CLL have been reported and the phenomenon of genetic anticipation, i.e. the earlier onset of the disease in successive generations, has been described [18]. In a report from the National Cancer Institute Familial Registry, the mean age at diagnosis among familial cases was approximately 10 years younger than that of sporadic cases [19]. Apart from the difference in age at presentation, familial CLL is essentially indistinguishable from sporadic CLL, having the same overall survival and same risk of transformation in high-risk lymphoma. This observation favors a genetic basis to disease development in general rather than a simple environmental etiology. More recently, six susceptibility sites were identified by a genome-wide association study, providing the first evidence for the existence of common, low-penetrance susceptibility to CLL [20]. Further insights into the biology of CLL development will be hopefully provided by future studies identifying additional variants associated with CLL predisposition. Finally, recent research indicates that CLL may be partly an antigen (Ag)-driven or autoimmune disease, as discussed in the following chapter.

1.4 PATHOGENESIS

1.4.1 BCR response and IgHV mutation

Both in normal and malignant cells, the B cell response to Ag stimulation is mediated through the B cell receptor (BCR). Each B cell displays a distinct BCR that is formed through variable combinations of V, D and J segments for the Immunoglobulin (Ig) heavy chain and V and J gene segments for the light chain. In addition to the combinatorial diversity of the different segments, the BCR repertoire is expanded by the introduction of somatic mutations through the somatic hypermutation (SHM) process during the germinal centre (GC) reaction (reviewed in [21]). BCR surface expression is usually weak in CLL [22].

It has been demonstrated that CLL patients can be divided in two subgroups characterized by the presence or absence of somatic mutations in the variable regions of the Ig heavy chain genes of the CLL clone [23-25]. The percentage of homology of the Ig heavy chain V (IgHV) genes in CLL with the germline which in most studies is taken as cut-off value is 98%.

The IgHV mutation status is currently a well recognized prognostic factor in CLL, with unmutated IgHV genes consistently associated with poorer clinical outcome [25-27].

The two CLL subgroups, with or without mutated IgHV genes, display also a number of other important biological differences. Compared to the CLL cases with mutated IgHV genes, the cases with unmutated IgHV genes have higher expression of the protein tyrosine kinase zeta-associated protein 70 (ZAP70) and CD38, have increased activation of key signal transduction pathways, have shorter telomeres and are genetically more unstable [23, 26-32]. Moreover, CLL cases with mutated IgHV genes have rather weak BCR signaling and are rather anergic [33-36].

The BCR structure, too, is different between the two CLL subgroups. Analysis of the IgHV gene usage has shown that some Ig gene segments, such as *IgHV1-69*, *IgHV4-34*, *IgHV3-7* and *IgHV3-21* are overrepresented in CLL [24, 37]. However, SHM does not occur uniformly among IgHV genes: *IgHV1-69*, for example, constantly carries very few mutations as opposed to *IgHV3-7*, *IgHV3-23* and *IgHV4-34* genes, which are usually mutated.

More frequently than the IgHV-mutated cases, the IgHV unmutated cases carry stereotyped rearrangements of the V, D and J segments that have very similar complementarity-determining region (CDR) 3 regions and display stereotyped light chains and biased somatic mutation patterns [38-40].

Overall, more than 20% of CLL cases carry stereotyped B cell receptors and in 1% of the cases the Igs are nearly identical, suggesting that common Ags are recognized in many patients with CLL [39-43]. In CLL cases with unmutated IgHV the BCR is usually polyreactive to Ags derived from endogenous or exogenous proteins or lipids generated, among others, by oxidative stress [44-47]. On the other hand, CLL cases with mutated IgHV genes usually display oligo- or monoreactive (non-autoreactive) BCRs. However, it has been shown that once BCR sequences carrying IgHV mutations are reverted to their unmutated counterpart, they also become auto- and polyreactive. This would suggest that IGHV-mutated and unmutated CLL derive from a common autoreactive precursor [45].

The observation that a stereotyped BCR, such as *IgHV3-21*, is associated with worse prognosis even if mutated further indicates a possible role of an unknown common Ag, but the role of Ag drive in CLL pathogenesis has yet to be clarified. Intensive research is ongoing to define those Ags, which may be autoAg or exogenous [48-50] and a picture of CLL as an Ag-driven or partly autoimmune disease is emerging. Ags currently discussed include cytoskeletal proteins vimentin, filamin B and cofilin-1, together with phosphorylcholine-containing Ags (eg. Streptococcus pneumoniae polysaccharides). Additional new Ags identified are cardiolipin and proline-rich acidic protein-1. Importantly, these Ags represent molecular motifs exposed on apoptotic cells and bacteria [46].

Whether CLL with and CLL without mutations in the IgHV genes have a common cellular origin or not is still matter of debate (Figure 1). The observation that the cells have undergone the SHM process, which typically occurs in GC B cells, and other observations indicate that IgHV-mutated CLLs are derived from post-GC memory B cells. However, the possibility that they might derive from B cells that accumulate

mutations in a T cell-independent (TI) immune response that does not involve the GC or during a primary, Ag-independent BCR diversification process is also considered. IgHV-unmutated CLL cases most likely derive from Ag-experienced B cells that acquire characteristics of memory B cells. It is still unclear, though, whether these are activated conventional naive B cells, $CD5^+$ B cells or marginal zone (MZ)-like B cells. Whether this activation takes place as part of a TI or T cell-dependent (TD) immune response is also yet to be understood. A TD immune response could induce an abortive GC reaction of autoreactive B cells [51].

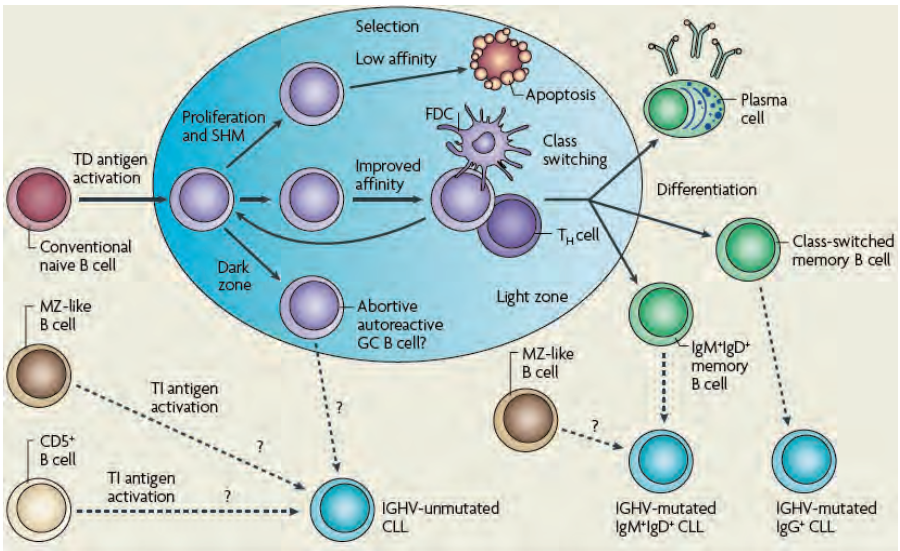

Figure 1. Germinal center reaction and possible cellular origins of IgHV mutated and unmutated CLL. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS CANCER] (Zenz et al.) [51], copyright (2010).

1.4.2 Genomic aberrations and gene mutations

Genomic aberrations are found in 80% of CLL cases. The chromosomal regions involved are few and affected both in IgHV mutated and unmutated CLL. High-risk aberrations, though, are found more frequently in unmutated CLL.

Deletion of 13q14 is the most frequent one and, when it is found as sole aberration, it is associated with better prognosis [52]. There is strong experimental evidence that two microRNA genes, *mir-15a* and *mir-16-1*, located in the crucial 13q14 region, might be implicated in the pathogenesis of CLL [53-55].

Deletions of 11q22–q23 are found in 10% of early-disease patients and in 25% of patients with refractory CLL. The deletions involve a minimal consensus region in chromosome bands 11q22.3–q23.1, a region harboring the ataxia telangiectasia-mutated (ATM) gene. ATM is a protein kinase involved in cellular response to DNA double-strand breaks. Trisomy 12 is present in 10-20% of CLL cases, with stable incidence in different disease phases. No genes possibly implicated in CLL pathogenesis have been yet identified in chromosome 12 [51]. Finally, deletion of 17p13, where the tumor

suppressor gene TP53 is located, was found in 4–9% of newly diagnosed patients or at the time of initiation of the first treatment [52, 56-58]. In 80% of the cases with monoallelic 17p13 deletion, the remaining *TP53* allele is mutated [59]. Notably, genetic complexity increases with the evolution of the disease, with TP53 mutations in particular becoming a more frequent finding and reaching approximately 40% frequency in patients with advanced phase CLL.

1.4.3 Epigenetic alterations

DNA methylation leads to transcriptional gene silencing and aberrant DNA methylation has been shown to play an important role in tumorigenesis. Global hypomethylation and regional hypermethylation of tumor suppressor gene promoters are characteristic features of cancer cells [60].

Genome-wide hypomethylation has been shown in CLL patients compared to healthy controls. Aberrant methylation was found in 2.5-8.1% of the GpG islands [61] and a strong correlation was found between promoter methylation and transcriptional silencing of certain individual gene promoters, such as DAPK1,TWIST2, ZAP70, and HoxA4 [62-64].

In a recent study, global methylation profiles were studied in 23 CLL patients by applying high-resolution methylation microarrays and significant differences in methylation profiles were found between IgHV mutated and IgHV unmutated cases. In the IgHV unmutated group, 7 known or candidate tumor suppressor genes (eg, VHL, ABI3, and IGSF4) were found to be methylated, while 8 genes involved in cell proliferation and tumor progression (eg, ADORA3 and PRF1) were unmethylated. In contrast, these latter genes were silenced by methylation in IGHV mutated patients [65]. Genes such as ADORA3 and PRF1 enhance the NF-kB and mitogen-activated protein kinase (MAPK) pathways, respectively, which are known to be dysregulated in CLL and lead to activation of anti-apoptotic pathways. Epigenetic modifications are an attractive therapeutic target because they are reversible by demethylating agents such as histone deacetylase (HDAC) inhibitors, but results obtained in CLL up to now are less encouraging compared to other leukemias [66].

1.4.4 Microenvironment

The proliferation compartment of CLL is essentially represented by prolymphocytes and paraimmunoblasts that cluster to form the pseudofollicular proliferation centers (PC). These are focal aggregates of variable size found in lymph nodes and to a lesser extent in the bone marrow (BM) [67, 68]. Here the growth of leukemic cells may be favored by T-cell help and by the interaction with stromal cells. Several findings suggest the possibility that T cells provide a short-term support to CLL cells, while stromal cells and accessory cells would provide a long-term support prolonging tumor cell survival and favoring the accumulation of leukemic cells [69-71].

An increased number of $CD3^+$ cells, mostly $CD4^+$CD40ligand $(CD40L)^+$ have been reported to cluster in and around the pseudofollicles [72]. CD40/CD40L interaction synergizes with BCR signaling [70] and in turn induces several anti-apoptotic signaling pathways, including the caspase inhibitor survivin [72] and NF-kB [73].

The progressive accumulation of the neoplastic cells is also supported by a chemokine production by the leukemic clone induced by $CD4^+$ T cells [74, 75]. Conversely, CLL cells induce phenotypic changes in T cells, which culminate in the formation of an impaired immune synapse [76].

Moreover, it has been shown that CD38 often expressed on the surface of CLL cells interacts with CD31 expressed on some large, round, fibroblast-like adherent cells named "nurse-like cells" (NLCs) [77]. NLCs may differentiate from blood monocytes *in vitro*, but it is likely that *in vivo* they represent a distinctive hematopoietic cell type [78]. NLCs also express stromal cell-derived factor-1 (SDF-1) and the TNF family ligands B-cell activation factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) that protect CLL cells from spontaneous apoptosis [69, 79, 80]. Co-culture of CLL cells with NLCs induced high-level expression of two T-cell chemokines (CCL3, CCL4) by CLL cells through BCR stimulation [74].

1.5 CLINICAL MANIFESTATIONS

The disease is often diagnosed in asymptomatic patients at a complete blood count (CBC) when lymphocytosis $>5.0x10^9$/L (5000/μL) is detected. Clinical manifestations occur in patients with more advanced disease. The accumulation of the leukemic cells in lymphoid organs leads to painless lymphadenopathies, often symmetrical, to splenomegaly (66%) and hepatomegaly. Symptoms of BM failure due to progressive BM infiltration are anemia, neutropenia and thrombocytopenia. Acquired hypogammaglobulinemia facilitates recurrent infections, especially pneumonias. Patients with very advanced disease may experience weight loss, night sweats and general malaise. Finally, autommune phenomena may arise. Autoimmune thrombocytopenia occurs in 1-2% of the cases and autoimmune hemolytic anemia (AIHA) in 50% of CLL patients with positive direct antiglobulin test (10-20% of all patients) [1].

1.6 DIAGNOSIS AND CLINICAL STAGING

The World Health Organization classification of hematopoietic neoplasias describes CLL as leukemic, lymphocytic lymphoma, being only distinguishable from small lymphocytic lymphoma (SLL) by its leukemic appearance [81]. CLL is always a B cell neoplasm and the entity formerly known as T-CLL has been now reclassified T-cell prolymphocytic leukemia (T-PLL) [82].

The diagnosis of CLL is made by evaluating the blood count, the blood smear and the immune phenotype of the circulating lymphoid cells. It requires the presence of at least $5.0x10^9$/L (5000/μL) in the peripheral blood (PB). The finding of fewer than this number of B cells in the absence of lymphadenopathy or disease-related symptoms is now defined as "monoclonal B-lymphocytosis" (MBL) [83]. In a prospective cohort study prediagnostic B-cell clones were found in 98% of the patients in PB obtained up to 77 months before CLL diagnosis [84]. MBL may progress to frank CLL at a rate of 1-2% per year [85]. Conversely, when the number of B lymphocytes in the PB is $<5.0x10^9$/L in the presence of lymphadenopathy and/or splenomegaly the diagnosis is SLL.

The leukemic cells found in the blood smear are characteristically small, mature lymphocytes which can be found admixed with larger atypical cells or prolymphocytes.

The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry. CLL cells coexpress CD5, CD19, CD20 and CD23. The levels of surface Ig, CD20 and CD79b are characteristically low compared with those of normal B cells [86, 87]. Each clone of leukemia cells is restricted to expression of either κ or λ Ig light chains [87]. The diagnostic criteria for CLL are summarized in Table 1.

A BM aspirate and biopsy generally are not required for the diagnosis of CLL, but recommended when treatment is initiated [88] or in newly diagnosed patients presenting with cytopenias to evaluate whether these are autoimmune or due to BM replacement [4]. The BM infiltrate may be nodular, interstitial, or diffuse or may show a combination of these patterns.

Table 1. Diagnostic criteria for CLL.

Clonal expansion of abnormal B lymphocytes in PB
>5.0x10^9/L (5000/μL)
Lymphoid cells ≤55% atypical/immature
Low density of surface Ig (IgM or IgD) with κ or λ light chains
B-cell surface antigens (CD19, CD20^{dim}, CD23)
CD5 surface antigen

Two staging systems are commonly used, the Rai system [89] and the Binet system [90]. After the number of prognostic groups in the Rai system has been modified from the five original to three [91], both systems define three patient subgroups with distinct clinical outcomes. Both simply rely on physical examination and standard laboratory tests.

Table 2. Clinical staging systems for CLL.

System	Clinical features	% of newly diagnosed patients	Median OS (y)
Rai stage (simplified 3-stage)			
0 (low risk)	lymphocytosis in blood and BM only	25	>10
I and II (intermediate risk)	lymphadenopathy, splenomegaly +/- hepatomegaly	50	7
III and IV (high risk)	anemia, thrombocytopenia [b]	25	0.75-4
Binet stage			
A	< 3 areas of lymphadenopathy[a]; no anemia or thrombocytopenia	60	12
B	>3 areas of lymphadenopathy[a]; no anemia or thrombocytopenia	30	7
C	hemoglobin <10 g/dL, platelets <100x10^3/dL[b]	10	2-4

[a] Lymphoid areas considered are: unilateral or bilateral cervical, axillary and inguinal lymph nodes, spleen and liver.
[b] with exclusion of haemolysis and unrelated causes of anemia or thrombocytopenia.

