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# T Cells in Patients with B-cell Chronic Lymphocytic Leukemia (B-CLL) and Multiple Myeloma (MM)

## An Immunological Study

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*To the people who taught me,*
*and to my family.*

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# ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| Allo-SCT | Allogeneic Stem Cell Transplantation |
| APC | Antigen Presenting Cell |
| APRIL | A Proliferation Inducing Ligand |
| ASCT | Autologous Stem Cell Transplantation |
| ATM | Ataxia Telangiectasia Mutated |
| β2m | β2 Microglobulin |
| BAFF | B cell-activating factor of the tumor necrosis factor family |
| BCR | B Cell Receptor |
| BM | Bone Marrow |
| CD | Cluster of Differentiation |
| CDR | Complementarity Determining Region |
| CLL (B-CLL) | Chronic Lymphocytic Leukemia |
| CMV | Cytomegalovirus |
| CR | Complete Response |
| CTL | Cytotoxic T Lymphocyte |
| CTLA-4 | Cytotoxic T Lymphocyte Antigen 4 |
| DAG | Diacylglycerol |
| DC | Dendritic Cell |
| EBV | Epstein-Barr Virus |
| Erk | Extracellular-regulated Kinase |
| FACS | Fluorescence-Activated Cell Sorting |
| FC | Fludarabine + Cyclophosphamide |
| FCR | Fludarabine + Cyclophosphamide + Rituximab |
| FDC | Follicular DC |
| FluCam | Fludarabine + alemtuzumab |
| HDT | High-Dose Therapy |
| HGF | Hepatocyte Growth Factor |
| HRD | Hyperdiploid myeloma |
| HSV | Herpes Simplex Virus |
| GC | Germinal Center |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IGF | Insulin-like Growth Factor |
| IL | Interleukin |
| IP3 | Inositol Triphosphate |
| iNOS | Inducible Nitric Oxide |

| | |
|---|---|
| ITAM | Immunoreceptor Tyrosine-based Activation Motif |
| KLF6 | Krupple-Like Factor 6 |
| LAT | Linker of Activation in T cells |
| LDH | Lactate Dehydrogenase |
| LDT | Lymphocyte Doubling Time |
| mAb | Monoclonal Ab |
| MFI | Mean Fluorescent Intensity |
| MGUS | Monoclonal Gammopathy of Undetermined Significance |
| MHC | Major Histocompatibility Antigen |
| MIP | Macrophage Inflammatory Protein |
| MM | Multiple Myeloma |
| NFκB | Nuclear Transcription Factor κB |
| NHRD | Non-hyperdiploid |
| NK | Natural Killer |
| NKT | Natural Killer T |
| NLC | Nurse-Like Cell |
| NO | Nitric Oxide |
| OPG | Osteoprotegerin |
| OS | Overall Survival |
| PBMC | Peripheral Blood Mononuclear Cell |
| PI3-K | Phosphatidyl-Inositol-3 Kinase |
| PIP2 | Phosphatidyl Inositol Bisphosphate |
| PCR | Polymerase Chain Reaction |
| PHA | Phytohemagglutinin |
| PPD | Tuberculin Purified Protein Derivative |
| QRT-PCR | Quantitative Reverse Transcribed-PCR |
| RANKL | Receptor Activator of NFκB Ligand |
| SDF-1 | Stromal Cell Derived Fcator-1 |
| SEB | Staphylococcal Enterotoxin B |
| Tc | Cytotoxic T Cell |
| TCL-1 | T Cell Leukemia-1 |
| TCR | T Cell Receptor |
| Th | Helper T Cell |
| TGF-β | Transforming Growth Factor-β |
| TLR | Toll-Like Receptor |
| T-PLL | T-cell Prolymphocytic Leukemia |
| TNF | Tumor Necrosis Factor |
| TRAF | TNF Receptor Associated Factor |
| VEGF | Vascular Endothelial Growth Factor |
| $V_H$ | Variable of Heavy Chain |
| ZAP-70 | ζ-associated protein 70 |

# ABSTRACT

There are several lines of evidence that T cells in patients with Multiple Myeloma (MM) and B-cell Chronic Lymphocytic Leukemia (B-CLL) are phenotypically aberrant. The overall aim of this thesis was to study T cells with a focus on TCR-signaling pathways in these two B-cell malignancies. The role of T-cells in B-CLL etiology was also examined.

In the first study the expression of the TCRαβ, CD28, CD152, CD154 and the signal transduction molecules CD3ζ, Lck, Fyn, ZAP-70 and PI3-kinase was studied. In addition, the cytokines IFN-γ, IL-4 and IL-2 in unstimulated and superantigen-stimulated T cells of MM patients at different stages of the disease was examined by intracellular staining and flow cytometry. The results of this study demonstrated multiple abnormalities both in freshly isolated T cells and following *in vitro* activation. The CD3ζ-chain, Lck, Fyn and ZAP-70 were all generally downregulated and did not respond normally to a TCR-activating signal. The aberrations increased with advancing disease stage and tumor burden.

The second study was undertaken to examine the TCR-signaling components as well as cytokine production in T cells of CLL patients with indolent or progressive disease. The cumulative data of this study suggest that several but not all T cell signaling molecules may be normal or even overexpressed in B-CLL patients in comparison to normal T cells. This observation was especially true in patients with indolent CLL. In addition, the expressions of CD3-ζ chain and ZAP-70, which are key molecules in the initiating of intracellular TCR signaling pathway, as well as IFN-γ and IL-4 were overexpressed to a greater extent in indolent patients than in progressive patients. The overall impression collected from these results suggest that the T cells in B-CLL demonstrate a state of chronic stimulation although this activation does not result in spontaneous anti-leukemic effector activity.

Fludarabine is a purine analogue and alemtuzumab a humanized anti-CD52 monoclonal Ab that are used for treatment of B-CLL. Treatment with either of these agents results in significant reduction of T cells and inhibition of cell mediated immunity. In the next study we investigated the expression of signaling molecules and cytokine production by T-cells of B-CLL patients who were in long-term unmaintained remission/plateau phase following fludarabine or alemtuzumab treatment. T-cell function was assessed after stimulation with the recall antigen, tuberculin purified protein derivative (PPD) or the polyclonal mitogen phytohemagglutinin (PHA). The results of this study demonstrated that T-cell functions might be relatively well preserved long-term after treatment with fludarabine and alemtuzumab.

In the fourth work we have analyzed global gene expression profiles of T cells from the blood of indolent B-CLL patients in an attempt to delineate T cell factors that may potentially influence the malignant B-CLL cells. We have also attempted to identify genes that may contribute to expansion and aberrant functions of T-cells in B-CLL patients. The results of this study demonstrate the expression of a large number (356) of genes that are involved in different cellular pathways and activities including signaling, proliferation control, apoptosis, metabolism, immune response, and cytoskeleton formation are dysregulated. Three genes that demonstrated the greatest upregulation were the chemokines XCL1, XCL2, and the cytokine IFN-γ. CCL4 and CCL5 are two other important chemokines that also was found to be specifically upregulated in T cells of B-CLL patients.

Collectively, our results indicate that T cells of MM and B-CLL patients exhibit a variety of anomalies and aberrations in their phenotype and function, which are exacerbated with progressive disease. The preservation of T cell function observed in patients on long term follow up following treatment with these agents may be due to a small population of noncycling memory T cells that remain relatively unaffected by the treatment. The results of the microarray analysis demonstrate that T-cells in B-CLL potentially produce several factors that may have a supportive and antiapoptotic function on the leukemic clone giving credence to the hypothesis that T cells in B-CLL may contribute to the etiology of the malignancy. The therapeutic potential of agents like fludarabine and alemtuzumab may partially be attributable to their ability towards reducing these abnormal T cells.

**Key words:** T cell, B-cell Chronic Lymphocytic Leukemia, Multiple Myeloma, signaling, gene expression

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# LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text by their Roman numbers.

I. Mozaffari F, Hansson* L, **Kiaii* S**, Ju X, Rossmann ED, Rabbani H, Mellstedt H, Österborg A. Signalling molecules and cytokine production in T cells of multiple myeloma -increased abnormalities with advancing stage. Br J Haematol 2004; 124: 315-24. (*, contributed equally).

II. **Kiaii S**, Choudhury A, Mozaffari F, Kimby E, Österborg A, Mellstedt H. Signaling Molecules and Cytokine Production in T Cells of Patients with B-Cell Chronic Lymphocytic Leukemia (B-CLL): Comparison of Indolent and Progressive Disease. Med Oncol 2005; 22: 291-302.

III. **Kiaii S**, Choudhury A, Mozaffari F, Rezvany R, Lundin J, Mellstedt H, Österborg A. Signaling molecules and cytokine production in T cells of patients with B-cell chronic lymphocytic leukemia: long-term effects of fludarabine and alemtuzumab treatment. Leuk Lymphoma 2006;47: 1229-38.