1.7 PROGNOSTIC FACTORS

Together with some clinical features predictive of poor prognosis, such as advanced stage at diagnosis, advanced age, diffuse pattern of BM infiltration and short lymphocyte doubling time, a number of molecular biomarkers allow nowadays to predict time to

progression, time to need for therapy, and overall survival in cohorts of patients. A molecular profile can be built from the assessment of these biomarkers in individual patients, but it should be underlined that at present there is no indication to treat patients based on these markers if standard criteria for treatment [88] are not yet met. Indeed, there is no evidence that patients presenting with high-risk features anyhow benefit from earlier treatment.

The most important biomarkers are cytogenetic analysis by FISH, IgHV mutational status, IgHV usage, ZAP-70, lipoprotein lipase and CD38 expression.

High-risk features predictive of disease progression include deletion of the long arm of chromosome 11 (del 11q) and, in particular, del 17p identified at fluorescence in situ hybridization (FISH) analysis, IgHV unmutated status, *IgHV3-21* usage, high levels of CD38 and ZAP-70 expression. Conversely, del 13q as a sole abnormality is associated with better prognosis. However, since cytogenetic abnormalities evolve over time, it is recommended by the Swedish CLL guidelines (www.swecll.org/Nationella-riktlinjer) that FISH analysis is performed before initiation of each line of therapy. Finally, when coming to treatment decisions it should be considered that patients with del 17p or p53 mutation, the proportion of which increases over time, should be treated with agents acting independently of p53.

1.8 TREATMENT

1.8.1 Treatment indications

Newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy. Indeed, it has been shown by several studies that the use of alkylating agents in patients with early-stage disease does not prolong survival [92-95].
Patients at intermediate and high risk according to the modified Rai classification or at Binet stage C usually benefit from the initiation of treatment, even though some of them may be monitored without treatment till development of progressive disease.

At least one of the criteria listed in Table 3 should be met for documentation of active disease [88].

Table 3. Criteria for definition of active disease.

Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia
Massive (i.e., >6 cm below the left costal margin) or progressive or symptomatic splenomegaly
Massive nodes (i.e., >10 cm in longest diameter) or progressive or symptomatic lymphadenopathy
Progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) <6 months
Autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapy
Constitutional symptoms, defined as any one or more of the following: • unintentional weight loss ≥10% in the previous 6 months; • significant fatigue (i.e., Eastern Cooperative Oncology Group (ECOG) PS ≥2; inability to work or perform usual activities); • fevers higher 38.0°C for ≥2 weeks without other evidence of infection; • night sweats for ≥1 month without evidence of infection

Lymphocyte doubling time (LDT) can be obtained by linear regression extrapolation of absolute lymphocyte counts obtained at intervals of 2 weeks over an observation period of 2-3 months. It should be considered that in patients with initial blood lymphocyte counts < $30x10^9$/L (30000/µL) LDT should not be used as a single parameter to determine treatment indication. In general, the absolute lymphocyte count should not be used as the sole indicator for treatment.

1.8.2 Cytostatic agents

For several decades chlorambucil as monotherapy was used as front-line therapy for CLL. The drug achieves 60–70% partial response (PR) in previously untreated patients, but no significant complete response (CR) and its use has now been largely replaced by combination chemotherapy and chemoimmunotherapy. Even today, though, chlorambucil can still be appropriate for elderly or less fit patients, given its advantages such as low toxicity, low cost and convenience and with results comparable to those of fludarabine alone [96]. However, chlorambucil as monotherapy is possibly associated with a shorter survival compared to fludarabine, as evidenced by a long-term analysis [97]. Response rates gained by combination chemotherapy such as cyclophosphamide, adriamycine, prednisone (CAP) or cyclophosphamide, adriamycine, vincrinstine, prednisone (CHOP) may be slightly higher than with chlorambucil, but survival is not improved [98].

Purine analogues act by inhibiting the DNA polymerase and the ribonucleotide reductase, finally promoting apoptosis [99]. Three purine analogues are currently used in CLL: fludarabine, pentostatin, and cladribine. Among these, fludarabine is the one most extensively studied. Its superiority as a single agent to older chemotherapy regimens (chlorambucil or CAP or CHOP) in terms of achievement of complete remissions and prolongation of progression-free survival (PFS) was assessed by three large randomized studies [100-102] which, though, evidenced no statistically significant advantage in overall survival except when compared with chlorambucil [97].

Cladribine was found to achieve similar responses as fludarabine in both previously treated and untreated patients, but no advantage was seen in overall survival compared to chlorambucil/prednisone [103].

Fludarabine has been evaluated in a variety of combination regimens, in particular with cyclophosphamide. *In vitro* data had in fact showed a synergic cytotoxic effect of this combination on CLL cells [104, 105]. Three randomized trials have shown that the fludarabine/cyclophosphamide (FC) combination clearly improves the CR and overall response (OR) rate and PFS as compared with fludarabine monotherapy [56, 106, 107]. Importantly, the trials showed that the FC did not increase the rate of severe infections despite inducing more grade 3-4 neutropenias.

The combination of cladribine with cyclophosphamide has been investigated in a randomized trial with a control arm represented by the combination of cyclophosphamide and mitroxantrone and did not show any superiority in terms of OR, PFS and OS [108]. Based on these results, cladribine combination therapies do not seem to offer a major advantage as first-line treatment for CLL.

In 2008, bendamustine, a hybrid between an alkylator and a purine analog, was approved by the US Food and Drug Administration (FDA) for the treatment of CLL, after its superiority to chlorambucil in terms of OR and PFS was shown in a randomized trial [109]. Whether cross-resistance exists between FC and bendamustine is an important clinical question still unanswered since only very limited clinical data are available [110].

1.8.3 Monoclonal antibodies as single agents

Alemtuzumab is a CDR-grafted IgG monoclonal Ab (mAb) against the CD52 antigen expressed on normal and leukemic B and T lymphocytes, macrophages and monocytes. It is assumed to exert its anti-tumor activity through antibody-dependent cell-mediated cytotoxicity (ADCC), complement activation and possibly also apoptosis induction [111]. An overall response rate of about 40% has been demonstrated in refractory CLL [112, 113] with particularly prominent effects in patients with del 17p [114]. After efficacy was also shown as first-line therapy in an exploratory phase II study [115], a phase III trial further demonstrated that it induced significantly higher OR (83% vs 55%), CR (24% vs 2%) and longer PFS compared to chlorambucil [116]. Following these observations, the drug has been approved in Europe also as front-line therapy for CLL and is routinely used in first-line in patients with del 17p. The most common complications associated with alemtuzumab therapy are immunosuppression and risk of infections. Infections by Pneumocystis jiroveci and Herpes zoster are substantially reduced by prophylactic treatment with antibiotics and antivirals. Moreover, cytomegalovirus (CMV) reactivation occurs in approximately 20% of patients, typically after 3–8 weeks of alemtuzumab therapy and it is recommended that patients are closely monitored for CMV reactivation during alemtuzumab therapy.

Rituximab is a chimeric anti-CD20 mAb, very active as single agent for the treatment of follicular lymphoma. Its cytotoxic activity occurs through various mechanisms, encompassing complement-mediated lysis, ADCC and direct induction of apoptosis [117]. Used as single agent, though, it has proved to be in CLL much less effective than in follicular lymphoma [118, 119].

Ofatumumab is a human mAb that targets an epitope on CD20 different from the one targeted by rituximab and has shown to induce more complement-mediated CLL cell lysis *in vitro* [120, 121]. In a recent study, an overall response rate of 50% was obtained in refractory CLL [122], which led to approval of ofatumumab by the FDA and by the European Medical Agency (EMEA).

1.8.4 Combination chemo-immunotherapy

On the basis of preclinical studies showing evidence for a synergy between rituximab and fludarabine [123], the association of rituximab with fludarabine-regimens was investigated in a randomized phase II trial [124]. A significantly higher CR rate for the concurrent administration of fludarabine and rituximab (FR) (47% CR) versus the sequential treatment (F followed by R) (28% CR) was seen. Moreover, an indication of advantage in terms of PFS and OS given by the FR combination compared to fludarabine alone was shown by a retrospective comparison of the CALGB 9712 and 9011 trials [125].

The combination of FC with rituximab (FCR) was investigated in a phase II trial on 300 patients with previously untreated CLL. FCR resulted in an OR rate of 95%, with CR in 72% [126]. An advantage of FCR vs FC in terms of PFS (2-yrs PFS 51.8% vs 32.8%, respectively) as well as OS (84.1% in the FCR arm vs 79.0 % in the FC arm at 38 months follow-up, p=0.01) was finally showed by a German randomized trial [127], which led to approval of rituximab in combination with chemotherapy for CLL in both the United States and Europe.

The results of the above mentioned randomized trials showing major advances in CLL treatment are shown in Table 4.

Table 4. Advances in the first-line treatment of CLL.

Reference	Regimen	N	CR, %	OR, %	PFS, mo
Rai (2000) [102]*	Chlorambucil	193	4	37	14
	Fludarabine	179	20	63	20
Catovsky (2007) [56]	Chlorambucil	387	7	72	20
	Fludarabine	194	15	80	23
	Fludarabine/cyclophospahmide	196	38	94	43
Flinn (2007) [107]	Fludarabine	137	5	59	19
	Fludarabine/cyclophospahmide	141	23	74	32
Eichhorst (2006) [106]	Fludarabine	182	7	83	20
	Fludarabine/cyclophospahmide	180	24	95	48
Hallek (2009) [3]**	Fludarabine/cyclophosphamide	408	23	85	33
	Fludarabine/cyclophosphamide/rituximab	409	45	95	40

*Longer OS with fludarabine in long-term follow-up [97];

**Longer OS with FCR [3]

In order to reduce the myelotoxicity of the FCR regimen (34% of patients experiencing grade 3-4 neutropenia), fludarabine was substituted with pentostatin in a phase III randomized trial, but no difference was seen in either clinical outcome or toxicity rate [128].

Finally the combination of bendamustine with rituximab has been explored in a phase II first-line trial giving a 91% OR rate with 33% CR, apparently with the induction of fewer neutropenias than FCR [129]. The two treatments are therefore now directly compared in an ongoing phase III study of the German CLL Study Group (GCLLSC).

The combination of alemtuzumab and fludarabine (FA) was investigated in a phase II trial enrolling patients with relapsed CLL and proved to be feasible, safe and very effective, with an OR rate of 83% including 30% CR [130]. To validate these observations, a Phase III randomized study was conducted to compare the efficacy and safety of FA vs fludarabine alone as second-line therapy for patients with relapsed or refractory CLL. The FA combination resulted in significantly higher OR (85% vs 68%) and CR rates (30% vs 16%) without increased toxicity [131]. The combination of FC with alemtuzumab (FCA) was being compared with FCR in a randomized phase III trial [132] which had to close prematurely due to the higher toxicity observed in the FCA arm. Another trial, conducted by the HOVON group, comparing FC with FCA and using a lower alemtuzumab dose is still recruiting.

Finally, the association of FCR and alemtuzumab (CFAR) was investigated both as frontline therapy [133] and in previously treated patients [134], showing good activity but at the expense of greater myelosuppression and leaving the open question whether all effective drugs should be used upfront or some spared for subsequent treatments.

In conclusion, the selection of the best treatment option should be based on (Figure 2):

a) the physical condition (fitness and comorbidity) of the patient;
b) genetic and other prognostic factors of the disease;
c) disease stage.

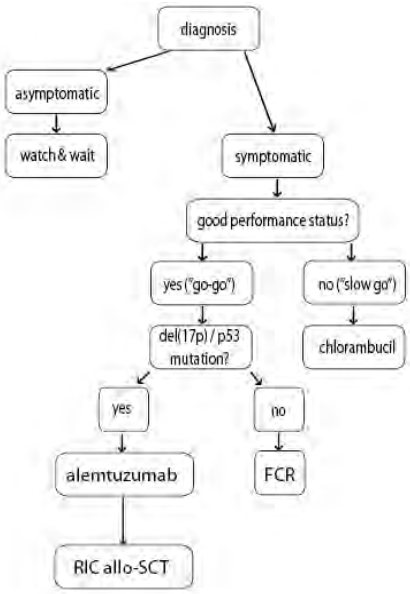

Fig. 2. Front-line treatment of CLL patients outside clinical trials (adapted from Gribben) [4].

The indications for second-line treatment are the same as for front-line therapy, which is active, symptomatic disease. Factors predictive of response to subsequent lines of treatment are, together with the above mentioned prognostic factors, the number of prior therapy lines and response to previous therapy. In general, if the duration of the first remission is > 12 months, the first-line treatment may be repeated.

1.8.5 Novel agents

A large number of mAbs is currently under investigation. Among these, mAbs targeting CD19, such as the BiTE Ab blinatumomab, or CD20 (e.g. GA101, veltuzumab, PRO131921) or CD80 (galiximab) or CD74 (milatuzumab) or CD40 (dacetuzumab). Lumiliximab is a primatized anti-CD23 antibody which showed its activity (65% OR of which 52% CR) in combination with FCR in a phase I/II trial [135]; the following multicenter, randomized study, nevertheless, failed to show any advantage of the combination vs FCR and was prematurely closed (http://clinicaltrials.gov).

Similarly, many small molecule inhibitors are under investigation, including Bcl2 antagonists, such as oblimersen and ABT-263, cyclin-dependent kinase (CDK) inhibitors, such as flavopiridol and many others. Oblimersen was added to the FC regimen (FCO) and compared to FC in a randomized study on 241 pretreated patients. The overall RR was 45% (of which 9% CR) in the FCO arm vs 41% (3% CR) in the FC arm. The addition of oblimersen significantly improved PFS in those patients achieving at least a PR [136].

1.8.6 Stem cell transplantation

No randomized trials are available that compare standard chemotherapy with either autologous or allogeneic hematopoietic stem cell transplantation (SCT), but several trials have evaluated this approach in selected patients. Due to the advanced age and extremely indolent clinical course of the disease in the majority of CLL patients, SCT cannot be considered a treatment option in most cases. Some indications for SCT in CLL are provided by the European Bone Marrow Transplant guidelines, which recommend allogeneic SCT for younger patients who do not respond or relapse early (<12 months) after first-line combination chemotherapy or in patients with p53 abnormalities requiring treatment [137]. Autologous SCT is no longer recommended in CLL.

The approach of choice is usually reduced-intensity conditioning (RIC) allogeneic SCT [138], which is suitable also for more elderly patients and attempts to exploit the graft-versus-leukemia (GVL) effect that exists in CLL.

2 TUMOR IMMUNOLOGY

2.1 TUMOR IMMUNOSURVEILLANCE AND CANCER IMMUNOEDITING

The concept of tumor *immunosurveillance* is based on the hypothesis that the immune system is able to recognize primary developing tumors and eliminate them before they become clinically evident. This hypothesis, first postulated by Paul Erlich in 1909 [139], was re-formulated in the late 1950s by Burnet and Thomas [140, 141] and has been the subject of intense debate in the past decades. To date, a huge amount of data from animal models, together with a number of observations from human patients, indicate that a functional cancer immunosurveillance process indeed exists. Following the development of gene targeting and transgenic mouse technologies and the ability to produce highly specific blocking monoclonal Abs to particular immune components, the immunosurveillance hypothesis could be tested in murine models of immunodeficiency. An increased susceptibility to develop spontaneous or chemically induced tumors was shown, for example, in mice lacking essential components of the innate or adaptive immune system, such as: the RAG-2 gene, which results in absence of T cells, B cells and natural killer T (NKT) cells; interferon-γ (IFN-γ) receptor; perforin; tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (reviewed in [142]).

In humans, the immunosurveillance hypothesis is supported by at least three observations. First, the incidence of cancers with no apparent viral origin in immunosuppressed transplant recipients is increased compared to age-matched controls [143-145]. Second, a large amount of data indicates that human cancer patients indeed develop immune responses to tumor-associated Ags (TAA) [146-148]. Third, tumor infiltration by T lymphocytes and NK cells was shown to correlate with a favorable clinical prognosis [149-151].

Nevertheless, the occurrence of cancer in spite of tumor immunosurveillance indicates that cancer cells somehow manage to escape the immune system.

Recently, Schreiber and colleagues [142, 152] introduced the concept of *immunoediting*, a dynamic process by which cancer cells can escape immune recognition by selection of tumor-cell variants with reduced immunogenicity. The term “immunoediting” was meant to emphasize the dual role of immunity which, on the one hand, protects the host against tumor development, and, on the other, imprints the tumor to facilitate its growth. The proposed model comprises three different phases. In the first phase (*elimination*), when immunosurveillance is still effective, the immune system succeeds in eliminating the cancer cells. In the second phase (*equilibrium*), a selective pressure by the immune system sorts out a new population of tumor clones with reduced immunogenicity that are more likely to survive in an immunocompetent host. Finally, in the third phase (*escape*) the selected tumor cell variants proliferate and the tumor becomes clinically evident.

Cancer would therefore develop as a consequence of a two-hit process, the first hit being the cell-intrinsic oncogenic event, and the second being an impaired immune recognition or effector function against the tumor cells.

The ability of malignant cells to evade the extrinsic tumor suppressor functions of the immune system would be therefore as crucial for tumor development as the other six characteristics (capacity to grow autonomously, insensitivity to negative growth regulation, evasion of intrinsic apoptotic signals, unlimited replicative potential, angiogenesis induction and ability to metastasize) proposed as hallmarks of cancer by Hanahan and Weinberg [153].

Understanding of immunoediting mechanisms has important implications for cancer immunotherapy in humans. Indeed, the cellular and molecular processes active in all the three phases can be exploited to strengthen the immune response against tumors, by directly potentiating immune effector functions or by increasing the immunogenicity of tumors or by counteracting immune escape mechanisms.

2.2 TUMOR-INDUCED IMMUNE RESPONSES

A large amount of data indicates that cancer patients develop immune responses to their tumors. Immunologic recognition of a developing tumor likely requires an integrated response involving both the innate and adaptive arms of the immune system [154]. In the model proposed by Schreiber and colleagues [155], initiation of the antitumor immune response occurs when the cells of the innate immune system become alerted to the presence of a growing tumor, at least in part owing to the local tissue disruption concomitant to tumor development. The process of stromal remodeling would in fact induce the release of proinflammatory molecules that, together with chemokines produced by the tumor itself [156], attract the cells of the innate immune system [157, 158]. At the tumor site, NKT cells, γδ T cells, NK cells and macrophages may recognize molecules on tumor cells and induce the production of IFN-γ, a central event for progression of antitumor response. IFN-γ amplifies the effects of innate immune recognition and recruits more cells of the innate immune system to the tumor site and stimulates antiproliferative [159], proapoptotic [160] and angiostatic [161-163] effects that could result in partial elimination of the tumor. Furthermore, macrophages release reactive oxygen and reactive nitrogen intermediates [164, 165], which are toxic for tumor cells, while NK cells activated either by IFN-γ or through their activating receptors can kill tumor cells via TRAIL-[166] or perforin-dependent [167] mechanisms, respectively. As a result, a number of tumor Ags become available and the adaptive immune system is therefore recruited. Immature DCs recruited to the tumor site become then activated in response to cytokines present in the tumor microenvironment or by interaction with NK cells [168]. Once activated, DC can acquire tumor Ags either by ingestion of tumor cell debris or by transfer of heat shock proteins/tumor Ag complexes [169, 170]. Ag-bearing DCs then migrate into the draining lymph nodes [171], where they activate naïve Th1 $CD4^+$ T cells, which in turn facilitate the development of tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) induced by cross-presentation of tumor Ags in the context of human leukocyte antigen (HLA)-I molecules on DCs [172-175]. Following this activation, tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site and eliminate tumor cells by diverse mechanisms. Activated $CD4^+$ cells produce interleukin (IL-2) which, together with IL-15 present in the microenvironment, maintains the function and viability of $CD8^+$ T cells. Moreover, activated $CD4^+$ cells recognize tumor-associated macrophages

(TAMs) in an MHC class-II-dependent manner and convert M1 macrophages, which produce IL-10, into IFN-γ-producing M2 macrophages.

On the other hand, $CD8^+$ T cells can kill tumor cells through direct and indirect mechanisms. The granule exocytosis pathway utilizes perforin to traffic the granzymes to appropriate locations in tumor cells, where they cleave critical substrates that initiate DNA fragmentation and cell apoptosis [176]. A number of cell-surface receptors, such as TNR receptor 1, CD95 (also known as FAS), TRAIL receptor 1 and 2 can mediate cell death following ligand-induced trimerization. Moreover, $CD8^+$ cells will also produce IFN-γ, which will cause tumor cell killing by the above described mechanisms: cell cycle inhibition, apoptosis, angiostasis, and induction of macrophage tumoricidal activity.

Finally, both activated NK and NKT cells secrete IFN-γ and can kill tumor cells by TRAIL- or perforin-dependent pathway, as described above. An overview of the described immune surveillance mechanisms is depicted in Figure 3.

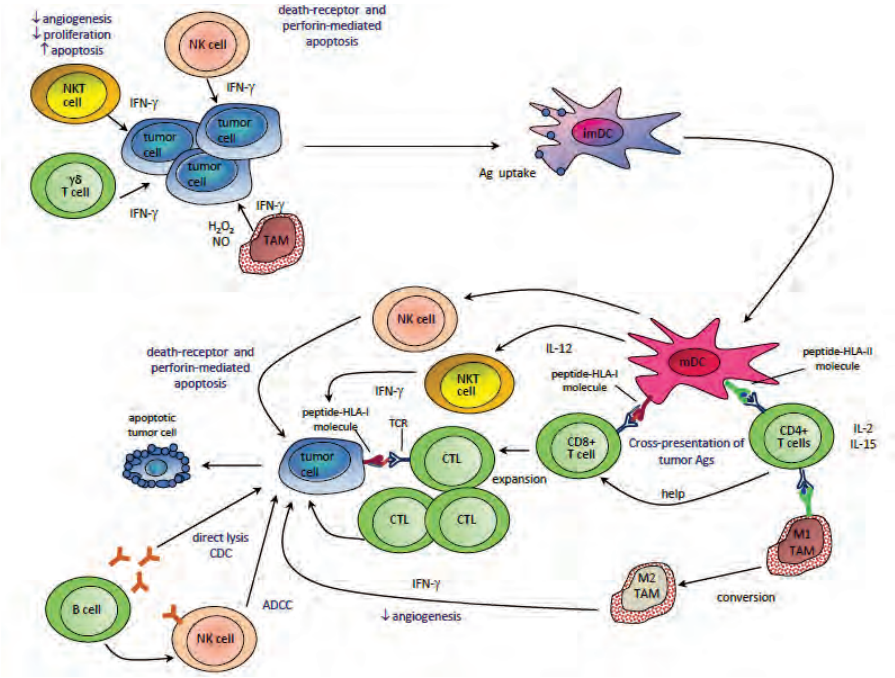

Fig. 3. Cancer immunosurveillance mechanisms.
imDC: immature DC; mDC: mature DC; TAM: tumor-associated macrophage; CTL: cytotoxic T lymphocyte; NK cell: natural killer cell; NKT cell: natural killer T cell; CDC: complement-dependent cytotoxicity; ADCC: antibody-dependent cytotoxicity; NO: nitric oxide; H_2O_2: hydrogen peroxide.

2.2.1 Tumor antigens

A large array of immunogenic Ags associated with a wide variety of cancers has now been identified [177, 178], which can be potentially targeted to elicit a therapeutic immune response. These molecules are called tumor-associated Ags (TAAs) and can be either proteins usually expressed at certain stages of differentiation, e.g. melanocyte differentiation antigens, Melan-A/MART-1, tyrosinase, gp-100; or only by certain

differentiation lineages, e.g. alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA); or proteins expressed at low levels in normal cells and at higher levels in cancer cells, e.g., Human Epidermal growth factor Receptor 2 (HER-2)/neu; or viral Ags, e.g., Epstein Barr virus (EBV) and Human papillomavirus (HPV); or proteins encoded by oncogenes (e.g., abnormal forms of p53, ras, human telomerase reverse transcriptase (hTERT)). A spontaneous immune response against the TAA, and so against cancer cells, does not occur always or, at least, not to such an extent that it counteracts and stops cancer proliferation. The reason for this is that tumor cells have evolved different mechanisms to evade immune surveillance, which are described in the following paragraph. To have an immune response against TAA, which in most cases are self Ags, it is also essential that the immune response is shifted from tolerance to immunity. The goal of a successful vaccine is to induce potent tumor-specific immunity and long-lasting immunological memory. This can be achieved by directing the cellular arm of the immune system towards the recognition of TAA, breaking self-tolerance and side-stepping tumor escape mechanisms.

2.3 TUMOR IMMUNE ESCAPE MECHANISMS

Immune escape mechanisms encompass both intrinsic modifications of the cancer cell to reduce tumor recognition by the immune system and the active suppression of the immune response by the tumor, a process known as *immunosubversion*.

Genomic instability gives rise to genetic diversity in tumors. Tumor-cell variants with reduced immunogenicity are naturally selected by differential propagation of tumor subclones in the microenvironment. Among the alterations impairing tumor recognition by immune effector cells, downregulation of TAAs [179-181], loss of HLA class I components [182, 183], shedding of ligands that activate NK cell receptors (NKR) such as NKG2D [184] and resistance to IFN-γ [185] have been described. Impaired Ag presentation can also be the consequence of down modulation of molecules involved in Ag processing and presentation, such as transporter associated with antigen processing 1 (TAP1), low-molecular-mass protein 2 (LMP2), LMP7 and tapasin [186]. Moreover, co-stimulatory factors such as IL-2, IL-12, IL-15 or type 1 IFNs required for activation of resting NK cells by DCs [168] may be lacking in the tumor microenvironment. Finally, tumors can acquire modifications which ultimately make them resistant to immune cell-mediated killing, such as defects at multiple sites in death-receptor signaling pathways [187] or expression of antiapoptotic signals [188]. As an example, overexpression of the caspase-8 inhibitor cellular FLICE-inhibitory protein (cFLIP) has been observed in various tumors and can contribute to immune resistance to T cells *in vivo* [189]. Similarly, cancer cells can circumvent TRAIL-mediated apoptosis by loss of expression of all TRAIL receptors by various mechanisms [190].

As mentioned above, immunosubversion is the second mechanism of tumor escape. It can occur both through the release of immunosuppressive cytokines or by involvement of cell populations with immunosuppressive activities.

Some tumors or the tumor-associated myeloid cells produce nitric oxide (NO) and have augmented arginase activity, both of which inhibit T-cell function [191]. More importantly, indoleamine 2,3-dyoxigenase (IDO) constitutively produced by tumors

prevents proliferation of $CD8^+$ T cells at the tumor site [192, 193] and induce apoptosis of $CD4^+$ T cells [193].

Tumor cells produce a variety of cytokines with angiogenic and growth factor functions that can also negatively affect the immune response. Vascular endothelial growth factor (VEGF) inhibits DC differentiation and maturation by suppressing the transcription factor NF-kB in hematopoietic stem cells [194]. IL-10 can impair DC function both by inhibiting their differentiation from stem cell precursor [195], compromising their maturation and functionality and enhancing their apoptosis [196]. IL-10 can also inhibit Ag presentation, IL-12 production and T helper type I responses *in vivo* [197, 198]. The proinflammatory factor prostaglandin E_2 (PGE_2) increases the production of IL-10 by lymphocytes and macrophages and inhibits IL-12 production by macrophages [199]. TGF-β inhibits the activation, proliferation and activity of lymphocytes [200] and can directly inhibit NK cell activation and function, as macrophage migration inhibitory factor (MIF) and IL-10 also do [201].

In conclusion, many mechanism have been described by which the tumor can circumvent the immune response, but which of these affects oncogenesis and cancer progression in humans is still an open question. A schematic overview of immune escape mechanisms is depicted in Figure 4.

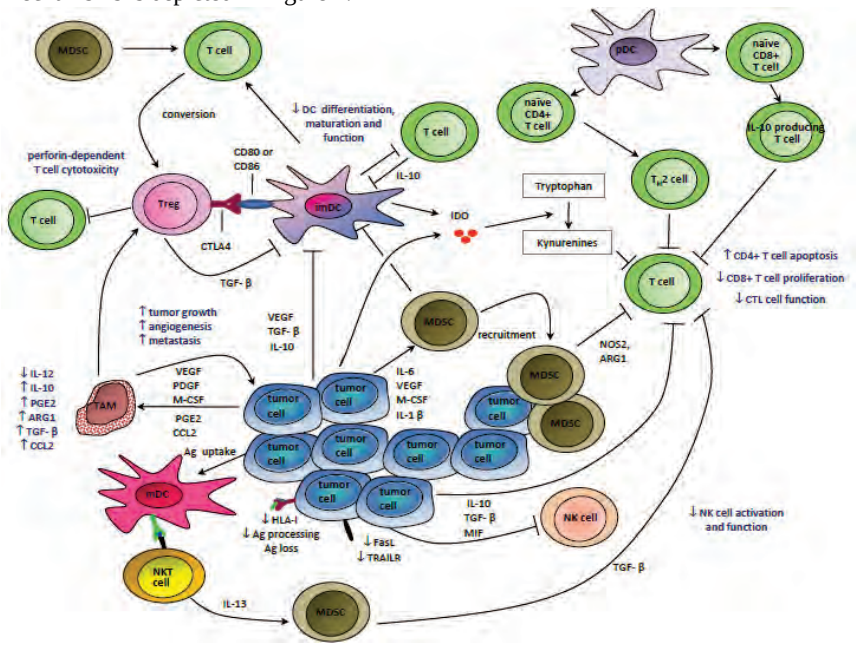

Fig. 4. Tumor immune escape mechanisms. pDC: plasmocytoid DC; ARG-1: arginase-1; NOS_2: nitric-oxide synthase 2; IDO: indoleamine 2,3-dyoxigenase; M-CSF: macrophage colony stimulating factor; PDGF: Platelet-derived growth factor; VEGF: Vascular endothelial growth factor; PGE_2: Prostaglandin E_2; MIF: macrophage migration inhibitory factor.

2.3.1 Suppressive cell populations

Immunosubversion occurs also by involvement in the tumor stroma of cell populations with immunosuppressive activities, such as immature or tolerogenic DCs, TAMs,

myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T_{regs}). DCs recruited to the tumor microenvironment undergo changes that endow them with regulatory functions favorable for the tumor. Different factors in the tumor microenvironment suppress the differentiation and maturation of myeloid DCs. Whereas functional mature myeloid DCs can induce potent TAA-specific immunity *in vivo* [202, 203], immature or partially differentiated myeloid DCs induce either suppressive T_{regs} [203-205] or T-cell unresponsiveness [206]. Plasmocytoid DCs (pDCs) can also be functionally modulated in the tumor microenvironment and exert immunosuppressive functions, such as inducing IL-10 production by T cells, which in turn inhibits the Ag-specific T cell functions induced by myeloid DCs [207].

MDSCs derive from immature myeloid cells (IMCs), which in normal conditions, migrate to different peripheral organs, where they differentiate into macrophages, DCs or granulocytes. IMCs accumulate at the tumor site in response to cytokines and soluble factors present in the tumor microenvironment, which also prevent their differentiation and induce their activation. Their activated state is characterized by an increased production of reactive oxygen and nitrogen species and of arginase-1, which mediate T-cell suppression and inhibit myeloid-cell differentiation. In response to IL-13 produced by $CD4^+$ NKT cells, MDSCs secrete TGF-β, whose immunosuppressive functions have been described above. The ability of MDSCs to induce T_{regs} *in vivo* has also been recently described [208]. MDSCs accumulate in the blood, lymph nodes, and BM and at tumor sites in most cancer patients and are typically $CD11b^+CD33^+CD34^+CD14^-HLA\text{-}DR^-$. It is likely that different tumors induce different subtypes of MDSCs [209] and this incomplete delineation has limited up to now the full functional characterization of this cell population.

TAMs originate from blood monocytes recruited at the tumor site [210] by a number of chemokines produced by tumor or stromal cells, such as CCL2, CCL5, CCL7, CXCL8, CXCL12, as well as cytokines such as VEGF, platelet-derived growth factor (PDGF) and the macrophage colony stimulating factor (M-CSF) [211, 212]. The main player in the TAMs recruitment process and key regulator of their function is nevertheless CCL2. MDSCs within the tumor microenvironment can also differentiate into TAMs. TAMs mainly belong to the M2 class of macrophages (the so-called "alternatively activated" macrophages) and therefore produce IL-10, arginase-1, TGF-β and PGE_2 and favor T helper 2 (Th2) cell responses [213].
Finally, TAMs mediate trafficking of T_{regs} to the tumor via CCL2 receptor CCR4 [214] and promote tumor cell proliferation and angiogenesis by secretion of growth factors such as VEGF, PDGF, TGF-β and members of the Fibroblast growth factor (FGF) family [215, 216].