IV. **Kiaii S**, Österborg A, Mashayekhi K, Moshfegh A, Choudhury A, and Mellstedt H. Gene expression profiling of peripheral blood T cells in patients with indolent B-CLL. (Manuscript)

# 1 THE IMMUNE SYSTEM

The immune system is the integrated body system of organs, tissues, cells, and cell products that is responsible for defending the body from potentially pathogenic organisms or substances. It consists of trillions of cells and is widely dispersed throughout the body. The continuous recirculation of cells facilitates rapid detection of invading pathogens and provides effective countermeasures in various tissue environments. This system consists of two parts: innate and adaptive immune and a continuous interplay between both parts are required.

## 1.1 Innate (non-specific) immunity

The innate system, which forms early barriers to infectious diseases, is comprised of all the mechanisms that defend an organism in non-specific manner. It evolved long before the adaptive system and with a similar defense strategy in plants, invertebrates, and vertebrates [1, 2]. The innate immune mechanisms act immediately, destroys invaders within minutes or hours but do not generate long-lasting protective immunity or memory. Innate immunity depends upon germline-encoded receptors to recognize features that are common to many pathogens, which discriminates very effectively between host (self) cells and pathogens (non-self) [3, 4].

### 1.1.1 Physical barriers

Physical barriers are an organism's first line of defense against infection such as the skin and the mucus membranes. Saliva, tears and mucous secretions (that contain various soluble anti-bacterial agents) act to wash away potential invaders and also contain antibacterial or antiviral substances [3, 4].

### 1.1.2 Physiological barriers

A variety of factors, such as temperature, pH, and oxygen tension are important in this respect. Various soluble factors may also contribute such as lysozymes, interferons, phospholipase A, histatins, digestive enzymes, bile salts, fatty acids, lysolipids, α-defensins, β-defensines, and complement elements as well as normal flora of nonpathogenic bacteria in epithelial surfaces [3, 4].

### 1.1.3 Phagocytes

Phagocytes are neutrophils, monocytes or macrophages that engulf foreign particles or microorganisms. They are originating from common myeloid progenitor cells.

Neutrophils are the most numerous and most important cellular component of the innate immune response: hereditary deficiencies in neutrophils lead to overwhelming bacterial infection [3]. The invading microorganism is transported inside a vacuole that merges with lysosomes, vacuoles rich in enzymes and acids, which digest the particle or organism. Phagocytosis is also an important part of the cleaning process after cellular destruction following infection, tissue trauma, exposure to toxins, or any other process that leads to cellular death. Neutrophils and macrophages are the most abundant cells in areas of inflammation [3, 4].

### 1.1.4 Natural Killer (NK) cells and Natural Killer T (NKT) cells

Natural killer cells or NK cells, which originate from common lymphoid progenitor cells, are distinctive in that NK cells are lymphocytes that attack cells that have been infected by microbes, but not specifically the microbes themselves. NK cells may also display activity against tumor cells. NK cells recognize targets that have "missing self" i.e., display non-self or low levels of self MHC (major histocompatibility complex) class I cell surface marker molecules [3, 4]. NK cells have the phenotype $CD16^+/CD56^+/CD3^-$. NK cells have two types of receptors: one is homologues to c-type lectins and is called the NK receptor complex (NKC). The other is composed with immunoglobulin (Ig)-like domains, which is called killer cell Ig-like receptors (KIRs). Apart from antibody-dependent cell-mediated cytotoxicity (ADCC), NK cells are very important for defense against certain bacteria and parasites [5, 6] because of their ability to produce IFN-γ, which enhances the activity of phagocytic cells, especially in the early phase of infection. NK cells have also been found to be important for the defense against cytomegalovirus (CMV), Epstein-Barr Virus (EBV), and herpes simplex virus (HSV) [7-9]. Importantly, NK cells also play an important role in T cell priming, and thus triggers and shapes the adaptive immune system [10]. During activation of an immune response, NK cells home to the lymph nodes especially in Ag-stimulated draining lymph nodes [10]. This process might be promoted by signaling through Toll-Like Receptors (TLRs) on innate immune cells. Further cross-talk between NK cells, DCs, and T-cells sustain immune responses against pathogens and tumors [11]. In this process, NK cells are an important source of IFN-γ which is necessary for $T_H1$ T cell responses [10].

Some lymphocytes display a mixed phenotype of both NK and T cells ($CD16^+/CD56^+/CD3^+$) and are therefore called NKT cells. NKT cells are a specialized lineage of T cells that recognize glycolipid antigens presented by the MHC class I-like molecule CD1d [12]. NKT cells are derived from the small fraction of thymocytes that have randomly generated CD1d-reactive T cell receptors (typically comprising Vα14- Jα18 combined with either Vβ8.2, Vβ7, or Vβ2) [13]. When these cells encounter CD1d, they differentiate to the NKT cell lineage [13].

The TCRs of NKT cells are semi-invariant. Thus, the NKT cell receptor resembles more closely the conserved pattern recognition receptors of innate immunity than the diverse antigen-specific receptors of adaptive immunity [14].

NKT cells become activated during a variety of infections and contribute to protective host immune responses. For Gram-negative bacteria containing LPS, recognition of microbial products by TLRs on DCs results in NKT cell activation. Alternatively, for certain Gram negative bacteria that lack LPS, NKT cells may be activated by specific microbial glycosphingolipids [14]. The same lytic mechanisms for NK cells have been observed in NKT cells, but it seems that the Fas/Fas-ligand is the preferred system [15].

The activation of NKT cells sometimes lead to suppression of immune responses, and it is not clear what conditions lead to suppression or activation of the immune system [16].

### 1.1.5 Discrimination of pathogens by the innate system

Although not antigen-specific, the innate system is able to discriminate foreign molecules from self. Phagocytes have receptors with lectin-like activity which recognize structures termed “pathogen-associated molecular patterns” present on microbes, but not host cells. Examples are lipopolysaccharides, lipotechoic acid, and mannans on gram negative, gram positive, and yeast cell walls, respectively. The pattern-recognition receptor molecules fall into three groups depending on function; those inducing endocytosis and thus enhancing antigen presentation; those initiating nuclear factor transduction and cell activation (toll-like receptors) and those, for example mannan binding lectin, which are secreted acting as opsonins. The increasing knowledge of these recognition pathways highlights the close relation between innate and adoptive immunity. A pattern recognition receptor presents the processed product to antigen-specific T cells. The interactions allowing the innate response to eradicate infectious agents, such as phagocytosis, opsonisation, and complement-mediated lysis, require exposure to the surface of the microbe. The response is therefore largely confined to eradicating extracellular organisms, mostly bacteria. This system is not able to detect intracellular organisms, notably viruses, mycobacteria, some fungi, protozoa, or other facultative intracellular pathogens [3, 4]. Activation of TLRs triggers the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules [17]. Activation of NFκB by toll pathway leads to production of several important mediators of innate immune system such as, IL-1, IL-6, IL-8, IL-12 and TNF-α [17].

## 1.2 Adaptive (specific) immune system

The adaptive immunity is characterized by applying specific immune responses against invaders and non-self antigens through antigen-specific receptors on T and B cells. First, the antigen is presented to and recognized by the T or B cell leading to cell priming, activation, and differentiation, which usually occurs within the lymphoid tissue. Second, the effector phase takes place, either due to the activated T cells homing to the target site, and/or due to the release of specific antibodies from activated B cells (plasma cells) into the blood. As there is a delay of 4-7 days before the initial adaptive immune response becomes effective, the innate immunity play a critical role during this early period [3, 4].

### 1.2.1 Formation of antigen-specific receptors on T and B cells

B and T lymphocytes originate from progenitor cells within the bone marrow. B cells (bone marrow derived) remain within the marrow for the development, but T cells (thymus derived) migrate to the thymus at an early stage [3]. The production of antigen-specific receptors on both cell types is the result of random rearrangement and splicing on multiple DNA segments that encode for the antigen-binding areas of the receptors (complementary-determining regions, CDRs). This step-wise gene rearrangement occurs early in the development of the cells, before exposure to antigen and leads to the production of a repertoire of over $10^8$ T-cell receptors (TCRs) and $10^{10}$ antibody specificities, sufficient to cover pathogens likely to be encountered in life. The process for B-cell receptor (BCR) rearrangement is similar for the TCR. There are four segments of genes involved in receptor formation called the variable (V), diversity (D), joining (J), and constant (C) regions. These are found on different chromosomes within the developing cell. The segments are cut out by nucleases and spliced together using ligases (a product of the recombination activation genes, RAG-1 and RAG-2). This forms the final gene sequence from which protein will be transcribed to form the receptor molecule. There are several ways in which clonal diversity occurs. First, there is a multiplicity of all these regions within the DNA (V=25-100 genes, D~25 genes, and J~50 genes). Second, there is combinational freedom in that any one of the genes can join with any one other to form the final VDJ region. Third, the splicing is inaccurate and frame shift in base pairs leads to the production of a different amino acid (junctional diversity). Fourth, the enzyme deoxyribonucleotidyltransferase can insert nucleotides to further alter the sequence. An even greater repertoire of BCR is produced as further immunoglobulin (Ig) gene rearrangement occurs during B-cell division after antigen stimulation (somatic hypermutation).