2.3.1.1 Regulatory T cells

Different T-cell populations with regulatory functions coexist and enhance immunosuppression. The $CD4^+CD25^{high}$ T_{regs} comprise indeed three different T_{regs} populations: the naturally occurring T_{regs}, the induced T_{regs}, as Tr1 and TH3 cells, and the T_{regs} developing in the periphery by conversion of $CD4^+CD25^-$ T cells [217-220].

Globally, $CD4^+CD25^+$ T_{regs} represent 5-10% of human $CD4^+$ T cells and play a pivotal role in maintenance of immunologic self-tolerance [218, 221, 222]. Since CD25 is also expressed on activated effector T cells, expression of the transcription factor FoxP3, identified as a key regulator of T_{regs} development [223, 224], is used as T_{regs} specific marker. Caution regarding its specificity is nevertheless warranted due to reports of FoxP3 induction in conventional activated T cells [225, 226].

Characteristics of $CD4^+CD25^{high}FOXP3^+$ T_{regs} are their anergic state and their capacity to actively inhibit $CD4^+CD25^-$ T cells, $CD8^+$ T cells, DCs, NK cells, NKT cells and B cells in a cell-to-cell contact and dose-dependent manner [214]. T_{regs} characteristically produce IL-10 and TGF-β [227], but their immunosuppressive activity is not mediated only by these cytokines. Once activated through their T-cell receptor (TCR), they can suppress both cytotoxic and helper T-cell activation in an Ag-independent manner [228], by competitive usage of IL-2 [229] and by displaying perforin-dependent cytotoxicity [230]. Moreover, the interaction between cytotoxic T-lymphocyte antigen 4 (CTLA4), constitutively expressed on their surface, and CD80 or CD86 at the surface of DCs induces production of IDO and IFN-γ by DCs, which ultimately leads to T cell killing through the reduction of tryptophans and the generation of kynurenines [192, 193]. Signaling through the glucocorticoid-induced TNF receptor family-related gene (GITR), largely expressed on T_{regs}, is also critical for mediation of their suppressor functions [231]. T_{regs} can also suppress cytokine secretion, proliferation and cytotoxic activity of NKT cells [232].

High T_{regs} frequencies have been described both in the tumor microenvironment and in the peripheral blood of patients with solid tumors of different origins (reviewed in [214]). There are indications that T_{regs} numbers increase with progression of disease and a correlation of higher T_{regs} frequencies with shorter overall survival has been shown for some tumor types, such as gastric [233, 234] and ovarian carcinoma [235]. In CLL, elevated T_{regs} numbers have been shown to correlate with advanced disease stage and unfavourable cytogenetics [236].

The increasing understanding of T regulatory functions will hopefully give hints as to how enhance the antitumor effect of immunotherapeutic interventions.

3 ANTICANCER IMMUNOTHERAPY

What we have before us are some breathtaking opportunities disguised as insoluble problems.
John Gardner, U.S. Secretary of Health, Education & Welfare, 1965

3.1 GENERAL CONSIDERATIONS

The conspicuous increase of knowledge of the immune system and its regulation has led in the past decades to a revival of interest in the immunologic approach to cancer therapy. The identification of an increasing number of TAAs has contributed to this renewed interest. Indeed, the possibility to marshal the exquisite specificity of the immune system to selectively target cancer cells without harming normal cells is probably the main appeal of this therapeutic approach.

The optimal immunotherapy strategy needs to accomplish three functions: provision of appropriate immune activating signals, elimination of inhibitory factors and neutralization of immune escape mechanisms. The purpose of cancer vaccines is to increase the number of tumor-reactive immune effector cells to promote tumor clearance and to maintain protracted activity to prevent relapse.

Current immunotherapy approaches for cancer treatment are either specific or non-specific. Non-specific approaches take advantage of the immunostimulatory activity of cytokines (e.g. IL-2, IFN-α), heat-shock proteins or attenuated bacilli such as Bacillus Calmette-Guerin (BCG) to indirectly potentiate the immune response to the tumor.

Specific immunotherapy targets a defined TAA and can be either passive or active. Passive specific immunotherapy provides “ready to use” immune mediators or molecules and is typically represented by mAbs. Active specific immunotherapy, on the other hand, aims at activating the patient´s immune system to recognize and eliminate the tumor. Adoptive therapy with T cells and NK cells that have been expanded and manipulated *ex vivo* to have augmented antitumor activity have features that are evocative of both active and passive immunotherapy.

3.2 VACCINATION STRATEGIES

The greater part of cancer vaccine strategies investigated hitherto have focused on the induction of tumor-specific CTLs. As discussed in the previous section, the combined action of CTL and IFN-γ- secreting $CD4^+$ T cells, in fact, is thought to be the most effective immune response to eradicate tumors *in vivo*.

Activation of $CD8^+$ T cell response may be accomplished by a variety of methods. Figure 5 provides a schematic diagram of the various approaches that may be used to deliver tumor Ags for recognition by the immune system.

Whole tumor cells, either autologous or allogenic, can be administered following irradiation. Allogenic tumour cell lines treated similarly may also be utilized. Moreover, to enhance the differentiation and activation of the patient’s Ag-presenting cells (APCs), tumor cells can be genetically engineered to secrete proteins, such as GM-CSF, that promote the activation of the APC. DC can be expanded and activated by *in vitro* culture and loaded with the TAA-peptide derivatives, or tumor-derived fractions such as apoptotic

bodies or tumor cell lysate or exosomes. DC can also be fused with tumor cells or transfected with tumor-derived DNA or RNA which combines single or multiple TAA delivery together with accessory molecules and costimulatory pathways critical for Ag presentation.

The identification of HLA class I restricted epitopes derived from tumor Ags gave a major impulse to the development of peptide vaccines [237]. Tumor-derived peptides can be reproducibly and reliably manufactured synthetically for use as immunogens. CTLs recognize in conjunction with specific class I HLA alleles short peptides, 8–10 amino acid residues in length that arise from proteasomal degradation of intracellular proteins.

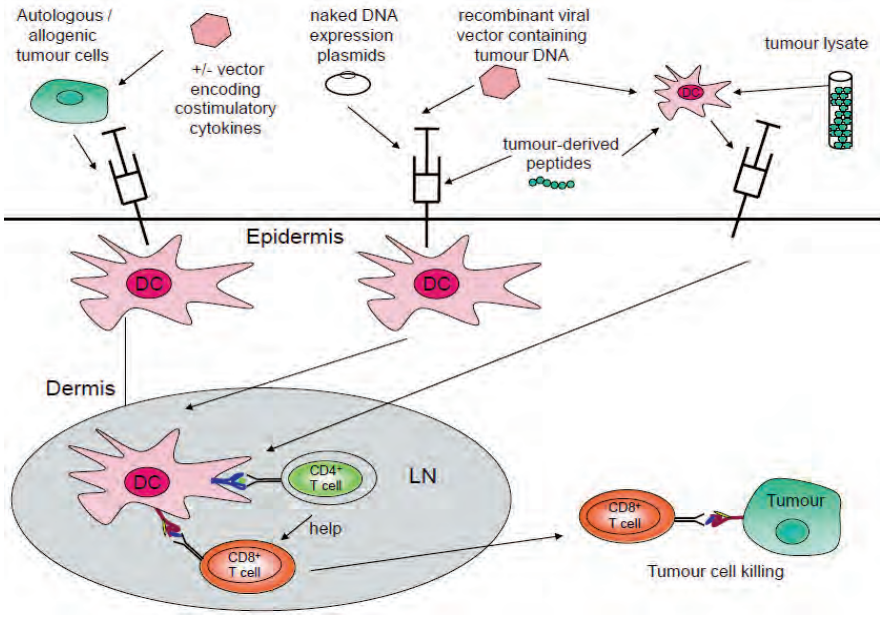

Figure 5. Different approaches for delivering TAA as vaccines for cancer immunotherapy
Reproduced from [Palma et al. 2007 [238]]

Given the prevalence of the HLA-A*02 haplotype in the North American and European populations [239], the majority of peptides used in clinical trials are HLA-A*02–restricted. Such short peptides are limited in use to patients with a particular haplotype. Conversely, long polyepitopic peptides that are 16 amino acids or more can be endocytosed and processed by DC for presentation in conjunction with different HLA class I and II restrictions. These peptides with multiple defined and undefined epitopes may enable all patients, irrespective of HLA type, to present one or more immunogenic epitopes to the effector cells. Similarly, immunization with a whole protein or a truncated version allows multiple epitopes to be presented to $CD4^+$ and $CD8^+$ T cells and is potentially pertinent to all patients regardless of their HLA haplotype.

Tumor-derived peptides can also be combined with immunostimulatory molecules or have modifications to the native amino acid sequence of the epitope. These modified

peptides known as “heteroclitic” peptides increase the immunogenicity of the TAA which are innately weak Ags since by and large they are self Ags. Heteroclitic modifications improve vaccine induced immune responses by increasing the affinity of the peptide for HLA molecules as well as affinity for the cognate TCR or by inhibiting proteolysis of the peptide (epitope enhancement).

Finally, DNA vaccines are an attractive strategy, providing DC with tumor Ag genes to be processed and presented to CTLs. A DNA sequence derived from the TAA can be directly injected as a plasmid or inserted into a recombinant viral vector unable to replicate in the host. Plasmid DNA have been demonstrated to produce immune responses but typically are weakly immunogenic when used alone. Packaging in viral vectors improves delivery and immunogenicity but repeated immunizations with the same viral construct bear the risk of competition with the viral vector epitopes and rapid elimination of the vaccine due to systemic immunity to the viral vector.

A summary of the advantages and disadvantages of the different vaccination strategies investigated in clinical trials is provided in Table 5.

Table 5. Advantages and disadvantages of the different anti-cancer vaccination strategies.

Vaccine	Advantages	Disadvantages
Whole tumour cells	• can be processed to enhance Ag presentation • all relevant TAA virtually expressed • Ag do not need to be defined	• require availability of autologous tumor or allogenic cell line sharing the relevant TAA
Gene-modified tumour cells	• same as whole tumour cells • can be engineered to coexpress immunostimulatory molecules	• same as whole tumor cells • need for *ex vivo* cell culture • cost, time and labor intensive
Peptides	• epitope enhancement possible • easy to produce and stable • different peptides can be combined together and/or with immunostimulatory molecules	• knowledge of the specific epitope needed • HLA restriction
DC	• powerful APC • large scale production of clinical grade DC feasible • can be loaded with relevant TAA in many ways (e.g. pulsed with tumour-derived peptides or whole proteins, tumour cell lysate; transfected with tumour-derived DNA or RNA)	• need for *ex vivo* cell culture • cost, time and labour intensive • optimal technique for Ag loading still to be defined • Criteria for standardization of final product still to be defined
Viral gene transfer vectors	• engineered to express the relevant TAA • can be engineered to coexpress immunostimulatory molecules • several vectors available (adenovirus, poxvirus, etc)	• competition with the viral vector epitopes • preexisting immunity against viral vectors may have detrimental effect on antitumor response
Plasmid (naked) DNA	• constructed to express the relevant TAA • easy to produce and stable	• detailed knowledge of the Ag DNA sequence necessary • immune response likely to be of Th2-type • high doses required to generate immune responses

Adapted by permission from Informa Healthcare: [HANDBOOK OF PRINCIPLES OF TRANSLATIONAL RESEARCH] [238], copyright (2007)

A great many number of preclinical and clinical studies over the years have established that vaccine therapy against cancer overall has very low associated toxicity. Systemic reactions are generally rare and adverse effects are normally limited to local reactions, transient mild fever and erythema. The vaccine-associated risk of acute or chronic autoimmune reactions is minimal. In general, documented clinical responses with vaccine therapy, though, have been infrequent. The fact that the majority of these trials recruited patients with advanced disease as well as the inability of active immunotherapy to debulk large tumors, which is an

important factor in most standardized criteria for documenting clinical responses, may have contributed to lack of significant clinical improvements [240]. Moreover, immune responses and clinical responses were often found to be discrepant, which highlights the necessity for optimization of the design of clinical trials. In this regard, patient selection and inclusion of other evaluation criteria such as improvement in overall survival and progression-free intervals may be critical factors.

3.2.1 DC vaccines

The discovery that myeloid DCs can be easily generated from monocytes and $CD34^+$ precursors [241] has greatly facilitated the implementation of DC-based vaccination. Dendritic cells for cellular vaccines can also be directly obtained from PB, albeit DCs are only 0.2% of white blood cells and therefore require substantial enrichment as well as an activation step by a short exposure *in vitro* to activating factors such as CD40L or Toll-like receptors (TLRs) prior to re-infusion [242]. Unactivated or incompletely activated DCs are known to induce tolerance rather than immunity [243].

The majority of clinical trials presently utilize monocyte-derived DCs. When cultured in the presence of GM-CSF and IL-4 for 3-5 days, these PB monocytes develop into immature DCs, which can be terminally matured with 1-2 days of further culture using different stimuli, such as TNF-α [241, 244]. *Ex-vivo* cultured DCs have been administered intravenously, intradermally or directly injected in the tumor-draining lymph nodes (LNs) in various animal models and clinical studies. It has been shown that immature DCs migrate less than mature DCs into the draining LNs and that inflammatory cytokines [245, 246], matrix metalloproteases and TLR ligands [247] can stimulate DC migration. Direct injection of DCs into the LNs or lymphatic circulation may circumvent the need for migration from the site of intradermal injection [248], but the immunostimulatory efficacy of intranodal administration compared to intradermal vaccination remains to be verified.

The observation that a single DC is capable of interacting with a large number of T cells elucidates how DCs can efficiently scan the T-cell repertoire in search of naturally occurring TAA-specific T-cells, ultimately promoting an efficient anti-tumor $CD8^+$ T cell response [249, 250].

Appropriate induction, expansion and maintenance of CTL responses is achieved through delicate interactions between $CD4^+$ T cells, DC and $CD8^+$ T cells involving several ligand-receptor pairs. $CD4^+$ T-helper cells mainly act through up-regulation of CD40L, which then interacts with CD40 on DC causing DC maturation [251]. Other molecular triggers of DC activation that can support induction of powerful CTL responses include TLR ligands, which bind TLR at the DC surface or intracellularly [252], and interaction with NKT cells, as shown by murine studies [253]. To be able to efficiently induce CTLs, CD80/CD86 on activated DC interact with the CD28 receptor on $CD8^+$ T cells providing critical costimulatory signals.

In general, DC vaccines have proved to be safe and associated with minimal toxicity [203, 248, 254-260], but clinical results have been limited. Several variables may have contributed to the potential of DC vaccines being undervalued in early clinical trials. First, several studies were performed using immature DCs, although subsequently it was demonstrated that only mature DCs succeeded in stimulating T-cell responses [248, 259,

260]. Second, as mentioned previously, in most cases, DC vaccine efficacy has been investigated in late-stage patients. Nevertheless, long-lasting clinical responses were noted which in some patients coincided with a specific cytotoxic T-cell response. These observations encouraged further investigation for the development of DC vaccination in cancer, in particular in lymphoid malignancies. Indeed, lymphoid tumors have been reported to be more responsive than solid tumors to DC vaccines [261], probably because they are more easily accessed by immune effector cells and often express co-stimulatory molecules. Still, there is a great need for standardization and quality control of DC vaccine immunotherapy. Additional research is needed to identify the optimal conditioning and activation stimuli, the optimal route of administration and the optimal dose and vaccination schedule. Moreover, several early published studies lack sufficient information about quality control for the vaccines and about differences between individual vaccine preparations. Clinical responses are often not reported using accepted tumor response criteria and immune responses are rarely monitored appropriately. For this reason, a number of minimum quality criteria to be met when designing DC vaccination studies have been suggested by Figdor et al. [262]. These criteria encompass: description of vaccine preparation according to good manufacturing practice (GMP) guidelines, quality control for *ex-vivo* generated DCs, description of patient characteristics, description of trial design, clear documentation and definition of clinical response, description of clinical outcome of all patients, description of immunological evaluation before and after vaccination.

3.2.2 Adjuvants

Immunological adjuvants are substances administered along with the vaccine to potentiate the immune response. These can be either chemicals, such as aluminium-based salts (alum) and incomplete Freund's adjuvant (IFA), or products of microorganisms, such as BCG, lipopolysaccharide (LPS), or recombinant cytokines, such as GM-CSF. With the exception of IFA, whose toxicity profile is not optimal, all these adjuvants have been used in clinical trials and proved to be safe.

Their mechanisms of action range from acting as a depot for the vaccine (alum) to promoting the secretion of cytokines and/or DC maturation (chemical adjuvants) or recruiting professional APCs and/or effector cells (cytokine adjuvants).

GM-CSF is the most used. GM-CSF is a pleiotropic growth factor that promotes the maturation, migration and functional activation of DCs, thereby enhancing Ag presentation. Its ability to augment both humoral and cellular immunity via multiple mechanisms is well established. GM-CSF, on the other hand, may also induce immune suppression, an effect most commonly seen when it is used at high doses [263, 264].

3.3 IMMUNOMODULATING STRATEGIES

3.3.1 Depletion of regulatory T cells

As discussed in the previous chapter, $CD4^{+}CD25^{high}FOXP3^{+}$ T_{regs} can impair immune effector functions by actively inhibiting several immune cell populations [214]. Hence, the selective elimination of T_{regs} could importantly contribute to induce vaccine-induced immune response to tumors.

Several approaches have been explored to selective deplete T_{regs}. Targeting CD25, the α chain of the IL-2 receptor constitutively expressed on T_{regs}, represents one such approach. When IL-2 diphtheria toxin conjugate DAB(389)IL-2 (denileukin diftitox; ONTAK) was administered to metastatic renal carcinoma patients, it reduced the number of T_{regs} in the PB and abrogated T_{regs}-mediated immunosuppressive activity *in vivo* [265].

In the 1980s studies demonstrated that low doses cyclophosphamide (CTX) could potentiate delayed-type hypersensitivity (DTH) responses [266] and that this occurred by its selective effect on a population of tumor-induced suppressor cells [267, 268]. The exact mechanism by which this effect occurred was elucidated only recently. It was in fact shown that both the frequency and the inhibitory function of T_{regs} were decreased by downregulating FoxP3 and GITR, critical mediators of T_{regs} development and suppressor function [269].

Following demonstration that a single low dose of CTX could dramatically reduce $CD4^+CD25^+$ T_{regs} accumulation in tumor bearing animals, thus restoring the efficiency of immunotherapy against established tumors, Ghiringhelli et al investigated the effects of iterative low dosing ("metronomic", 50 mg twice a day every other week) of oral CTX in 9 advanced cancer patients. These results showed that metronomic CTX selectively depletes T_{regs} in humans while preserving other lymphocyte subsets in number and function. This metronomic CTX regimen not only did not inhibit T and NK cell functions but actually boosted them dramatically. When higher doses of CTX (200 mg per day instead of 100 mg) were used in another series of 6 cancer patients, a profound decrease of all lymphocyte subpopulations was observed, without any selective activity on T_{regs}, indicating that only low doses of CTX succeed in obtaining T_{regs} depletion [270]. However, a phase I study in which advanced cancer patients were treated with non-specific (BCG) immunotherapy and a single dose (250, 500 or 750 mg/m^2), of intravenous CTX reached the opposite conclusion [271].

In CLL, elevated numbers of T_{regs} have been described [236], but the effect of low-dose CTX has never been investigated. Reduced frequencies of functionally impaired T_{regs} have been observed after fludarabine treatment, which nevertheless had also a strong impact on other lymphocyte subpopulations [272].

Finally, it is noteworthy that both lenalidomide and pomalidomide were shown to strongly inhibit T_{regs} proliferation and suppressor-function *in vitro* [273]. Recently, a reduction in T_{regs} frequency was reported following lenalidomide administration in CLL patients [274], an observation that needs validation with a larger series of CLL patients.

3.4 ENDPOINTS OF CANCER VACCINES

3.4.1 Clinical endpoints

Extending survival is the main endpoint of all anticancer treatments; secondary endpoints are longer time to progression (TTP), tumor regression and improvement of quality of life. Presently the majority of clinical trials evaluating cancer treatments for objective response in solid tumors use the Response Evaluation Criteria in Solid Tumors (RECIST), a set of criteria published in the year 2000 by an international collaboration including the European Organization for Research and Treatment of Cancer (EORTC), National Cancer Institute of the United States, and the National Cancer Institute of Canada Clinical Trials Group. For

hematological malignancies different response criteria are used depending on the type of malignancy. For CLL, the criteria indicated by the International Workshop on CLL (IWCLL) [88] are applied.

While adherence to these guidelines is essential for comparing the results of different treatment protocols [275], deviation from these and/or inclusion of other criteria may be appropriate when evaluating the anti-tumor effect of treatments, such as vaccines. Some of these criteria include overall survival (OS), disease-free survival (DFS) and TTP. Nevertheless, whenever new response criteria are applied, these should be predefined and clearly described. In many published vaccine trials, in fact, the exact criteria used for definition of clinical response were not clearly specified and "soft" subjective criteria (e.g. "shrinkage of some lesions", "symptom improvement" or "survival longer than expected") were used, which may have caused considerable confusion in the interpretation of the results [240].

3.4.2 Surrogate endpoints

A surrogate endpoint is expected to be an indirect measure of the clinical effect of a treatment. The induction of a vaccine-induced, tumor-specific immune response can be regarded as a surrogate endpoint for clinical outcome after vaccination. In phase I and II clinical trials immunologic monitoring is not a protocol-mandated requirement, but it is still important because it may be able to correlate clinical responses to a specific immune mechanism, to predict responsiveness to therapy or help estimate the survival [276]. Both B-cell and T-cell activity in standard *in vitro* assays have been have been used for the assessment of vaccine-induced humoral and cellular immunity. A pre-vaccine to post-vaccine comparison of antitumor immune response gives the information of the immunological efficacy of the vaccine (vaccine potency). However, analogous to other such surrogate markers (e.g. elimination of micrometastasis and changes in tumor markers), the clinical relevance of a vaccine-induced immune response has not yet been established. Efforts are underway by regulatory agencies such as the US FDA task force with the International Society for Biological Therapy of cancer (iSBTc) and other European organizations like EMEA working together with Association for Immunotherapy of Cancer ("CIMT") [277, 278] for harmonization of assay protocols and results interpretation that would allow implementation of uniform standards to immunological testing in cancer vaccine trials.

It is generally believed that the antitumor immune response should be Th1-type, involving both humoral and cellular arms and activate the innate as well as adaptive immune system. Unlike vaccines against infectious diseases, which are primarily measured using humoral markers, the success of an anticancer vaccination approach in general is essentially assessed by cellular immunological biomarkers. This is because T-cell mediated immune response is thought to be the most effective to eradicate tumors *in vivo*. Unlike Ab responses, though, which can be easily detected and titrated in PB, Ag-specific lymphocytes may not be easily detectable in the PB. Although it is proven that Ag-specific lymphocytes home to the tumor tissue, BM and regional LNs, PB is the only compartment typically accessible for serial analyses in a clinical setting.

3.4.2.1 Immune monitoring of vaccine clinical trials

The only *in vivo* measure of Ag-specific immunity is the DTH test. The majority of other assays are performed *in vitro*. The first generation of *in vitro* T-cell assays includes lymphoproliferation assay and cytotoxicity assays against tumor targets using radioactive isotopes of chromium (Cr^{51}). Like DTH, these assays measure overall T-cell response before and after vaccination.

Second-generation T-cell assays, on the other hand, measure the immune response at the individual immune cell level. Detecting single-cell events, they provide quantitative and qualitative data, i.e. provide individual cell phenotypic and functional information. They include peptide HLA tetramers and detection of cytokines by different assays, of which the most common are ELISPOT (Enzyme-linked Immunospot), CFC (Cytokine Flow Cytometry), Real-time PCR and the multiplex bead immunoassay. This latter utilizes sets of distinct fluorescently labelled microspheres, each linked to a cytokine-specific Ab, allowing the simultaneous assessment of several pro-inflammatory cytokines [279, 280]. Moreover, T cell degranulation via cell surface exposure of CD107 provides an indirect measure of T-cell- mediated cytotoxicity [281]. In general, these assays are very sensitive to variations in assay conditions and their sensitivity can be impaired by non-specific background cytokine production, but ELISPOT and CFC been now extensively used and provide information which can be quite reliably correlated to clinical efficacy of the treatment.

Third-generation assays represent an evolution of the second-generation ones. After detecting Ag- specific T cells with tetramers or CFC, they characterize T cells properties, such as cytotoxic, proliferative and migratory capacity, regulatory functions and their ability to interact with other cells of the immune system, such as APCs. They allow the functional evaluation of different T-cell subpopulations (e.g. memory, helper and cytotoxic T cells) as well as their cytokine production in response to Ag exposure. Nevertheless, as no specific correlation has yet been found between any of these T-cell properties and clinical vaccine efficacy, third-generation assays need further validation.

Advantages and disadvantages of these assays are shown in Table 6.

Some immunological parameters have been shown to correlate with improved prognosis. Among these, HLA class II expression on tumor cells [282], extensive intratumoral infiltration of $CD8^+$ T cells [283-285], NK cells [151], and the presence of $CD8^+$ tumor-infiltrating lymphocytes (TIL) showing proliferative activity and IFN-γ secretion [150]. At present, no single validated and quality assured method has been proved to be an accurate and reproducible indicator of clinical prognosis following anticancer vaccination therapy. Serological tests are undoubtedly less problematic and analysis of IgG subclass response might be useful, but, for the reasons mentioned above, they are less informative than cellular tests with regard to antitumor immune response. At present, for immune monitoring of vaccine clinical trials it is recommended using a combination of two or more second-generation assays and their cumulative findings be utilized as indicators of immunological activation following vaccine therapy [286].

The field of biomarkers for immunological monitoring is yet nascent and rapidly developing. The key issues are the delineation of *in vitro* assays that demonstrate accurate and reproducible correlation to clinical prognosis, standardization of the assay protocols to

eliminate the laboratory-to-laboratory variability and resolution of the issues with regard to sampling cells in the PB and other compartments. It is anticipated that large scale studies and new and improved approaches to sampling immune cells and assaying immune biomarkers may lead to the development of a set of standard criteria for measuring immunological function after anticancer vaccination.

Table 6. Assays used for immune monitoring in vaccine clinical trials.

Assay	Brief description	Advantages	Disadvantages
DTH	Ag as soluble protein is injected i.d. and the diameter of erythema or induration is measured after 48-72 h	• only *in vivo* measure available • easy to perform	• no standardized cut-off for a positive response • no standardized dose for DTH testing • Ag-specificity is questionable
Lymphoproliferation assay	Lymphocytes are cultured with the Ag studied. ^{3}H thymidine is added to the culture medium. Proliferating (dividing) cells incorporate ^{3}H thymidine, which is quantitated using a beta scintillation counter	• easy to perform • reliable • sensitive • reproducible	• can be influenced by the non-specific immune function of the patient • can be influenced by the *in vitro* stimulation procedure • not qualitative • not quantitative • no information about responding cell population (CD4+, CD8+, etc)
Cytotoxicity assay	Lymphocytes previously sensitized to the Ag present on the target cells are cocultured with the target cells. Percentage of lysis of target cells is quantitated by ^{57}Cr release assayor at Flow Cytometry	• functional assay • measures the ability of direct tumor lysis	• low sensitivity • often involves multiple *in vitro* stimulations • not quantitative • often other targets than autologous tumor are used, which may not reflect the capability of effector cells to lyse autologous tumor cells *in vivo*
ELISPOT	Lymphocytes are cultured with the Ag studied in a micro-titer plate coated with a monoclonal Ab to a specific soluble factor (e.g. IFN-γ, IL-4, IL-10, TNF-α). The cells and the Ag are then washed from the wells and replaced with secondary Ab conjugated to a detection reagent. The plate is developed with a chromogen and spots appear where there was a cell secreting the soluble factor being investigated.	• functional assay • allows to measure individual soluble factors secreted by activated T cells and identify the pathway of the immune system activated by the vaccine • Lowest limit of detection (1/100000 Ag-specific T cells) • considerably reliable • relatively rapid	• provides no information on cell phenotype
Tetramer staining	Tetramers are composed of four MHC-I molecules, each bound to the epitope of interest. The tag is a fluorescent label, which allows to measure the binding of the tetramer to the TCR at flow-cytometry.	• sensitive (1/100000 Ag-specific T cells) • T cell subset analysis is optimal • allows to identify the peptide sequence or epitopes that bind to the highest number of TCR in a naïve individual • allows to identify the phenotype of the T-cell to which the tetramer binds • allows to measure the change in the number of T-cells displaying a particular TCR before and after vaccination	• requires knowledge of the epitope • requires availability of the tetramer for the respective epitope/HLA allele • unable to distinguish between functional and dysfunctional T cells
Cytokine Flow Cytometry	Lymphocytes are cultured with the Ag studied and the presence of intra-cellular cytokines is detected by fluorescein-labelled Mab. The phenotype of the lymphoid cells (CD4+, CD8+, etc) is identified with a second set of fluorescein-labelled Mabs.	• functional assay • sensitive (1/10000 Ag-specific T cells) • provides additional information on cell phenotypes • relatively rapid	• non-specific background staining
Real time PCR	Detection and quantitation of cytokines at the DNA/RNA level	• very sensitive (1/20000 – 1/50000 Ag-specific T cells) • quantitative • flexible • least specimen required • does not require *in vitro* expansion	• lack of standardization • does not measures cytokines at protein level • cannot quantitate T cell frequencies or characterize T cells • time-consuming

Reproduced from [Palma et al. 2007 (403)]

4 IMMUNOTHERAPY OF CHRONIC LYMPHOCYTIC LEUKEMIA

The subjects who volunteer for clinical trials are the true heroes of modern clinical research. It is the responsibility of our profession to serve these persons to the best of our ability.
E.E. Slater, 2002.

4.1 RATIONALE

CLL represents an attractive model in which to investigate immunotherapy approaches for a number of reasons. First, its indolence allows in principle enough time for the generation of an immune response against the cancer cells. Secondly, tumor cells are easily accessible from the PB where they circulate in high numbers. Thirdly, although deficient in critical costimulatory molecules, CLL cells express both HLA-I and HLA-II molecules and can potentially serve as Ag-presenting cells.

Anecdotal reports of spontaneous remissions of CLL [287] as well as established "graft-versus-leukemia" effect after hematopoietic stem cells transplant or donor lymphocyte infusion [288-291] and clinical responses to immunomodulatory cytokines [292] may indicate that CLL is responsive to immune effector functions. Furthermore, the presence of CLL-reactive autologous and allogeneic T cells has been demonstrated in several reports [293-297] and **Paper I**). Expression of oligoclonal TCR Vβ expansion has been shown by our group [298] and others [299, 300], possibly implying repeated antigenic stimulation perhaps by CLL Ags.

Both $CD4^+$ and $CD8^+$ may kill tumor B cells [301, 302] and may potentially mediate therapeutic responses if their numbers and activation states can be sufficiently increased by tumor vaccines and their activity maintained for long enough to clear tumor cells.

4.2 THE IMMUNE SYSTEM IN CLL

Although CLL cells express tumor Ags that can be presented in the context of HLA class I and class II molecules, there is no naturally occurring, effective autologous immune response against the tumor cells [303]. Three different mechanisms can contribute to this condition. The ability of CLL cells to act as effective APCs is limited. Secondly, defects induced by CLL cells impede normal T cell effector activity and, finally, an increased T_{reg} frequency is often noted in CLL patients.

Despite normal expression of HLA class I and II molecules as well as adhesion molecule (ICAM)-1 (CD54), CD27 and CD40, CLL cells lack completely or express very low levels of the critical costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) [304]. Moreover, no upregulation of HLA-I molecules occurs following IFN-γ stimulation [305].

Immune dysfunction is an associated attribute of CLL and both humoral and T-cellular defects are known to increase with disease severity. The CD4/CD8 ratio is inverted [306] with an increase in absolute numbers of phenotypically activated $CD4^+$ and $CD8^+$ cells [307]. Moreover, CLL cells are resistant to FAS (CD95) ligand-dependent apoptosis [308].

CLL cells express immune-modulating factors, including TGF-β, IL-10 and IL-4 [309-311], which skew the immune system toward a Th2–type response, thereby

suppressing T-cell activation, expansion, and effector functions by HLA class I–presented Ags [309, 311-313].