In T lymphocytes the receptor has two forms. The most common consists of a heterodimer of an $\alpha$ and a $\beta$ chain, each with a constant and variable domain. The

other form (<10% of T cells) has γ and δ chains (the function of this type of TCR bearing cell remains uncertain). The TCR forms a complex with the CD3 molecule, with its associated signaling molecules (Figure 1). In B cells the gene product is a membrane bound form of IgM, initially expressed alone and later with IgD. Early in B-cell development this molecule acts as the antigen receptor, being able to induce signal transduction in a similar way to the TCR. The membrane bound molecule can also internalize antigen, inducing processing, and re-expression for antigen presentation to T cells. After B-cell activation the secreted form of BCR (antibody) is produced by plasma cells. Despite the similarities in gene rearrangement processes, the T and B cell receptors recognize antigen differently. The TCR binds linear peptides usually of eight to nine amino acids (see below). This generally means antigen that has been broken down by intracellular processing by antigen presenting cells. The BCR and antibody recognize the conformational structure of epitopes, and such antigens do not require processing. New clones of T and B cells continues to emerge through life, but slows after the 25-30 years of age [3, 4].

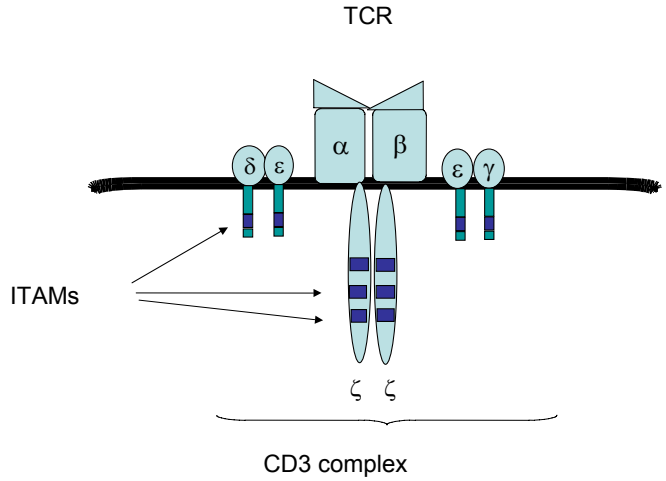


**Figure 1.** TCR, CD3-complex, and ITAMs of TCR.

### 1.2.2 Lymphoid organs

In the lymphoid organs, the lymphocytes interact with nonlymphoid cells which is important either to lymphocyte development, to the initiation of adaptive immune responses, or to their survival and maintenance. Lymphoid organs are divided to central or primary lymphoid organs, and peripheral or secondary lymphoid organs. The cells that emerge from the thymus and bone marrow (primary lymphoid organs) having undergone gene rearrangement are naïve, i.e. they have not yet encountered their specific antigen. The secondary lymphoid tissues (lymph nodes, spleen, tonsils,

and mucosa associated lymphoid tissue) provide the microenvironment that brings the naïve T and B cells as well as antigen presenting cells (APCs) together, where adaptive immune responses are initiated and where lymphocytes are maintained [3, 4].

Although about 95% of T lymphocytes are sequestered within the lymphoid tissue, they are not static but circulate continuously between the lymphoid tissues via the blood or lymph in 1-2 days. When the T cell meets an APC bearing its specific antigen, activation occurs over the next 2-3 days. The antigen is brought to the lymphoid tissue directly in the lymphatics, or within dendritic (or other APCs) cells that have captured the antigen locally. Antigens in the blood are taken to the spleen, in the tissues to the lymph nodes, and from the mucosae to the mucosa-associated lymphoid tissue [3, 4].

### 1.2.3 Antigen presentation and the MHC molecules

To mount an immune response, antigens have to be processed and presented by an APC to the T cell within the peptide-binding groove of a self-MHC molecule.

Depending on the antigen, loading onto MHC can occur in two different ways [3, 4]. Endogenous such as viral or tumor proteins are combined with MHC class I intracellularly. Exogenous antigens are taken up by endocytosis by specialized professional APCs which include DCs, B cells, and macrophages. Exogenous antigens are expressed with MHC class II on the APCs. There are about 100,000 MHC molecules on the surface of each cell [3, 4]. Macrophages and DCs also present exogenous antigens through MHC class I pathway. This relatively new finding is called “cross presentation” [18, 19]. The exact mechanism of cross presentation pathway is not clear. However, there are two proposed ways which might not be mutually exclusive. One model suggests the processed antigenic peptides within the phagolysosome is exchanged with the peptides bound to MHC class I molecules internalized from the cell surface into the same phagolysosome. Alternatively the antigenic peptide might be processed within the phagolysosome that might have transient, regulated continuity with the ER. In this fashion, nascent class I molecules are loaded with the peptides derived from exogenous antigens by a process identical to normal peptide loading within the ER [20]. Cross presentation is involved in responses to transplantation, viral infections as well as malignant cells.

The induction of an adaptive immune response begins when an immature DC ingests a pathogen. These specialized phagocytic cells are resident in most tissues and are long-lived compared to other white blood cells [3, 4]. Eventually, the tissue resident DCs migrate to the regional lymph nodes where they interact with recirculating naive lymphocytes. On activation, the DC matures and undergoes

changes that enable it to activate the specific lymphocytes and secretes cytokines that influence both innate and adaptive immune responses.

### 1.2.4 Antigen recognition by T cells

CD4 lymphocytes recognize antigen presented with MHC class II and CD8 cells with MHC class I. Endogenous antigens accompanied with MHC class I molecules activate $CD8^+$ cytotoxic T cells. Because almost all nucleated cells express MHC class I, this means that any such cell that is infected with a virus or other intracellular pathogen, or is producing abnormal antigens e.g. tumor antigen, can present these antigens with class I and can be targeted by cytotoxic attack. Whereas these CD8 responses are highly targeted to the cell that they recognize, CD4 activation leads to production of cytokines that in turn activate a wide range of cells around them. The reaction therefore needs to be kept under control, which is achieved by only a small number of class II expressing APCs. The need for intracellular processing and expression with MHC ensures that only antigens derived from what is regarded as foreign molecules are recognized [3, 4].

### 1.2.5 TCR signaling

The TCR is made up of clonally variable Ag-binding chains (α and β) that are associated with invariant accessory proteins. The invariant chains are required both for transport of the receptors to the cell surface and for the initiation of signals when TCR binds to an Ag:self-MHC complex.

The TCR also contains different accessory chains such as CD3γ, CD3δ, CD3ε (that make up the CD3 complex), and the ζ chain, which is present mainly as an intracytoplasmic homodimer (Figure 1) [21-23]. Immunoreceptor tyrosine-based activation motifs (ITAMs) are amino acid sequences that are composed of two tyrosine residues separated by about 9-12 amino acids. The TCR complex in total contains ten ITAMs (Figure 1). Many receptors employ ITAMs, such as BCR, NK cell receptors, and Fc receptors on mast cell, macrophages, and monocytes. When Ag binds to TCR, the tyrosines in these ITAMs become phosphorylated and are then able to bind with high affinity to the members of tyrosine kinases. Phosphorylation of tyrosines in ITAMs serves as the first intracellular signal indicating that the T cell has recognized its specific Ag (Figure 2) [21-23]. The initial events in TCR are implemented by two Src-family kinases, Lck and Fyn. Lck is constitutively associated with ζ and CD3ε chains upon receptor clustering [21-23].

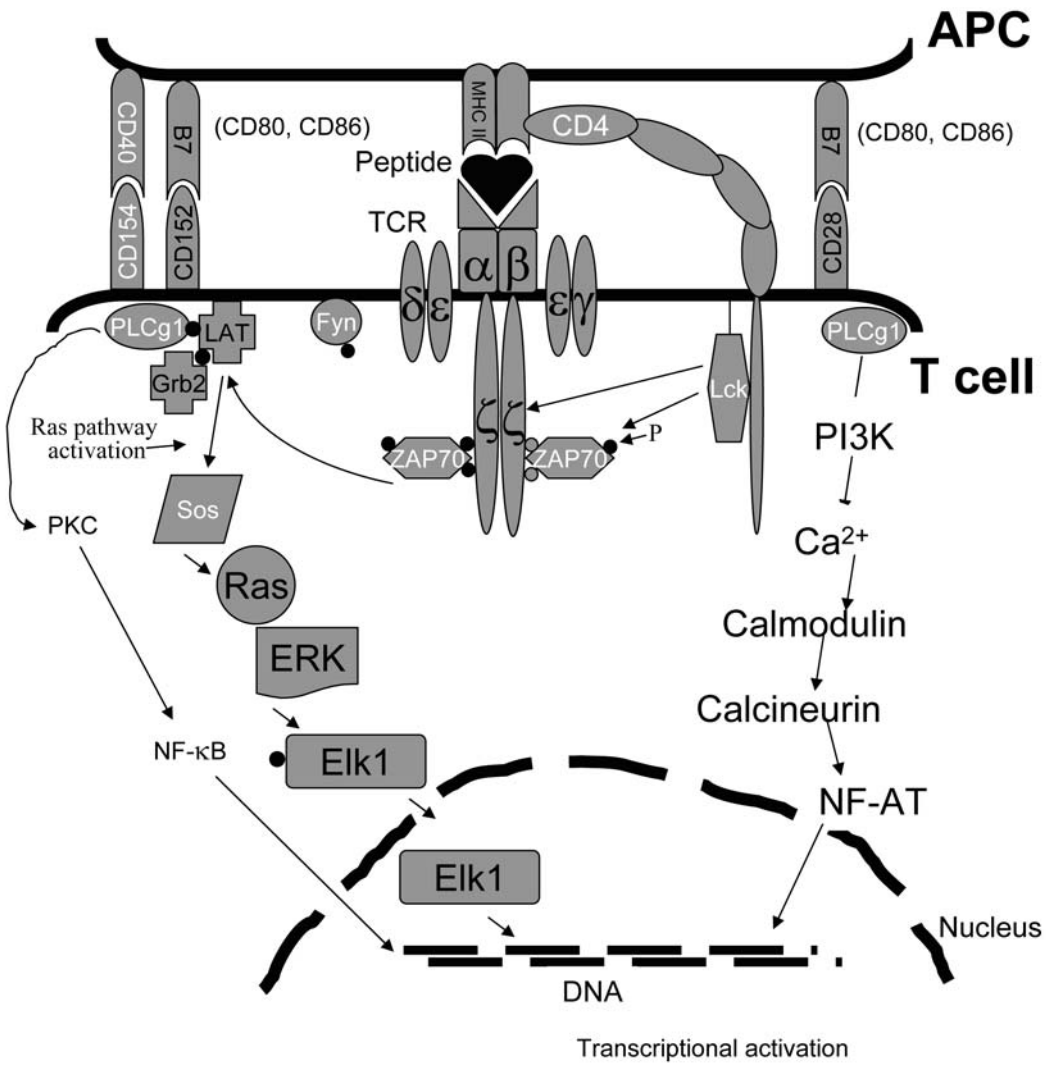


**Figure 2.** TCR, CD3-complex, and the signaling pathway through TCR.

Aggregation of TCR:peptide:MHC with the coreceptor brings the Lck in proximity of ITAMs in cytoplasmic tails of TCR complex that leads to phosphorylation of ITAMs. Coreceptors on the surface of the T cell that transmit signals, referred to signal 2, cause activation if the TCR is also engaged. Without signal 2, the cell will either become anergic (non-reactive) or go into apoptosis [3]. The main coreceptors for T-cell activation are CD80 (B7-1), CD86 (B7-2), and CD40 that bind CD28, CTLA-4, and CD40 ligand on the T cell, respectively [3]. Activated DCs are the most potent stimulators of naive T cells, bearing large amounts of CD80, CD86, and CD40. Inflammatory mediators induce the upregulation of costimulatory molecules,

therefore a T cell is much more likely to be activated if it encounters its specific Ag via an APC pre-exposed to an inflammatory environment [3].

The double phosphotyrosines in ITAMs acts as binding site for another kinase, ζ-associated protein 70 (ZAP-70). Following binding, ZAP-70 becomes phosphorylated and thereby activated by Lck (Figure 2). Once activated, ZAP-70 phosphorylates the substrate LAT (linker of activation in T cells) and a protein called SLP-76 [21-23].

The next step in the signaling pathway is to amplify the signal at the cell membrane, and finally transfer it through the cytoplasm to the nucleus. Several classes of proteins participate in signal amplification. These involve the enzyme PLC-γ, which initiates two of the main signaling pathways that lead to the nucleus. Activated PLC-γ cleaves phosphatidyl inositol bisphosphate (PIP2) to generate the intracellular second messenger diacylglycerol (DAG) and inositol triphosphate (IP3). The other main pathway is generated by activation of the small G protein Ras. These three pathways amplify the signal from the activated receptors to the nucleus by many mediators, leading to cell proliferation and differentiation (Figure 2) [21-23].

The ultimate response of T cells to extracellular signals is the induction of expression of new genes. This is achieved through the activation of transcription factors. Several transcription factors are involved in T cell responses to Ags, such as extracellular-regulated kinase 1(Erk1), Erk2, nuclear factor of activated T cells (NFAT), and nuclear factor κB (NFκB) [21-23]. NFAT, for instance, is released from the cytosol by the action of the enzyme calcineurin. Calcineurin is itself activated by the increase of intracellular free $Ca^{2+}$ that accompanies lymphocyte activation, the free $Ca^{2+}$ is bound by calmodulin within the cell and the $Ca^{2+}$-calmodulin complex binds to and activates calcineurin. Once NFAT has been dephosphorylated by calcineurin it enters the nucleus, where it can act as a transcriptional regulatory protein [21-23]. Vav1, a 95-kDa protein expressed in all hemopoietic cells has been found to be involved in the TCR signaling pathway [24]. Vav1-deficient T cells are defective in TCR-induced proliferation and cytokine production. Vav1 is required to transduce signals to the activation of a calcium flux, ERK and the NF-κB transcription factor. Vav1 has also been shown to control the activation of PLCγ via both PI3K-dependent and -independent pathways [24].

Upon activation clonal expansion of each T cell (and B cell) produces up to 1000 (2-4 divisions/day for 3-5 days) progeny of identical specificity [3]. Most of them are armed effector cells, which upregulate receptors, leave the lymphoid tissue and are guided to the site of inflammation by chemokines. Adhesion molecules attract both the effector and memory cells to the objective site [25], where the T cells will recognize target cells expressing the specific foreign antigen with MHC and initiate either a cytotoxic attack, or stimulate an inflammatory response. Some of the activated T cells remain in the lymph nodes as central memory cells which may live

for 10 years or more [3, 4]. They react more quickly on re-exposure to the same Ag. It is immunological memory that enables successful vaccination.

## 1.2.6 Effector T cells

Two major types of effector T cells have been identified, T helper (Th) and T cytotoxic (Tc), having either CD4 or CD8 molecules on their surface, respectively. CD4 Th cells are the leading cells of the immune response, recognizing foreign antigen, and activating other parts of the cell-mediated immune response. They also play a major part in activation of B cells. CD8 cytotoxic cells are involved in antiviral and anti-tumor reactivity. Both types have a major role in the control of intracellular pathogens [3, 4].

### *1.2.6.1 Helper T cells*

Th cells are subdivided functionally by the pattern of cytokines they produce. Upon stimulation, Th0 lymphocytes differentiate to either Th1 or Th2 cells. Th1 cells produce interleukin (IL)-2, which induces T cell proliferation (including that of $CD4^+$ cells in an autocrine response). IL-2 also stimulates $CD8^+$ T cell proliferation and cytotoxicity. Another cytokine produced by Th1 cells, interferon-γ (IFN-γ), activates macrophages and NK cells. The Th1 cytokines therefore induce mainly a cell-mediated inflammatory response. There is a positive feedback loop as interferon-γ stimulates other Th0 cells to become Th1 and inhibits Th2 differentiation. A Th1 response is essential for fighting against pathogens, but possibly contributes to the pathogenesis of autoimmune diseases. On the contrary, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 that favor antibody production. IL-4 induces class-switching in B cells to IgE production and IL-5 promotes the growth of eosinophils. IL-4 provides positive feedback to induce further Th2 responses and suppress Th1 differentiation. The Th2 response is also associated with allergic disease [3, 4].

### *1.2.6.2 Cytotoxic T cells*

CD8 T cells are directly cytotoxic to cells presenting their specific antigen. Thus these cells are also called cytotoxic T lymphocytes (CTL). Following binding to the target cell, they may release perforins and granzymes. These molecules via activating caspase enzymes, induce DNA fragmentation and cell apoptosis. CTL also binds target cell surface Fas (death inducing) molecules by their Fas ligand (FasL). Fas-FasL interaction also triggers apoptosis in the target cell [3, 4].

### 1.2.7 Regulatory T cells ($T_{reg}$)

Gershon and Kondo in 1971 were able to transfer Ag-specific tolerance to naive animals by transferring Ag-experienced T cells [26], which they called "suppressor" cells. Until the late 1980s there was not agreement about the concept of "suppressor" T cells. However, reports describing T cells responsible for antitumor immune suppression both in mice and human, clearly suggested the existence of *in vivo* mechanisms of tumor-driven cellular immune suppression [27].

Sakaguchi et al have found a population of CD4 T cells expressing high amounts of surface CD25 and inhibiting autoimmunity in a murine model [28]. Several reports in the following years explained major aspects of now called "regulatory" T cells ($T_{reg}$ cells) and characterized different T-cell subpopulations with regulatory properties [27]. There are many different T-cell subpopulations with regulatory function that coexist and contribute to the immune suppression [29-32]. $CD25^{high}$ is one of the first markers introduced for $T_{reg}$ cells in human however, since humans are constantly exposed to foreign antigens, leading to a significant fraction of recently activated CD25 effector T cells, CD25 may not be the optimal marker for $T_{reg}$. The transcription factor FoxP3 has been suggested as a more specific $T_{reg}$ marker but some recent reports in humans showed $FoxP3^{+}$ T cells without suppressive activity [27].