In T cells from CLL patients CD40 ligand (CD154) [314], the ζ chain of the TCR and CD28 [315] are expressed at lower levels compared to T cells from healthy individuals. Downmodulation of CD154, in particular, abrogates expression of co-stimulatory molecules such as CD80 and CD86 on CLL cells, and thereby interferes with effective Ag presentation. Moreover, T cells exhibit variable expression of the low affinity and non-specific adhesion molecules leukocyte function–associated antigen (LFA)-1 and ICAM-1, reduced CD28 and CD152 expression and reduced IL2 and CD25 (IL2R) expression [316]. All these observations indicate that the T cell in CLL may be unable to start and maintain an immune response to the malignant B cell, but the exact mechanisms by which this occurs have still to be completely elucidated. Indications about the aberrant function and phenotype of T cells in CLL was noted in a study in which global gene expression of highly purified $CD4^+$ and $CD8^+$ T cells from peripheral blood from individuals with CLL was compared with age-matched healthy donors [317]. The analysis revealed an altered expression of genes mainly involved in cell differentiation in CD4 cells and defects in cytoskeletal formation, vesicle trafficking, and cytotoxicity in CD8 cells of CLL patients. A study by our group demonstrating the antiapoptotic effect of T cells from CLL patients on the leukemic B cells as well as dysregulation of global gene expression in the T cells compared to healthy donors and multiple myeloma patients is currently under review for publication [318]. T cell activation is regulated by complex cytoskeleton-dependent cellular processes which imply polarization of the actin cytoskeleton and accumulation of F-actin at the site of contact with the APC, termed "immunological synapse" [319, 320]. In a study by Ramsay et al., impairment of T cell conjugate formation and actin polymerization at the immunological synapse was shown in autologous $CD4^+$ and $CD8^+$ T cells from CLL patients. These changes could be induced in T cells from healthy donors in coculture experiments with CLL B cells. The occurrence of such changes seemed to be dependent on cell contact and not to be cytokine-mediated. Upon contact with CLL cells both T cells from CLL patients and healthy donors, recruitment of key regulatory proteins, such as LFA-1, TCR and Lck, to the immune synapse was in fact inhibited. These data indicate that the primary defect in the CLL T cells is a failure of cytoskeletal organization rather than a blockade of TCR-mediated signaling. This suggests that what occurs in CLL is T cell suppression rather than T cell anergy. It is noteworthy that treatment of autologous T cells and CLL cells with the immunomodulating drug lenalidomide repaired defective T-cell immune synapse formation [76]. A detailed description of the effects induced by CLL interaction on $CD4^+$ and $CD8^+$ cells is shown in Figure 6.

Finally, a significantly increased frequency and suppressive function of T_{regs} have been described in CLL, especially in untreated or progressing patients presenting with extended disease [272].

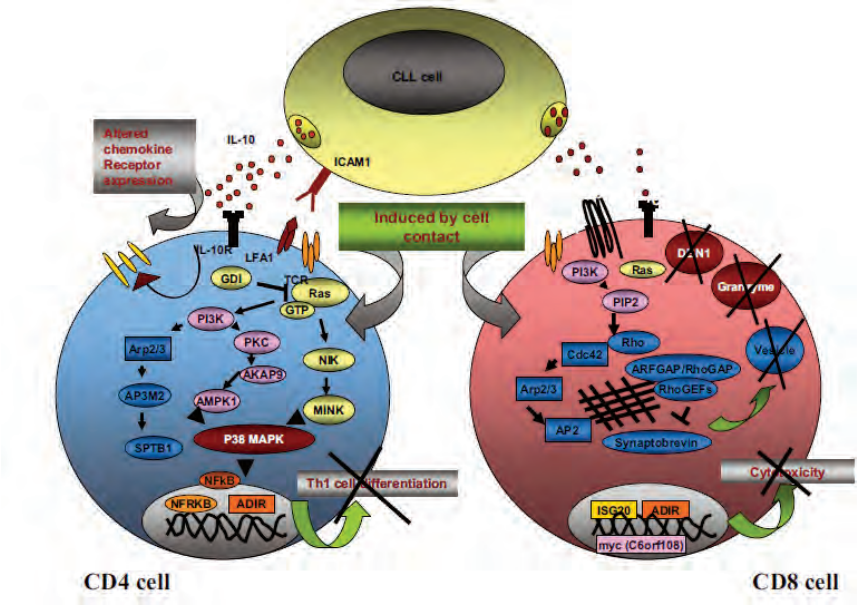

Figure 6. Interactions of CLL cells with T cells induces specific T-cell defects.
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4.3 TUMOR ANTIGENS IN CLL

In general, the ideal tumor Ag has three characteristics. It should be expressed uniquely on tumor cells and not on normal tissue in order to decrease the risk of autoimmune reaction; it should be expressed on all tumor cells; and it should be essential for cancer cell survival to prevent the emergence of Ag-negative variants that could escape the immune response.

In CLL, as in most cancers, there is no ubiquitous immunodominant tumor Ag that can be applied to all patients for vaccine development.

The best characterized CLL-specific Ag is the immunoglobulin idiotype (Id), which is patient-specific but poorly immunogenic. In a study by Krackhardt et al, 14 Ags were identified which showed aberrant expression in CLL cells using serological identification by recombinant expression cloning (SEREX). These Ags were named KW1-KW14 and cytotoxic T cells responses could be generated against peptides derived from these Ags [321]. Other identified CLL Ags are CD23 [322], fibromodulin [323, 324], formin-related protein in leukocytes 1 (FMNL1) [325, 326], murine double minute 2 oncoprotein (MDM2) [327], oncofetal antigen immature laminin receptor protein (OFA-iLRP), [296, 328], the orphan receptor type 1 tyrosine kinase ROR1 [329-331], receptor for hyaluronic acid-mediated motility (RHAMM)/CD168 [332], survivin [333, 334] and hTERT. This latter was investigated as potential target in **Paper I** of this thesis. Cytotoxic T-cell responses could be generated against peptides from all the above mentioned Ags. A list is reported in Table 7.

Table 7. Potential tumor antigens in CLL.

CLL Ag	Normal function	Expression in normal tissues	Expression in CLL	Immune responses in CLL	Ref.
Idiotype	Ig	none	Patient-specific (100% of CLL cells)	humoral, T cell	[37, 335-337]
CD23	low-affinity Ig E receptor	B cells, activated macrophages, eosinophils, follicular DC and platelets	100%	T cell	[322]
Fibromodulin	collagen-binding protein	connective tissue	60-100%	T cell	[323, 324]
FMNL-1	actin regulation	PBMC, thymus, lung and spleen	60%	humoral, T cell	[325, 326]
MDM2	cell growth , apoptosis and differentiation regulation	ubiquitous	75-100%	T cell	[327]
OFA-iLRP	participates in binding laminin to integrins	none	Up to 100% in advanced stage	T cell	[296, 328]
ROR1	organogenesis	none	100%	humoral, T cell	[329-331]
RHAMM/CD168	mitotic spindle organization and maintenance	thymus, testis, placenta	>75%	T cell	[332]
Survivin	Inhibition of apoptosis and cell division regulation	thymus, testis, placenta, endothelial cells	0-75% (induced with CD40 ligation)	T cell	[333, 334]
hTERT	catalytic subunit of telomerase	$CD34^+$ hematopoietic stem cells, epithelial cells of the colon crypts	75%	T cell	[338] (**Paper I**)

4.4 CELLULAR VACCINES

Given the difficulty in finding appropriate TAAs in CLL, novel approaches have been explored to elicit an antitumor response without requiring precise identification of antigenic targets. One such approach is the use of autologous tumor cell vaccines, which may have the advantage of targeting the whole repertoire of TAAs. Nevertheless, as discussed above, CLL cells are poor APCs and strategies to render them more immunogenic have to be implemented. One possibility is to subject CLL cells *ex vivo* to a combination of oxidative physicochemical stressors in order to induce the release of Ag-binding heat-shock proteins and free radicals that increase the immunogenicity of CLL cells *in vivo* [297].

Another option is to employ more powerful APCs, such as DCs, loaded with tumor cell lysate, tumor RNA or apoptotic bodies or directly fused with CLL cells (fusion hybrids). CLL-specific immune responses *in vitro* were generated by DC pulsed with tumor lysate [339], tumor RNA [340] and fusion hybrids [341]. In two preclinical studies,

these four different strategies were compared and it was shown that apoptotic bodies of leukemic cells were superior to the other three approaches for loading of whole tumour cells into autologous DCs [342, 343].

Alternatively, to enhance the immunogenicity of the cellular vaccine, genes encoding immune accessory surface molecules such as CD80 and CD40-ligand (CD154), and stimulatory cytokines such as IL-2 and IL-12 may be introduced in the CLL cells as transgenes. CD154 expression by T cells is induced by TCR activation. CD154 binds to CD40 on APCs triggering the expression of the co-stimulatory molecules CD80 and CD86 by APCs, which in turn bind to CD28 on T cells further promoting their activation and proliferation [344-346]. The immune gene approach typically uses a viral vector to transfer the transgene into the desired cell. The most extensively studied vector for transduction of CLL cells has been adenovirus. CLL cells infected with a replication-defective adenovirus vector encoding recombinant CD40 ligand, Ad-CD154, were able to stimulate and expand CTLs specific for autologous leukemia cells *in vitro* [304]. Similarly, CLL transduced with a modified vaccinia virus strain Ankara vector inducing the expression of CD80, ICAM-1 and LFA-3 (TRICOM) efficiently induced CTLs against autologous CLL cells *in vitro*. The combination of transduction of CD154 and IL-2 was evaluated in non-Hodgkin's lymphoma B cells and induced greater T-cell activation than either population alone [347].

An alternative form of Ag presentation makes use of trioma cells, which are lymphoma cells that have been fused to a hybridoma and have thereby been modified to express an Ig directed against surface receptors of APCs. The trioma cells express tumor-derived Ags and have anti-APC specificity. In a preclinical study, malignant cells from 11 patients with CLL were fused to anti-Fc receptor hybridomas. Trioma cells were successfully generated in seven cases and CLL-specific T-cell responses could be generated *in vitro* [348].

Finally, it was shown that CLL T cells activated and expanded *ex vivo* by culture with magnetic beads coated with mAbs against CD3 and CD28 in the presence of IL-2 (Xcellerate T cells) may recover their capacity to respond to Ags [349, 350].

4.4.1 Vaccine clinical trials in CLL

All vaccine clinical trials conducted in CLL used cell-based vaccines. Some of the strategies mentioned in the previous paragraph have been evaluated in clinical trials (summarized in Table 8).

Patients in the indolent phase of disease were vaccinated with oxidized autologous leukemia cells in a phase I trial. Partial responses were noted clinically in five of 18 patients that were associated with enhanced T-cell anti-tumor activity, but duration of such responses was rather short (1-4 months) [351].

In two studies from the another group of investigators, patients were vaccinated with either allogeneic DC loaded with tumor lysates or apoptotic bodies (n=9) [352] or autologous DC pulsed with tumor lysates (n=12) [353]. Increases in T-cells that reacted against the CLL Ags RHAMM and fibromodulin were detected in some patients and there were some minor clinical effects, but no significant clinical responses were observed.

A Phase I clinical trial was performed with a single dose of autologous Ad-CD154-transduced CLL cells to assess tolerability, toxicity and activity of this approach. Patients experienced flu-like symptoms and some had transient elevations in hepatic transaminases and transient thrombocytopenia. None of the treated patients experienced dose-limiting toxicity. The treatment significantly increased the absolute blood T-cell counts and the numbers of leukemia-specific T cells within 1 to 4 weeks of treatment and high plasma levels of IL-12 and IFN-γ were noted. These biologic effects were associated with reductions in leukemia cell counts and LN size [354]. In another clinical trial, subcutaneous administration of autologous CLL cells modified to express CD154 and IL-2 was evaluated as a vaccine strategy. Nine patients received treatment with autologous modified leukemia cells in this Phase l clinical trial. Seven out of nine of these patients had transient enhancement of T-cell reactivity against autologous CLL cells, but no clinical response was observed. High levels of T_{regs} were reported in all the patients [355].

Finally, Xcellerate T cells have been evaluated in a phase I/II dose-escalation clinical trial, in which doses up to $100x10^9$ autologous Xcellerated T cells have been administered as a single infusion to CLL patients. No dose-limiting toxicities were reported. The treatment resulted in consistent dose-dependent increases in blood T-cell counts and reduction in the LNs and spleen, but disappointingly no reductions in blood leukemia cell counts were observed [356]. In all the above mentioned studies, no vaccine adjuvants were used.

In conclusion, despite intense investigation on immunotherapy for patients with CLL conducted in the past decade, further studies are needed to obtain major advances in the field. The vaccine strategies investigated up to now provided the demonstration that immunization of CLL patients actually succeeds in inducing anti-leukemia immune responses, but with limited clinical benefit to patients. Further studies are warranted to better define the optimal vaccination strategy and best combination with immune enhancing/modulating drugs.

Table 8. Vaccine clinical trials in CLL.

Disease setting	Rai stage	No of pts	Vaccine	Adjuvant	Number of immunizations	Immune response	Clinical outcome	Duration of follow-up	Ref
Indolent phase	0-IV	18	oxidized autologous leukemia cells	none	Group 1: 12 in 6 wks; Group 2: 12 in 16 days; Group 3: 4 in 6 wks	9/18 pts had cellular response to autologous CLL cells[a]	5/18 PR 6/18 SD 7/18 PD	6 mo	[351]
Indolent phase	0-I	9	allogeneic DCs pulsed with tumor cell lysates or apoptotic bodies	none	5	1/9 had cellular response to RHAMM[b]; ↑ in Th1 and Th2 cytokines	1/9 PD 8/9 SD	12 mo	[352]
Indolent phase	0-II	12	autologous DCs pulsed with tumor cell lysates	none	5 every 4 wks (first 2 pts); 4 every 2 wks → 4 every 4 wks (10 pts)	4/12 had cellular response to RHAMM and fibromodulin[a,b]; ↓ Tregs in clinical responders	5/12 R[C] 3/12 SD 4/12 PR	12 mo	[353]
Progressive phase	II-IV	11	autologous Ad-CD154-transduced CLL cells	none	1	↑ leukemia-specific T cells; ↑ IFN-γ and IL-12	2/11 PD ; 9/11 ↓ WBC count and LN	4 wks	[354]
Progressive phase		17	Xcellerate T cells	none	1	↑T cell counts	↓ LN in 11/14	12 wks	[349]

PR: partial response; SD: stable disease; PD: progressive disease; R: response; WBC: white blood cells; LN: lymph nodes; mo: months; wks: weeks.

[a] T-cell proliferation.

[b] IFN-γ and granzyme B ELISpot assays.

[c] >25% decrease in WBC count.

5 AIMS OF THE THESIS

The general aim of this thesis was to develop immunotherapy for chronic lymphocytic leukemia by the identification of new potential immunotherapy targets and the generation of a cellular vaccine.

More specifically, the aims of the thesis were the following:

1. to evaluate the occurrence of hTERT-specific T cells in CLL patients, to investigate the possibility to expand these T cells *in vitro* by stimulation with DC pulsed with the hTERT (611-626) peptide and verify that the expanded T cells could mediate lysis of autologous tumor cells *in vitro*.

2. to analyze hTERT expression pattern in CLL patients and to investigate possible correlations with clinical characteristics and IgHV mutation status in order to identify a new potential immunotherapy target.

3. to study whether it was feasible to produce an autologous DC-based vaccine from CLL patients and to evaluate the safety and efficacy of this vaccine in a clinical trial.

6 RESULTS AND DISCUSSION

6.1 TELOMERASE AS POTENTIAL TUMOR ANTIGEN IN CLL

The replicative potential of eukaryotic cells is regulated through specialized DNA structures called telomeres which cap the ends of the chromosomes. Telomere length is maintained through the activity of the telomerase enzyme, a ribonucleoprotein complex made of two essential components: a catalytic subunit with reverse transcriptase activity (hTERT) and a RNA subunit (hTR). In normal cells, the activity of the enzyme compensates only to a limited extent for the loss of telomeric repeats occurring at each cell division, driving the cell to an irreversible growth arrest known as ‘‘replicative senescence’’. Programmed telomere shortening in human somatic cells can act as a tumor suppressor pathway [357]. Telomerase is therefore a key enzyme in the process of immortalization of cancer cells and has a pivotal role in carcinogenesis [358, 359]. High telomerase activity has been described in 85% of human cancers [360, 361] and hTERT expression was found to correlate with poor clinical outcome in most cancer types including hematological malignancies [362, 363].

Hence, hTERT could be an attractive universal target for anti-cancer immunotherapy [364], on condition that CTL precursors recognizing telomerase peptides in cancer patients can be expanded through immunization.

Accumulating evidence that peptides derived from hTERT are specifically recognized by $CD8^+$ cytotoxic T lymphocytes came from several studies in which a number of HLA-class I and class II epitopes were characterized [365-372]. All these epitopes were HLA-A2 or HLA-A3 restricted.

Induction of a specific CTL response by one of these peptides (hTERT 540–548) was also studied *in vivo* in two clinical trials [373, 374] and proved to be safe. Moreover, telomerase-specific $CD4^+$ and $CD8^+$ T-cell responses were also induced upon vaccination with DCs transfected with hTERT mRNA [375].