CD4 $CD25^{high}$FoxP3 $T_{reg}$ cells exhibits anergic status, ability to inhibit CD4 CD25 T cells, CD8 T cells, DCs, NK cells, NKT cells, and B cells in a cell-cell contact and dose-dependent manner [27, 33-38]. $T_{reg}$ cells are typically characterized as Ag experienced memory T cells, although some reports described them as naive cells. Cytotoxic T lymphocyte associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR) are surface markers that are associated with the CD4 $CD25^{high}$FoxP3 $T_{reg}$ cells [39, 40]. Functional importance of IL-10 and TGF-β for $T_{reg}$ cells *in vivo* have been suggested [41, 42]. Major interest of current studies on $T_{reg}$ cells are the description of $T_{reg}$ cells in autoimmune diseases and their role in malignancies, infectious diseases and tolerance in transplantation [43-46].

### 1.2.8 B lymphocytes

B cells are specialized cells to produce antibody (Ab) that is responsible to neutralize toxins, prevents organisms adhering to mucosal surfaces, activates complement, opsonises bacteria for phagocytosis, and sensitizes tumor and infected cells for Ab-dependent cell-mediated cytotoxic (ADCC) attack by killer cells. Thus Ab enhances elements of the innate system. BCR internalizes antigen and takes it for processing to act as an Ag-presenting cell for T-cell responses. During subsequent infections by the same pathogen, follicular dendritic cells, which bear Fc receptor, can activate B cells and complement receptors, bind immune complexes containing Ag, and trap this to

activate the B-cell response. There are five main heavy chain classes or isotypes: IgM, IgD, IgG, IgA, and IgE that confer the functional specialization of an Ab. The heavy chain segments encode these isotypes are μ, δ, γ, α, and ε, respectively. In humans there are four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) and two of IgA (IgA1 and IgA2) [3, 4].

#### *1.2.8.1 T-cell dependent responses*

Antigen recognized by the surface IgM of the B cell, is internalized, processed, and expressed on the MHC class II molecule of the B cell. This can then present the antigen to a primed specific T cell (which recognizes a different part of the same antigen). The T cell in turn produces cytokines leading to B cell division and maturation to antibody secreting cells. Further T-cell interactions, in particular the binding of CD40 on B cells with the CD40L on T cells induces isotype switching from the initial IgM response. A mature but naïve B cell, that has rearranged its *V(D)J* gene, will initially make an IgM response on primary antigen stimulation because this is the first constant chain to be translocated. IgG and other isotype responses develop later and require additional T cell help. The process of B-cell activation occurs mainly within the germinal centers of lymph nodes. At this site somatic hypermutation occurs, leading to a greater diversity of antibodies and therefore the antibody response matures with increased affinity. Once the switch from IgM to another isotype has occurred, some of the activated cells become long-lived memory cells. The memory cells react rapidly to rechallenge and the secondary response occurs. The activated B cells leave the lymphoid tissue as plasma cells and migrate to the bone marrow (BM) [3, 4].

#### *1.2.8.2 T-cell independent responses*

B cells can also respond to some Ags in a T-cell independent manner. This response is generated against such Ags which have repeating epitopes that bind multiple BCRs and activate the B cell directly. In this way no affinity maturation, class switching or generation of memory cells occurs. T-cell independent responses are therefore IgM limited, of poor specificity, and short-lived [3, 4].

## 1.3 Superantigens

Superantigens have a unique style of binding to both MHC and TCR molecules that enables them to stimulate very large numbers of T cells. Superantigens are produced by many different pathogens and the responses they cause are helpful to the pathogen rather than the host [3].

Superantigens are recognized by T cells without being processed into peptides. In addition to binding MHC class II molecules, superantigens bind to the Vβ region of many TCRs (Figure 3) [3]. Bacterial superantigens bind mainly to the Vβ CDR2 loop, and to a smaller extent to the Vβ CDR1 loop and an additional loop called the hypervariable 4 or HV4 loop [3]. Thus, the α-chain V region and the CDR3 of the β chain of the TCR have not vital effects on superantigen recognition. Each superantigen is specific for one or a few of the different Vβ gene segments, of which there are 20-50 in mice and humans; a superantigen can thus stimulate 2-20% of all T cells [3].

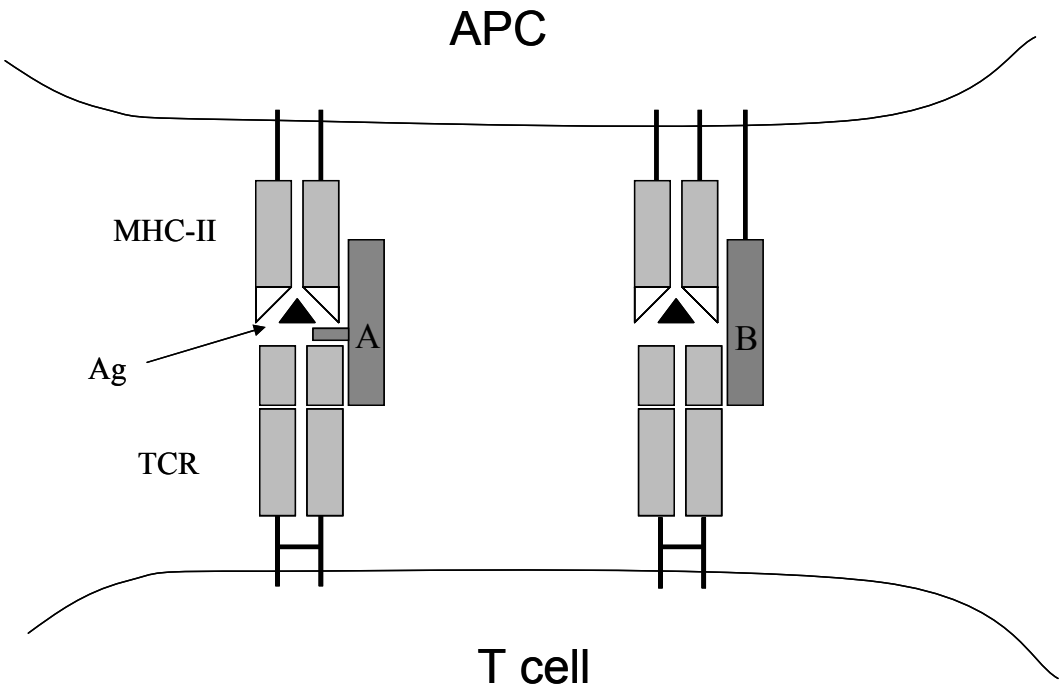


**Figure 3.** Superantigens bind directly to TCR and to MHC molecules. The panel A shows a staphylococcal enterotoxin (SE) superantigen, and the panel B shows viral superantigen.

# 2 MULTIPLE MYELOMA (MM)

MM is a lymphoproliferative disorder characterized by clonal proliferation of plasma-cells (PC) and post-germinal center B cells [47]. MM accounts for more than 10% of all hematological malignancies [48]. This disease is characterized by the presence of a monoclonal Ig (the M component) that is detected in the serum and/or urine. Most patients have clinical symptoms and abnormalities in physiological parameters including painful osteolytic lesions, hypercalcemia, anemia and renal impairment.

Despite advances in systemic therapy, MM is still an incurable disease. During the past decade, novel insights into the biology of the disease have provided new therapeutic agents such as bortezomib, thalidomide and lenalidomide that target the malignant cells and its bone marrow microenvironment.

## 2.1 Epidemiology

The incidence of MM is relatively stable in most countries and the incidence rates vary from 0.4 to 5 per 100,000 with higher rates in the Western word compared to Asian Countries [49, 50]. The median age of diagnosis is 68 and it is rare under the age of 40. One of the highest incidence rates has been reported in Sweden, with 304 new cases in men and 254 in women in 2004 [51]. Mortality from MM has increased gradually over the last few decades in USA and across Europe [50, 52]. However, the incidence of MM in Sweden has been relatively constant for several decades [53]. The median survival for the patients with MM ranges from 3.5 to 4 years [54]. However, the range is from less than 6 months to greater than 10 years [55] indicating heterogeneity in biology which may be critical for understanding the disease.

## 2.2 Etiology

The cause of myeloma is still mainly unknown. Several studies have indicated an increased risk of myeloma among workers in the nuclear industry [56, 57]. Environmental factors such as agricultural chemicals, pesticides, benzene, metals, petroleum products and, chronic antigenic stimulation have been speculated to be associated with MM [58-63]. Although direct genetic linkage has not been found, hereditary factors might impact on MM development [64, 65].

Two distinct pathways in the pathogenesis of MM has been suggested: one involving an early IgH translocation and mostly is associated with non-hyperdiploid chromosome content and a second, that infrequently involves early IgH translocation and is associated with hyperdiploid chromosome content and multiple trisomies [48]. Dysregulation of a cyclin D gene also appears to be early event [47].

## 2.3 Genetic alterations and pathogenesis

MM development depends on several genetic alterations. The translocations of the IgH gene (locus 14q32) is correlated with the disease stage and is present in about 50% of patients with monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic MM, 85% of cases with plasma cell leukemia and in >90% of human myeloma cell lines [66-68]. The major oncogenes that are involved in the chromosomal translocation process of the IgH genes, which are referred as primary IgH translocations, and are present in about 40% of MM and MGUS cases are; 11q13 (CCN D1) (15%), 4p16 (MMSET and usually FGFR3) (15%), 16q23 (c-MAF) (5%), 6p21 (CCN D3) (3%), and 20q11(MAFB)(2%) [69-71]. The apparent increased prevalence of IgH translocations of 4p16 and 16q23 in MM suggests that these translocations might be associated with rapid progression from MGUS to MM [47]. Secondary translocations that accounts for other 20-30% of MM cases, sometimes do not involve Ig loci, and are associated with unbalanced and more complex translocations. They are not mediated through the B-cell genome modification mechanisms and are related to other oncogenes, e.g. c-myc (8q24), and are frequently allied with tumor progression [69]. About 30% of MM and 45% of MGUS cases do not have either an IgH or IgL translocation [47]. Aneuploidy is another characteristics of MM. In MM two major ploidy categories have been proposed: the hyperdiploid myeloma (HRD), which is characterized by the increased prevalence of multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, and the non-hyperdiploid (NHRD) tumors which can be hypodiploid, pseudodiploid or subtetraploid [47, 67, 70]. Monosomy 13 is also present in 50-60% of MM cases.

Recent analyses from gene expression profiling suggest that in practically all MM and MGUS cases, regardless of IgH translocation, at least one of the cyclin D genes (D1, D2 or D3) is highly expressed [47].

## 2.4 Clinical manifestations

About 70% of MM patients suffer from bone pain, which is caused by the proliferation of tumor cells, activation of osteoclasts that destroy the bone, compression (osteoporosis) fractures of the spine, and pathologic fractures of the long bones.

Recurrent bacterial infections are the second most common clinical problem in patients with myeloma, in particular pneumonias and urinary tract infections. 50%-70% of the patients with MM die as a result of bacterial infections [72]. Diffuse hypogammaglobulinemia of all IgG, IgM and IgA subtypes is another feature of MM.

Renal impairment is also noted in approximately 50% of MM patients. Glomerular deposits of amyloid (large amounts of the variable portion or the

complete Ig light chain), hyperuricemia, recurrent infections, hypercalcemia and dehydration all may contribute to renal dysfunction [73].

The cause of symptomatic polyneuropathy, which is observed in 5-15% of MM patients, might be paraneural deposition of amyloid. About 80% of myeloma patients develop anemia, which is related to the inhibition of hematopoiesis by inflammatory cytokines as well as impaired endogenous erythropoietin (EPO) production [74].

## 2.5 Diagnostic criteria

Minimal criteria for the diagnosis of multiple myeloma include more than 10% plasma cells in BM, plus at least one of the following:

- A monoclonal protein in the serum, IgG >35 g/L, IgA >20 g/L
- A monoclonal protein in the urine, >1 g/24 hr
- Lytic bone lesions

Decrease of polyclonal immunoglobulins in serum (IgG <6 g/L, IgA <1 g/L, and IgM <0.5 g/L) is common [75].

A study at the Mayo Clinic on 1027 patients with newly diagnosed MM revealed a monoclonal protein in plasma with in 93% of patients. A monoclonal light chain was found in the urine of 78% of cases. Non-secretary myeloma was recognized in 3% and light chain myeloma (only Bence-Jones proteinuria) was present in 20% of patients [76].

Overt MM must be distinguished from MGUS and smoldering (asymptomatic) MM (SMM) [77]. MGUS is characterized by the absence of symptoms, an M-protein of < 30 g/L, < 10% of plasma cells in the BM and absence of lytic lesions, anemia, hypercalcemia, and renal insufficiency. SMM is characterized by an M-protein ≥ 30 g/L, or ≥ 10% plasma cells in BM. SMM and MGUS patients should not be treated, since they may remain stable for a prolonged period of time. Serum and urine M-protein should be measured periodically and clinical and other laboratory features should be examined [78]. MGUS progresses to MM at a rate of 1% per year [79].

## 2.6 Staging

Durie & Salmon staging system [80] was for a long time the most commonly used prognostic tool. Even though these criteria reflect the tumor burden, additional laboratory parameters, which may reflect also the biology of the disease, may be considered. Recently the new International Staging System (ISS) has given widespread use, with a three-stage system using serum β2-microglobulin and albumin (Table 1) [55].

**Table 1.** International Staging System

| Stage | Criteria | Median Survival (months) |
|---|---|---|
| I | Serum $\beta_2$-microglobulin <3.5 mg/L<br>Serum albumin ≥3.5 g/dL | 62 |
| II | Not stage I or III* | 44 |
| II | Serum $\beta_2$-microglobulin ≥5.5 g/L | 29 |

*There are two categories for stage II: serum $\beta_2$-microglobulin <3.5 mg/L but serum albumin <3.5 g/dL; or serum $\beta_2$-microglobulin 3.5 to <5.5 mg/L irrespective of the serum albumin level [55].

## 2.7 Prognostic factors

In 1980s, serum β2-microglobulin (β2M) emerged as the single most powerful prognostic factor and was considered a simple reliable predictor of survival [81]. Other prognostic factors, include serum level of C-reactive protein, serum albumin, and the plasma cell labeling index.

The recently introduced ISS, three-stage system based on serum β2m and albumin (Table 1) provides a simple method both for staging and prognosis of MM [55]. Median survival for stage I is 62 months; stage II, 44 months; and stage III, 29 months. Patient numbers are distributed in the following way: stage I, 29%; stage II, 37%; and stage III, 34%) [55].

Chromosomal abnormalities have also prognostic values. Del 13 is the most common and is associated with poor prognosis [82, 83]. Both 1q21 gain and increase gene expression level were found significantly associated with reduced survival of MM [84]. A study that used interphase FISH revealed that patients with t(4;14) (13% of patients) had a poor overall survival (23% at 80 months) [66]. In contrast, patients with t(11;14) (16% of patients) had a good prognosis (88% at 80 months).

Standal et al [85] have shown that serum insulin-like growth factor 1 (IGF-1) is a strong indicator of prognosis. Also a low expression of p27KIP1 (CDKN1B) has been reported as an adverse prognostic factor [86].

It has recently been reported that increased serum proteasome concentration correlates with advanced disease and is a statistically significant independent prognostic factor in MM [87]. Serum proteasome concentrations were found significantly increased in MM compared to healthy donors ($P < 0.001$), in MM compared to MGUS ($P = 0.03$) and in active (n=101) compared to smoldering (n=40) MM ($P < 0.001$). Thus proteasome concentration correlate with advanced disease and is an independent prognostic factor in MM [87].

## 2.8 Treatment of MM

There is no evidence that early treatment of non-symptomatic MM is advantageous. Younger patients with symptomic MM should be considered possible candidates for autologous stem cell transplantation. If they are deemed to be eligible, they should be treated for 3 to 4 months with therapy that does not damage the hematopoietic stem cells [88].

High-dose therapy (HDT) with stem-cell support increases the rate of complete response and extends event-free and overall survival [89, 90]. However, this approach is generally suitable for patients younger than 65 years, who represent only about a third of all myeloma patients. For patients older than 65 years, conventional chemotherapy with oral melphalan and prednisone has remained the treatment of choice since 1960 [91], a situation which now finally seems to change (see below).

As induction treatment for patients planned for HDT with autologous stem cell transplantation (ASCT), cyclophosphamide in combination with steroids is commonly used in Sweden. However, at some centers thalidomide plus steroids are used for induction therapy [88]. ASCT prolongs disease-free survival and overall survival. Melphalan, 200 mg/m$^2$, is the most widely used conditioning regimen [88]. Although allogeneic stem cell transplantation is attractive, the mortality rate (about 20%) is too high to recommend as standard therapy [88]. Patients with relapsed or refractory disease may be treated with dexamethasone in combination with thalidomide, bortezomib, or with lenalidomide.

The response rate of relapsed myeloma to thalidomide alone is around 30%. When thalidomide is used in combination with corticosteroids, the response rate increases to about 50% [92], and around 70% when used in combination with alkylating agents [93, 94]. Recently a randomized controlled clinical study showed that oral melphalan and prednisone plus thalidomide is an effective (prolonged survival) first-line treatment for elderly patients with multiple myeloma which should be considered for this patient group, despite that more side effects, such as somnolence, peripheral neuropathy, and venous thromboembolic complications, can occur [91].

Bortezomib inhibits proliferation and induces apoptosis of human myeloma cells *in vitro*. It also inhibits NFκB activation, overcomes drug resistance and adds to the antimyeloma-activity of dexamethasone, melphalan and doxorubicin *in vitro* [48]. In the large APEX trial, bortezomib was shown to be more effective than dexamethasone as far as response, time to progression and overall survival are concerned [95]. Whether bortezomib is better than thalidomide is still not known; it appears to have a better side effect profile but is also considerably more expensive.