6.1.1 hTERT 611-626 as TAA in CLL (Paper I)

hTERT (611–626) (GV1001) is a promiscuous HLA class II epitope, corresponding to a sequence of hTERT derived from its active site. It is capable of binding to molecules encoded by multiple alleles of all three loci of HLA class II and can also be further processed into CTL epitopes [376]. These characteristics of the hTERT (611-626) peptide might virtually enable all patients, irrespective of HLA type, to present one or more immunogenic epitopes to effector cells. Even though most tumors do not express HLA class II molecules on their surface, $CD4^+$ T lymphocytes are pivotal in generating optimal activation of $CD8^+$ T cells and in the maintenance of immune memory, and therefore their activation is critical for cancer vaccine efficacy [377].

Vaccination with the telomerase peptide GV1001 has recently shown to induce T-cell responses in patients with non-small cell lung cancer [376] and pancreatic cancer [378] vaccinated in two clinical trials. In one of these studies T-cell clones were generated from patients, which could lyse HLA-compatible hTERT-positive target cells [376].

Our study is the first investigating the existence of hTERT-specific immunity in CLL patients. We first analyzed hTERT expression at the mRNA level in 25 CLL patients by Reverse transcriptase polymerase chain reaction (RT-PCR). hTERT expression was detected in 19 of 25 (76%) patients. We then selected seven hTERT-positive and three hTERT-negative patients for the T-cell studies.

We generated DCs by culturing $CD14^+$ cells with GM-CSF and IL-4 for 5 days; on day 4 DCs were pulsed with either the hTERT (611-626) peptide (10 μg/mL) or with a 17-amino acid-long mutated Ras peptide (10 μg/mL) used as an irrelevat control. On day 5 TNF-α and poly I:C were added to DCs and the cells were further cultured for two more days. Peptide-loaded DCs obtained on day 7 were then used to stimulate fresh autologous T cells in a 5 days proliferation assay. The proliferative response of T cells (stimulation index, SI) from the seven hTERT-positive CLL patients stimulated once with peptide-loaded DC was 33.9±15.4 (mean±SEM) against hTERT and 13.2±5.6 (mean±SEM) against the irrelevant peptide Ras. The difference was statistically significant ($p=0.05$). The corresponding figures for the three hTERT-negative patients were 5.3±5.3 and 10.3±6.5 (not significant, n.s.), and for three healthy control donors 5.4±0.9 and 4.5±1. The hTERT-specific proliferative response could be inhibited by both HLA class I and II mAb. Percent inhibition was 52.2±6.95% (mean±SEM) for HLA class I mAb and 42.5±12.85% for HLA class II mAb. Inhibition with the irrelevant isotype-matched mAb was 13.3±3.95%.

Peptide-pulsed DCs were also used to stimulate T cells three times (on days 0, 7 and 14) and on day 21 T cells were utilized as cytotoxic effectors against autologous CLL cells. In six of seven hTERT-positive CLL patients, CTLs could be generated against the autologous leukemic cells, while this was not seen in T cells from three hTERT-negative patients. In the hTERT-positive patients, lysis at the highest E:T ratio was 49.75±9.3% (mean±SEM) of hTERT stimulated T cells as compared to 13.1±2.9% for Ras-stimulated T cells ($p<0.05$). In the three hTERT-negative patients, hTERT-expanded T cells mediated 3.5±1.8% lysis of CLL targets compared to 2.1±1.2% with the Ras peptide (n.s.). Cytotoxic activity of hTERT-expanded T cells could be blocked by mAb against HLA class I, but not HLA class II. Percent inhibition was 53.5±1% and 7.0±1.7%, respectively (n=4). Inhibition in the presence of the irrelevant isotype control antibody was 17±2.1%.

This is the first report demonstrating that CLL patients with hTERT-expressing leukemic cells spontaneously have naturally occurring, hTERT-specific T cells that can be expanded *in vitro* and used to lyse autologous tumor cells. Furthermore, hTERT-positive CLL patients also exhibited hTERT-specific HLA class I and class II-restricted T cells with a proliferative capability, which could not be detected in either CLL patients with hTERT-negative leukemic cells or healthy control donors.

In conclusion, these results indicate that the hTERT 611–626 peptide might be a suitable vaccine candidate in CLL. Whether vaccination of CLL patients with the hTERT (611-626) peptide is able to elicit immune responses *in vivo* will be investigated in a future clinical trial.

6.1.2 hTERT expression pattern in CLL (Paper II)

Several studies have investigated telomerase activity in CLL and an association of high telomerase activity with progressive disease [379] and with an unmutated IgHV profile [26] has been found. Concordantly, telomere length has been shown to be an independent predictor of survival and need for therapy in CLL [380] and a general telomere dysfunction was indicated by studies showing aberrant telomere structure [381] as well as altered expression of telomere maintenance genes [382].

It is well established that telomerase activity correlates with expression of hTERT [383-385], which is the rate-limiting component of the telomerase complex.

Previous studies investigating hTERT expression in CLL by RT-PCR-based assays used primers designed either to the region upstream [386] or downstream [382] of two main splicing sites named α and β. Such splicing sites cause a 36-bp deletion (α) within the conserved reverse transcriptase motif A [387, 388] and a 182-bp deletion (β) leading to a nonsense mutation [387, 388], respectively. Only the full-length transcript (α+β+, FL) containing the A and B reverse transcriptase motifs, translates into a functional protein [387-389]. The quantitative real-time PCR (qPCR) assays used in the above mentioned studies do not discriminate between the deletion variants. In another study, Terrin et al. [390] developed a qPCR assay to quantify the amount of hTERT functional transcript (hTERT-FL) and of all the transcripts (hTERT-AT) together. In this study hTERT expression was assessed by this assay in 134 CLL cases and its prognostic value was evaluated in relation with other known prognostic markers, such as IgHV mutation status, CD38 and ZAP-70 expression. The authors observed that the cumulative expression of all hTERT transcripts was higher in patients with unmutated IgHV genes.

The existence of multiple transcripts suggests that telomerase activity may be regulated by transcriptional control mechanisms as well as alternative splicing of hTERT [387, 391-393]. We therefore performed this study to analyze the full hTERT expression pattern including both the functional and the deletion variants in different disease phases of CLL. In order to completely characterize hTERT splice variants we designed a new qPCR assay which allowed discriminating between the following variants: full-length (FL, α+β+), with α deletion (del-α, α-β+), with β deletion (del-β, α+β-) or with both α and β deletions (del-αβ, α-β-).

We confirmed the previous observation [390] that the functional transcript of hTERT FL (α+β+) was expressed at higher levels in patients with unmutated than with mutated IgHV genes ($1.68x10^{-03}$ vs $3.36x10^{-04}$, p=0.0004). FL levels directly correlated with the percentage of IgHV homology (r=0.36, p= 0.005). Moreover, we found that the expression of FL is higher in progressive patients compared to non-progressive patients ($1.55x10^{-03}$ vs $3.36x10^{-04}$, p<0.0001) and healthy controls ($2.04x10^{-04}$, p=0.03). Subsequently, we analyzed IgHV mutated and unmutated patients separately. Notably, the difference between progressive and non-progressive patients was found only in the unmutated subgroup ($2.19x10^{-03}$ and $4.85x10^{-04}$, respectively), whereas in patients with mutated CLL no difference could be identified ($2.61x10^{-04}$ and $3.51x10^{-04}$, in progressive and non-progressive patients, respectively). These observations highlight the necessity of subgrouping patients according to IgHV mutation status when analyzing hTERT expression. Indeed, the higher expression of the functional transcript found in the

progressive patient population in previous studies might result from a higher frequency of unmutated patients in the progressive disease subgroup at the time of testing. On the other hand, an imbalance in the proportions of IgHV mutated vs unmutated cases in the population studied could as well contribute to underestimating hTERT expression levels. In a recent paper, Poncet et al [382] report a lower expression of the telomerase enzyme complex genes (hTERT, hTR, DYSKERIN) and of the genes of the sheltering complex (TRF1, hRAP1, POT1) in CLL patients compared to healthy donors. Of the 42 tested patients analyzed in this study only 10 had unmutated IgHV genes. Moreover, in this study as well, the qPCR assay used for hTERT did not discriminate between the deletion variants.

Additionally, we observered that the del-α (α-β+) and the del-β (α+β-) transcripts were expressed at higher levels in the unmutated patients than mutated patients ($2.77x10^{-03}$ vs $4.45x10^{-04}$, p=0.0004 and $8.78x10^{-04}$ vs $6.92x10^{-04}$, p=0.002, respectively), which suggests a general upregulation of hTERT transcription in unmutated patients. No difference between mutated and unmutated patients was noted for the del-αβ (α-β-) splice variant. Furthermore, expression of all the transcripts was higher in progressive compared to non-progressive patients. Mean expression levels for del-α were $2.5x10^{-03}$ vs $5.56x10^{-04}$ (p=0.0003), for del-β $4.89x10^{-04}$ vs $4.2x10^{-04}$ (p= 0.01) and for del-αβ $1.43x10^{-03}$ vs $2.16x10^{-03}$ (p<0.0001). In contrast to FL expression, no difference in expression of the other transcripts could be observed between progressive and non-progressive patients, when mutated and unmutated patients were analyzed separately, which suggests that alterations in the expression levels of these transcripts do not directly play a role in disease progression.

Cumulatively, the levels of expression of the full-length transcript FL significantly correlated with those of the other splice variants del-α (r=0.52, p<0.0001), del-β (r=0.50, p<0.0001), del-αβ (r=0.47, p<0.0001). Since we did not assess the percentage of expression of each splice variant, we are not able to establish which one had the greatest affect on the levels of all transcripts together (hTERT-AT). We therefore consider that it would be more appropriate to evaluate hTERT levels by an assay detecting its functional transcript only.

Finally, we tested two patients repeatedly in different disease phases. Both patients had mutated IgHV genes. We found an increase in the expression of the functional transcript at disease progression, while the del-α transcript expression levels decreased. The del-α variant has been shown to act as dominant negative modulator of telomerase activity [394, 395]. The higher FL levels and lower del-α levels we noted following disease progression suggest an ongoing process favoring the expression of the functional transcript and abating of the dominant negative modulator variant del-α during progression of CLL.

In conclusion, this first study analyzing hTERT mRNA splicing patterns in CLL highlights the necessity of focusing on the functional transcript when analyzing hTERT expression in CLL patients and of interpreting the results in various phases of the disease in the light of the IgHV mutational status. The function and biological role of hTERT splice variants is still to be clarified. Predominant splice variants patterns identified in tumors compared to normal cells could be used as diagnostic or prognostic biomarkers [396] or possibly as targets for therapy. Cancer-specific splicing events may generate novel epitopes that may serve as immunotherapy targets [397]. Since the correlation between hTERT full-length transcript expression and telomerase activity indicates that the hTERT-FL mRNA is

translated in leukemic cells, it may potentially give rise to tumor-specific Ags that may be targeted with immunotherapy.

6.2 A DC-BASED VACCINE FOR TREATMENT OF CLL PATIENTS

In CLL there is no established therapy to prevent or delay progression in the indolent phase of the disease or as maintenance treatment of patients in the response/plateau phase following chemotherapy; hence there is a great need to develop such therapies. Several lines of evidence indicate that immunotherapy could be a valuable therapeutic approach for CLL. In particular, non-progressive CLL presents an optimum disease setting for testing active immunotherapy approaches. The tumor burden is low and the slowly progressing disease may allow sufficient time for the induction of an effective immune response, which is typically deferred after initiation of vaccine therapy. Moreover, immune functions are better preserved in this setting than during the progressive phase or following initiation of immunosuppressive anti-tumor therapy. A number of observations, ranging from the presence of CLL-reactive autologous and allogeneic T cells ([293-297] and **Paper I**) to the GVL effect after hematopoietic stem cells transplant or donor lymphocyte infusion [288-291] indicate that CLL is responsive to immune effector functions.

Use of autologous tumor cells, rather than a single, defined protein or peptide as the immunogen provides the benefit of potentially presenting the entire repertoire of TAAs to the patient's immune system. Nevertheless CLL cells are poor APCs and for vaccination purposes they must be rendered more immunogenic. One option is to use powerful APCs, such as DCs.

Ex vivo matured DCs loaded with Ags express the whole array of co-stimulatory and adhesion molecules required for the activation of the innate and adaptive immune system to induce tumor-specific $CD4^+$ and $CD8^+$ T cells [255]. In previous studies [342, 343], a direct comparison of CLL tumor lysate, apoptotic bodies, tumor RNA and fusion hybrids, revealed that apoptotic bodies were the best approach to loading whole CLL tumor Ags into DC. We therefore selected this strategy, DC loaded with apoptotic tumor cells (Apo-DC), as a vaccine for an explorative clinical trial.

6.2.1 Generation of the Apo-DC vaccine (Papers III and IV)

To the purpose of generating DCs, monocytes collected from PB by apheresis can be cultured for 3-5 days in the presence of GM-CSF and IL-4 and then further matured by 1-2 days of additional culture with maturation stimuli such as TNF-α. The procedure is well established and it is used in the majority of DC vaccination studies nowadays. However, in CLL the production of a DC-based vaccine has practical constraints as the frequency of monocytes in the PB is very low amongst a huge number of leukemic cells. Moreover, *ex vivo* generation of monocyte-derived DC for clinical use has to be performed under GMP conditions. For enrichment of monocytes from PB cells discontinuous density gradients cannot be utilized because they can rarely be performed in a completely closed system. An alternative approach is counterflow centrifugal elutriation [398], a method which does not require a density gradient pre-enrichment step and can be performed in a closed system. For clinical applications, the elutriation technology has been utilized by us and others using the ELUTRA® platform. In a previous study by our group [399] a minimum

of 4% $CD14^+$ cells in the starting material was needed to achieve a satisfactory enrichment of monocyte precursors from cancer patients. This constraint of 4% monocyte in the starting material is a major limitation of the elutriation technology when dealing with samples of CLL patients.

We later showed in another study (**Related publication B**) that adequate numbers of functional DCs required for clinical therapy could be generated from CLL patients who have > 1% of $CD14^+$ monocytes in the leukapheresis product by using the CliniMACS® system. This procedure permitted to produce 5 or more doses of vaccine, each consisting of a minimum of $1x10^7$ cells, from a single leukapheresis product.

One of the primary objectives of the trial was to determine the feasibility of generating the Apo-DC vaccine from CLL patients. Mean lymphocyte count of the 16 patients at start of vaccination was $34.8x10^9$/L (range 3.6-$96.1x10^9$/L). At leukapheresis, the percentage of monocytes in the leukapheresis products (n=16) ranged from a minimum of 0.6% to a maximum of 3.3% (mean 1.6%). Percentages of monocytes, lymphocytes and granulocytes were comparable in PB and the leukapheresis product. After leukapheresis, $CD14^+$ and $CD19^+$ cells were isolated by immunomagnetic separation using the CliniMACS® affinity-based technology. In patients with ≤ 2% monocytes in the leukapheresis product, up to three separate CD14 selection columns and kits have been utilized to obtain adequate number of monocytes. For all the 16 patients accrued, $CD14^+$ selection resulted in a highly enriched cell population 93±1.7 % (mean ±SEM) (n=16).

In **Paper III** detailed specifications of the Apo-DC vaccines produced from the first 10 patients accrued in the trial are reported. Following positive selection with the Clini-MACS beads, the purity of $CD14^+$ cells was 91±2.2% (mean ± SEM) with contaminating lymphocytes and granulocytes decreasing to 3±0.6% and 4±1.3% (mean ± SEM), respectively. Monocytes recovered in the $CD14^+$ fraction were calculated as percentage yield relative to the total number in the initial leukapheresis product. Monocyte yield in the $CD14^+$ fraction was 48±5% (mean ± SEM) determined by $CD14^+/CD45^+$ positivity and 51±5.4% determined by $CD45^+$ cells versus side scatter (SSC).

For the first 5 patients, a single selection for $CD19^+$ cells was performed with the CliniMACS® system, which increased the purity of $CD19^+$ cells used for generating apoptotic bodies from 87±3.1% (mean ± SEM) in the leukapheresis product to 100±0.04% post-selection. For the remaining eleven patients the selection step was omitted as high percentage of $CD19^+$ cells in the initial leukapheresis product made further enrichment superfluous. The mean number of vaccine ampules produced for the 16 patients was 10 (range 5-23).