Lenalidomide is an analogue of thalidomide, which has similar mechanisms of action but with greater potency. Lenalidomide is up to 2000 times more potent in

stimulating T-cell proliferation and production of IFN-γ and IL-2. It also activates FAS-mediated apoptosis and reduces release of IL-6 and TNF-α [96]. One promising benefit of this agent is its more favorable side-effect profile compared with thalidomide [96].

Bisphosphonates should be given to all patients with symptomatic myeloma to prevent skeletal complications. This can be given either as monthly intravenous infusion (preferentially pamidronate to reduce risk of jaw osteonecrosis) or oral clodronate [97-99].

## 2.9 Bone marrow microenvironment

The survival, growth and differentiation of normal plasma cells and myeloma cells are dependent on the BM microenvironment. Various cytokines, soluble factors, receptors and adhesion molecules mediate reciprocal positive and negative interaction between myeloma cells and BM stromal cells.

### 2.9.1 Osteoclasts

There is a complicated interaction between soluble factors produced by both MM cells and stromal cells, which shifts the balance towards bone destruction without new bone formation. Factors produced by myeloma cells are involved in both bone destruction and impaired new bone formation. The factors produced by myeloma cells *in vivo* that can raise osteoclast activity include receptor activator of NFκB ligand (RANKL), macrophage inflammatory protein (MIP)-1α, IL-3 and IL-6 [100-102].

RANKL is a major factor involved in myeloma bone disease. When MM cells bind to stromal cells RANKL expression is increased, which results in enhanced osteoclast activity through binding of RANKL to its receptor, located on osteoclast precursor cells, promoting differentiation [103]. RANKL further plays a role in the inhibition of osteoclast apoptosis [104]. T lymphocytes also express RANKL within the MM marrow microenvironment. The proposed mechanism is through the release of a soluble factor by MM cells, which increases RANKL expression on the T lymphocytes and ultimately results in enhanced osteolytic bone destruction [105].

A soluble decoy for RANKL, known as osteoprotegerin (OPG), is produced by bone marrow stromal cells and inhibits the actions of RANKL in osteoclast activation. The proportion of RANKL to OPG determines the osteoclast activity. Some studies suggest that imbalance between RANKL expression and OPG levels favors osteoclastogenesis and osteoclast activation in MM [101, 106].

The chemokine MIP-1α, an important factor in osteoclastogenesis, is present in marrow samples from MM patients with active disease [100, 107, 108]. MIP-1α seems to enhance the growth of myeloma cells and may be associated with a poor prognosis [109].

IL-3 is also significantly elevated in marrow plasma from patients with MM as compared with normal controls [110]. Serum from MM patients with elevated IL-3 stimulates the growth of IL-3-dependent MM cell lines [111]. IL-3 induces osteoclast formation in human marrow cultures and probably in MM patients [110]. IL-3 also enhances the effects of RANKL and MIP-1α on the growth and development of osteoclasts, and directly stimulates MM cell growth [110]. Overall, IL-3 increases the number and activity of osteoclasts, leading to further bone destruction, and appears to be an osteoclast stimulatory factor in myeloma.

The role that IL-6 plays in MM is controversial. Most studies support the idea that cells such as osteoblasts, osteoclasts and stromal cells in the bone marrow microenvironment through contact with myeloma cells produce IL-6. IL-6 enhances the growth of myeloma cells and inhibits myeloma cell apoptosis [112, 113].

Multiple studies have reported that a proliferation inducing ligand (APRIL) and B cell-activating factor of the tumor necrosis factor family (BAFF) levels are elevated in the sera of patients with MM and both autocrine and paracrine APRIL and BAFF production exists in the MM [114, 115]. BAFF and APRIL can protect myeloma cells from apoptosis induced by IL-6 deprivation and/or dexamethasone [114].

### 2.9.2 Osteoblasts

Factors that are responsible for decreased osteoblast activity in MM include IL-3, dickkopf 1 (DKK1), secreted frizzled-related protein-2 (sFRP-2) and IL-7 [116-119]. Several of these affect the Wnt signaling pathway that is critical for osteoblast differentiation [120].

### 2.9.3 Other soluble factors

Other cytokines that affect the growth of MM cells, osteoclast or osteoblast activity have been identified in the marrow plasma of MM patients. These include hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), IL-1 and TNF-α [121]. HGF can exert proliferative and anti-apoptotic effects on MM cells and has been associated with osteoclast activation. Serum levels of HGF have been correlated with TNF-α and IL-6, levels both potent stimulators of osteoclast activity. HGF can induce IL-11, a stimulator of osteoclastogenesis, from osteoblasts [122, 123].

TNF-α has also been implicated in MM, although its role is yet unclear. Elevated serum levels of TNF-α have been detected in MM patients with advanced bone lesions when compared with those without significant bone disease or those with MGUS [124].

Both MM cells and BM stromal cells can secrete VEGF. VEGF stimulates BM angiogenesis and increases release of itself and IL-6 [125] which mediates myeloma cell growth and migration [126]. Osteoclasts produce IGF-1 which may cause proliferation and inhibition of apoptosis of MM cells [127]. Transforming growth factor-β (TGF-β) secreted by MM cells cause paracrine IL-6 secretion in BM stromal cells [128].

## 2.10 T cells in MM

There are some reports about finding of unusually large "expanded" $CD8^+$ T-cell clones in the blood of patients with MM and SMM [129-131]. These clones have been shown to persist over long periods, suggesting that they might be the result of chronic antigenic stimulation [130]. Monoclonality of $CD57^+CD8^+$-expanded T-cells in MM patients has also been reported [131]. Transient antigenic stimulation is known to be associated with short-lived expansion of $CD8^+$ T cell clones [132]. Importantly, presence of chronically expanded T cell clones correlated with a better overall survival [133]. The expanded $CD8^+$ T cell populations express high levels of CD57 [134-136]. Furthermore, most of the expanded T cells express low level of CD28, consistent with a history of prolonged stimulation and proliferation [137]. S-phase analysis has demonstrated that $CD57^+$ clonal T cells in MM patients have a low rate of turnover [131]. They also express lower levels of the apoptotic receptor CD95 (Fas) than their $CD57^-$ counterparts, providing a tentative explanation for the accumulation of clonally expanded $CD8^+CD57^+$ T cells [131].

Efforts to define the specificity of naturally arising expanded T cell clones in MM have not yet yielded clear answers. It has been suggested that the clones may be reacting to a persistent viral infection in a compromised host or to the tumor itself [138].

Significantly increased BM infiltration by T cells was found in MM patients [139]. T cell subsets were $CD4^+$ $CD8^-$, $CD4^-CD8^-$, $CD4^+$ $CD28^-$, and $CD8^+$ $CD28^-$ [139]. The increase of frequency of T cells secreting IFN-γ as well as a higher plasma concentration of IFN-γ was also found in the patients. Positive correlation between the proportion of $CD28^-$ and both the frequency of the IFN-γ-secreting T cells and the proportion of expanded TCR-Vβ lymphocytes within the total BM $CD4^+$ T cells, was noticed [139].

CD8 T cells specific for cancer germline gene antigens was found in many patients with MM, and their frequency were found to be correlated with disease burden [140]. As mentioned above, T lymphocytes express RANKL within the MM marrow microenvironment, through which they may possibly contribute to disease pathogenesis.

# 3 B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

The current view of the immunobiology of B cell chronic lymphocytic leukemia (B-CLL) has changed considerably in recent years. Previously, it was believed that the B-CLL cells were functionally immature [141], minimally self-renewing and with defective apoptotic mechanisms [142-144]. B-CLL is now viewed as a malignant disease of Ag-experienced mature B lymphocytes that have functional competency and escape death due to interactions with factors produced by other cells, including T cells [142-144].

## 3.1 Epidemiology

B-CLL represents 22-30% of all leukemia cases with a worldwide incidence projected to be between <1 and 5.5 per 100,000 people [145]. There is an average annual incidence of about 5/100,000 in Sweden (400-500 cases/year [146]). As shown in Figure 4, the incidence of CLL increases dramatically with age. In 2003 in the US, persons over the age of 65 had an incidence of 21.0 per 100,000, while those under the age of 65 had an incidence of 1.2 per 100,000 [147]. Because many B-CLL patients are asymptomatic, the true incidence is unknown. B-CLL presents in adults, at higher rates in males than in females and in whites than in blacks. Median age at diagnosis is 64-70 years [145]. The mortality rate for CLL in the US in 2003 was 1.5 deaths per 100,000. Males (2.2 per 100,000) had a higher mortality rate than females (1.04 per 100,000) in 2003 [148]. Persons with close relatives who have CLL are at an increased risk.

Active rheumatoid arthritis [149] and Sjögren's syndrome [150, 151] give a higher risk of developing CLL. Researchers have attempted to correlate environmental risk factors with the incidence of B-CLL, but the evidence has not been strong for any single factor yet. Exposure to pesticides, ionizing radiation, carcinogens, diets, alkylating agents, hepatitis C, and Epstein-Barr virus have been suspected, but not consistently associated with B-CLL [145]. Living in a farming community and the exposure to electromagnetic radiation has also been associated with B-CLL, but the evidence is not strong [145].