Monocyte viability was 97% immediately following CliniMACS® selection. The viability of mature Apo-DC was 98±0.5% (mean ± SEM) after 7 days of culture. Viability and recovery of Apo-DC after thawing and immediately prior to vaccination were 96±0.6% and 96±4.7% (mean ± SEM), respectively. Five Gy irradiation followed by overnight culture resulted in apoptosis induction in 80±4.7% (mean ± SEM) of the $CD19^+$ cells. Following coculture, 84±4.1% of the immature DCs had endocytosed apoptotic bodies (**Paper III**). For the remaining 6 patients in the third cohort, comparable values to those reported for the first two cohorts were found.

The Apo-DC phenotype after 7 days of culture was assessed by flow cytometric analysis. For all the 16 vaccine preparations (**Paper IV**) the Apo-DC showed a mature DC phenotype with the expression of CD80 (94.5±2%), CD86 (91.9±1.9%), CD83 (71.5±4.9%), DC-SIGN (87.1±4.3%) and CD1a (44±6.7%). The expression of other markers was as follows: ILT-3 (31.1±5.5%), HLA-DR (94.8±1.7%), CD14/CD45 (19.6±6.9%), CD20 (1±0.2%) and CCR-7 (18±6%) (Figure 7).

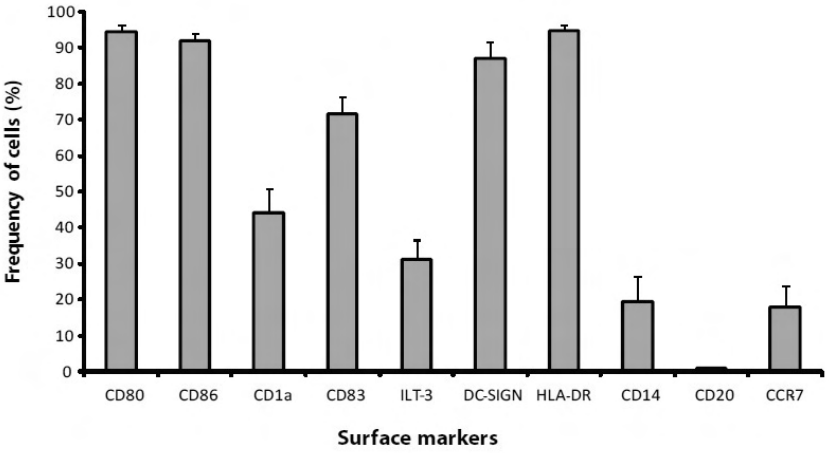

Fig. 7. Surface staining (mean ± SEM) of 7-day cultured Apo-DC of 16 CLL patients.

Cell viability and recovery of thawed Apo-DC was 96±0.2% and 98±4.7% (mean ± SEM) (n=101) respectively, with no significant differences beween the three cohorts. Vaccine production for the patient treated out of protocol (I-06) is not reported, but detailed specifications were comparable to those of the other patients.

Finally, for the first 10 patients (**Paper III**) we analyzed supernatants harvested from Apo-DC cultures on day 7 for secreted cytokines by multiplex bead array technology. High levels of IL-8 were noted in all patients. IL-6 was also secreted at high levels by virtually all patients. IL-12p40 was produced in 100–400 pg/ml range. The other cytokines (IL-2, IL-10, IL-12p70, IL-15, IL-17, IFN-γ, soluble CD40L) were detected in very low concentrations.

Production of the vaccine from a single leukapheresis product has at least three significant advantages: 1) discomfort to the patients is reduced since they are subjected to only a single apheresis procedure; 2): batch-to batch variations are eliminated; 3) the monocyte enrichment procedure, which is labor, time and cost intensive, has to be performed only once.

The use of multiple immunomagnetic columns increased the efficiency of the process, considered that a limitation of the immunomagnetic enrichment method we utilized is the number of cells from the starting leukapheresis product that can be loaded onto the $CD14^+$ CliniMACS® column (maximum loading capacity of $4x10^9$ $CD14^+$ with total a $20x10^9$ leukocytes). To avoid overloading of the system, we divided the initial leukapheresis products into three fractions loaded on separate columns.

Vaccination of CLL patients with autologous DCs has been investigated by another group of investigators. In this study, monocyte precursors were enriched using two different methods. For the first two patients, precursor monocytes were enriched from a leukapheresis product by plastic adherence. For the other 10 patients, precursor monocytes were immunomagnetically enriched from 150 ml of PB. The plastic adherence method yielded enough precursor monocytes only for a single vaccine dose from a leukapheresis product. The problem was partly ameliorated through immunomagnetic enrichment using the MACS system, but even then the patient had to be repeatedly phlebotomized for the 150 ml of PB required per vaccine dose and multiple vaccine batches were produced, which implies variability.

Figdor et al. proposed a list of quality criteria for DC vaccines, which include quality control of the individual steps in the production of the vaccine and proper product specification and documentation. Comprehensive information with regard to functional and phenotypic characteristics is in fact indispensible for accurate interpretation of safety and efficacy results following vaccination. In **Paper III** we provided detailed documentation of a number of phenotypic and functional characteristics of the Apo-DC vaccine, including frequency of endocytosis of the apoptotic bodies and secretion of cytokines by the Apo-DC following maturation. The cell viability and purity of the final Apo-DC product were ≥97% and 80%, respectively, higher than minimum acceptability criteria [262].

In conclusion, using a combination of leukapheresis and affinity-based technologies (CliniMACS®) for monocyte enrichment, we obtained a large number of clinical-grade, highly purified monocytes and were able to produce a sufficient amount of DC vaccine that met accepted and established quality criteria. The procedure was highly reproducible despite considerable variability in the starting material from different patients.

6.2.2 Vaccine administration and safety (Paper IV)

In this clinical trial the patients were accrued in three consecutive cohorts, each consisting of 5 patients. Cohort I received the Apo-DC vaccine alone as an intradermal injection at weeks 0, 2, 4, 6 and 14. At each vaccination a minimum of 10^7 viable Apo-DC were administered. Cohort II received the vaccine as above together with 75 µg/day of GM-CSF subcutaneously, for four consecutive days starting the same day as vaccine and administered at the same site as the vaccine [400].

Cohort III was treated according to the same schedule as cohort II but with the addition of CTX 300 mg/m^2 i.v. at day -2 at week 0, 6 and 14. One patient in cohort III received only four vaccine injections due withdrawal from the study and was replaced.

The mean number of Apo-DC administered at each vaccination was $13\pm0.3x10^6$ (n=25) in cohort I, $18\pm0.4x10^6$ (n=26) in cohort II and $17\pm0.9x10^6$ (n=28) in cohort III. The median total number of Apo-DC received in cohort I was $67x10^6$ cells (range: 35-89) (n=5); cohort II $88x10^6$ (range: 76-120) (n=5) and $83x10^6$ (range: 57-97) in cohort III (n=6).

As above mentioned, for the majority of the patients, the number of vaccine doses produced far exceed the five required as for protocol. Therefore, starting at week 52, the patients who had not progressed received additional immunizations every fourth weeks as

long as vaccine was available for a maximum of 1 year ("maintenance vaccination"). Five patients received maintenance vaccination, two in cohort II and three in cohort III. During maintenance vaccination the mean number of Apo-DC administered at each vaccination was $15\pm0.6x10^6$ (n=22) and mean total number of Apo-DC administered was $65\pm12x10^6$ (n=5).

Finally, one additional patient (I-06) was initially included but then treated outside the protocol due to hepatitis C positivity. The patient received Apo-DC alone at week 0, 2, 4 and 6 and then every 4 weeks up to week 22; thereafter Apo-DC + GM-CSF every 4 weeks to week 52 (totally 15 vaccinations), receiving a total of $195x10^6$ Apo-DC.

Adverse effects noted in the study were minimal and in cohort I, only one patient had a mild (grade 1) injection site reaction at the time of the first vaccination. In cohort II and III, injection site reactions occurred in all patients following GM-CSF administration. All reactions were grade 2 (moderate). Swelling and erythema was seen in all patients, pruritus in 8/11 and local pain in 2/11 patients. All reactions were transient and resolved within 48 hours. One patient had grade 2 fevers and grade 1 chills after the first vaccination. The dose of GM-CSF was reduced to one third and at the second vaccination only grade 1 fever occurred, but not subsequently. No cumulative toxicities were observed during the induction phase.

The five patients who received maintenance vaccination had more intense injection site reaction, but not greater than grade 2. No significant renal, hepatic, gastrointestinal, cardiac, hematologic or neurologic toxicities were considered attributable to the treatment were observed.

6.2.3 Immunological responses and clinical effects (Paper IV)

The patients were evaluated for clinical effects and immune response at weeks 8, 16, 28, 40 and 52. All patients had antileukemic immune reactivity before vaccination and 10/15 patients mounted a vaccine-induced immune response: 2/5 in cohort I, 3/5 in cohort II and 5/5 in cohort III.

Higher concentrations of IL-2, IL-5, IL-10, IFN-γ, GM-CSF in the proliferation assay supernatants were noted in immune responders while IL-4 and TNF-α showed higher values in non-immune responders. Significantly higher through values (all cytokine secretion values over time) were seen in immune responders for IL-2 and IFN-γ ($p=0.003$ and 0.0009, respectively), while marginally significant values for TNF-α were observed in the non-responders group.

No statistically significant difference between the three cohorts was seen in T_{regs} frequency at baseline, or during the 52 weeks follow-up period was noted. However, significantly lower levels of T_{regs} during the first year of follow-up ($p<0.0001$) were noted in immune responders compared to non-immune responders. T_{regs} levels correlated inversely to the proliferative response ($p<0.0001$). We also tested the effect of CTX administration on T_{regs} levels. T_{regs} were analyzed before CTX administration and 10 days later on nine occasions. At 7/9 testing times the levels remained stable and increased in the remaining two.

$CD8^+$ and $CD4^+$ cells degranulation (CD107 positivity) following incubation with autologous leukemic targets could be detected 11/11 pts in whom the test was performed at

≥ 2 timepoints. An increased frequency of $CD8^+CD107^+$, $CD4^+CD107^+$ and $CD16^+CD56^{dim}CD107^+$ cells after vaccination was detected in all the patients (n=7) in whom baseline values were available. In all patients a double positive $CD3^+CD19^+$ cell population was noted in all *in vitro* tests at a E/T cell ratio of 1:2. This population showed a high frequency of degranulating T cells, indicating that it may represent T/B cell aggregates, including T cells with a cytotoxic capability. The presence of such aggregates and destruction of the leukemic targets was visually confirmed by confocal microscopy.

No patient (except Pt I-06) fulfilled the iwCLL criteria for response [88]. Eleven out of 15 patients (73%) had a stable disease for 7 months and the majority remained stable for quite a longer time, which is in line with a previous report [351]. Among the 10 patients who showed a vaccine-induced immune response, 8/9 evaluable patients (88%) were clinically stable at the week 28 follow-up. In contrast, 3/6 patients who did not develop a vaccine-induced immune response had an early clinical progression (at week 8, 16 and 16, respectively). The median TTP in immune responders and immune non-responders was 14 months and 12 months, respectively (n.s.).

Patient I-06 experienced a gradual reduction of the lymphocyte count during the vaccination period, culminating in a CR in blood one year after the last vaccination. A BM aspirate performed 34 months after last vaccination showed 30% lymphocytes with remaining CLL cells (nodular PR). It seems unlikely that the concomitant HCV infection could have contributed to the anti-tumor response. Indeed, the viral load was unchanged during the 4-year follow-up, indicating that the patient did not mount an antiviral immune response. On the other hand, the patient, who did not show a tumor-specific immune response at baseline, developed a vaccine-induced proliferative response from week 8 onward throughout the whole vaccination period, continuing as far as 7 months after the last vaccination. The frequency of T_{regs} in this patient was very low during the whole follow-up period. The clinical response in patient I-06 may suggest that long-term vaccination may be a promising strategy, as also partly suggested by the results of Spaner [351]. Further insights on this issue will hopefully come from long-term follow-up of patients who received maintenance vaccination. Up to now, of the 5 patients who received maintenance vaccination, only 3 are evaluable and 2 of them developed an immune response.

In conclusion, our results demonstrate that vaccination with autologous DC loaded with apoptotic CLL cells is a feasible approach that can generate immune responses and potentially clinical responses. Whether this may indicate that the vaccine may delay disease progression can only be established in randomized trials. Disease stabilization with minimal toxicity would be an important therapeutic goal in CLL, as no maintenance therapy exists.

In two previous studied performed by the same group of investigators, CLL patients were vaccinated either with allogeneic DC loaded with tumor lysates or apoptotic bodies (n=9) [352] or autologous DC pulsed with tumor lysates (n=12) [353]. In these studies, no vaccine adjuvants were used. CLL-specific responses were detected and there was some minor clinical effects, but no partial responses were observed.

In our study, we also showed that adjuvant GM-CSF and low-dose CTX seem to enhance the frequency of immune responses. Indeed, the observations that all patients in

the third cohort (receiving Apo-DC + GM-CSF + CTX) mounted a CLL-specific T cell response and that T_{regs} levels were significantly lower in immune-responding patients are indirect indications that both adjuvant strategies concur to enhance immune responses. Notwithstanding the observation that CTX did not reduce the number of blood T_{regs}, which was also shown in another study [271], it should be considered that CTX could mediate an immune enhancing effect through other mechanisms than by reducing T_{regs} numbers. Indeed, chemotherapy-induced lymphopenia allows homeostasis-driven expansion of T cells, which could also play a role in potentiating specific immune responses [401]. Further studies are planned to better define the most suitable patient population, optimal vaccination schedule and best combination with immune enhancing/modulating drugs.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

The identification of new TAAs can substantially contribute to implement and refine immune therapy to cancer. The possibility to specifically target antigens on cancer cells without harming normal cells is in fact probably the main appeal of this therapeutic approach. The data presented in this thesis identify telomerase as a vaccine candidate in CLL. To investigate natural immunity against CLL we employed a promiscuous HLA class II epitope, hTERT (611–626) (GV1001) and showed that CLL patients with hTERT-expressing leukemic cells have naturally occurring hTERT-specific T cells. This study provides the rationale for a clinical trial to investigate whether immune responses can be elicited in CLL patients *in vivo* by vaccination with hTERT (611-626).

Furthermore, the analysis of hTERT mRNA splicing patterns by a newly designed quantitative PCR assay showed that the expression of the functional transcript of hTERT is independent from disease phase in IgHV mutated but not in unmutated patients, therefore highlighting the necessity of focusing on this transcript when analyzing hTERT expression and of interpreting the results in the light of the IgHV mutational status. These results encourage further studies to establish whether hTERT-FL mRNA may give rise to TAAs to be targeted with immunotherapy.

In this thesis, our clinical trial experience with the Apo-DC vaccine is also reported. By using a combination of leukapheresis and affinity-based technologies (CliniMACS®) for monocyte enrichment, we managed to produce sufficient amounts of the Apo-DC vaccine for vaccination of a total 17 CLL patients. We showed in a proof-of-principle/phase I clinical trial that the vaccine was well tolerated and increased leukemia-specific immunity in 66% of the patients. This is the first vaccination study in CLL which also investigated the effects of two adjuvants, GM-CSF and low-dose cyclophosphamide (CTX). At present, the elevated T_{regs} numbers associated with cancer is probably the best characterized immune suppressive mechanism and therapeutical interventions aiming at the selective elimination of T_{regs} need to be explored in clinical trials.

The trial design permitted to assess the additive effect of each adjuvant on the vaccine by direct comparison of three cohorts of patients. Even though the small number of patients warrants caution in the interpretation of the results, our data point out that both GM-CSF and low-dose CTX function as immunological adjuvants. Still, the observation that CTX did not directly reduce T_{regs} frequency suggests further investigation on other immune modulating mechanisms influenced by CTX.

In conclusion, we envision that vaccination, either with TAA-derived peptides or with tumor-cell loaded DCs, could be a valuable therapeutic strategy for CLL patients with slowly progressive disease or in the maintenance phase following chemotherapy. In this setting, in fact, disease stabilization with minimal toxicity would be an important therapeutic goal. Long-term vaccination may be a promising strategy, especially if associated with immunomodulating strategies to boost immune effector functions or to reduce T_{regs} numbers. To this regard, other approaches then CTX could be worth investigating, such as combination with CTLA-4 blocking Abs, which seem to directly

enhance T cells effector functions [402], or with lenalidomide, which was shown to reduce T_{regs} [273], in addition to stimulating $CD4^+$ and $CD8^+$ T cells.

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