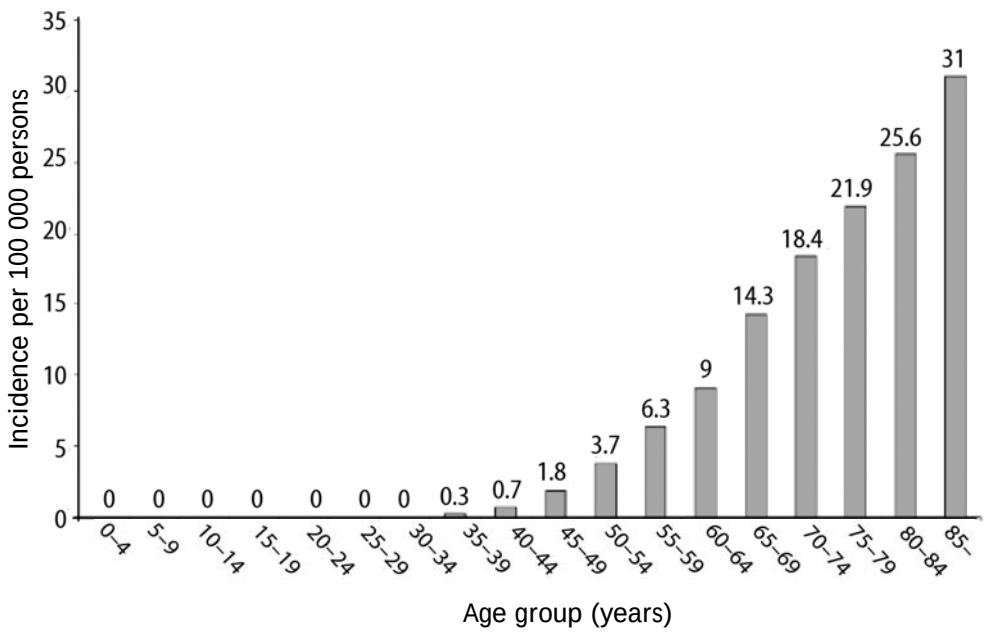


**Figure 4.** Age-specific incidence of chronic lymphocytic leukemia (1996–2000) [145].

## 3.2 Etiology

The cause of CLL is not yet known. Some studies of large series of CLL patients demonstrated that in CLL cells of about half of the cases the $IgV_H$ genes have undergone somatic mutations [152, 153] and the unmutated cases were correlated to a worse clinical outcome [154, 155]. The presence of somatic mutations suggests that, at least in the mutated patients, CLL clonal founder cells had encountered an Ag and that the procedure triggered by this encounter had likely occurred in a germinal center (GC) site [143, 156]. However, based on other evidences the unmutated cases could not be considered naive B-cell driven and are similar to mutated ones, except for $IgV_H$. Although slightly different, both subsets of CLL cells show a remarkable skewing in $IgV_H$ genes usage such as the overexpression of $IgV_H$ 1-69 and $IgV_H$ 3-21[152, 153, 155]. This bias appears to be disease related, therefore suggesting a role for specific Ag or Ag-like elements in the genesis of this subset of CLL [143]. In addition, both CLL subsets have surface phenotype of activated B cells similar to the phenotype of Ag-experienced cells (CD23, CD25, CD69, and CD79) [157]. The characteristics of CLL cells' BCR look like those observed in normal B cells upon Ag interaction [158, 159]. Finally, gene expression profiling revealed that both mutated and unmutated subsets share similarities with memory B cells [160, 161]. From all of these evidences this impression has arisen that in CLL, regardless of the $IgV_H$ genes mutational status, the cell of origin appears to be an "Ag-experienced" B cell [143]. It

has been suggested that the expression of the naive marker CD5 is perhaps related to activation events rather than the cellular origin [160].

Data of the complementary determining regions 3 (CDR3) sequences of the BCRs of the CLL cells are in line with the involvement of Ag or Ag-like element in the etiology of CLL. Several studies showed that unrelated patients in different parts of the world express very similar, if not identical, BCR [162-166]. Although the chances that the process of VDJ recombination may result in two almost identical sequences are few in a million, such similarities can be found in more than 10% of CLL patients [142]. These data plus the fact that both CLL subsets exhibit similar VDJ rearrangements, suggest involvement of an antigenic element able to bind to a particular Ag-pocket might be responsible for the selection and/or progression of the leukemic clone [142, 143, 167].

CLLU1 gene has been recently reported as disease-specific gene since its restricted expression pattern in unmutated CLL cells that is irrespective of trisomy 12 or large chromosomal rearrangements [168]. However, whether the CLLU1 is involved in the etiology of CLL is not clear yet.

It has also been reported that the T cell leukemia-1 (TCL-1) gene, which is involved in the pathogenesis of T-cell prolymphocytic leukemia [169], is expressed in CLL [170]. Overexpression of *Tcl-1* in B cells in mice leads to a leukemia or lymphoma of $CD5^+$ B cells that is suggestive for causing CLL [171]. However, abnormalities of *TCL-1* or its regulation have not been reported in patients with CLL yet.

Two different studies showed that CLL is characterized by genome-wide hypomethylation [172, 173]. Considering the fact that genome-wide hypomethylation results in chromosomal instability [174], it is possible that this feature is responsible for genetic aberrations observed in CLL cells (see below). It was also found that the overexpression of the Bcl-2, Mdr1, and Tcl1 genes could be due to promoter hypomethylation [175-177]. These results indicate that genome-wide and gene-specific DNA hypomethylation might have a role in the etiology of CLL diseases.

## 3.3 Clinical manifestations

CLL may have a wide range of symptoms with physical and laboratorial abnormalities at the time of its diagnosis or later. Common findings in patients requiring treatment include lymphadenopathy (87%), splenomegaly (54%), hepatomegaly (14%), total white blood cell count >100,000/μL (30%), hemoglobin concentration <11.0 g/dL (31%), and platelet count <100,000/μL (16%) [178]. Most patients are however diagnosed at routine health care visits without symptoms because of finding an elevated lymphocyte count.

Symptoms requiring initiation of therapy include weight loss (≥10% of the body weight within the previous six months), fever without the evidence of infection, night sweats, and fatigue (cannot work or unable to perform usual activities) [179, 180].

The presenting features may also relate to infections, autoimmune complications such as hemolytic anemia, or thrombocytopenia [179, 181].

## 3.4 Diagnostic criteria

In the World Health Organization (WHO) classification CLL is a disease of neoplastic B cells, whereas the entity previously called T-CLL is now uniformly included in the T-PLL (T-cell prolymphocytic leukemia) diagnosis [182].

It is important to distinguish CLL from other leukemic lymphoproliferative disease e.g., mantle cell lymphoma, splenic lymphoma with villous lymphocytes, marginal zone lymphoma, or hairy cell leukemia. Therefore a minimum of 2 examinations is required to diagnose CLL:

1) Evaluation of blood lymphocyte count
2) Immune phenotype of the leukemia cells in the blood [183].

A minimal panel of cell surface markers to distinguish CLL from other entities includes CD5, CD19, CD20, CD23, and surface Ig. Another characteristic of CLL is that the levels of CD20 and CD79b typically are lower than that of normal B cells. At least 5000 lymphocytes/μL is required for CLL for diagnosis [183].

Histological examination of the BM can assess the extent and pattern (diffuse, non-diffuse) of the BM infiltration by CLL but is not necessary for the diagnosis [179]. A marrow biopsy and flow cytometry is also indicated whether there is complete regression in the BM following treatment.

## 3.5 Staging

There are two traditional rather old clinical staging systems for B-CLL that is still in practice: the Rai system (1975) [184] and the Binet system (1981) [185]. These two classification systems are outlined in Table 2 and Table 3. Although the Rai and Binet systems by themselves are prognostic and help to guide treatment, there are other factors that recently have been shown to be superior predictive of disease prognosis (see below).

**Table 2.** Rai classification system for B-CLL [184].

| Risk level | Stage | Clinical features at diagnosis | Median survival time | % of patients |
|---|---|---|---|---|
| low | 0 | Blood and marrow lymphocytosis | ≥10 years | 30 |
| intermediate | I | Lymphocytosis and lymphadenopathy | 9 years | 35 |
| | II | Lymphocytosis and splenomegaly or hepatomegaly | 7 years | 25 |
| high | III | Lymphocytosis and anemia (hemoglobin <11 g/dl) | 5 years | 7 |
| | IV | Lymphocytosis and thrombocytopenia (platelets <100000/ml) | 5 years | 3 |

**Table 3.** Binet classification system for B-CLL [185].

| Stage | Clinical features at diagnosis | Median survival time | % of patients |
|---|---|---|---|
| A | Blood and marrow lymphocytosis and less than three areas of palpable lymphoid involvement | >7–10 years | 65 |
| B | Same with three or more areas of palpable lymphoid involvement | 5–7 years | 30 |
| C | Same plus anemia or thrombocytopenia | <2–5 years | 5 |

Lymph areas include cervical, axillary, inguiofemoral, liver, and spleen.

## 3.6 Prognostic factors

### 3.6.1 Conventional prognostic factors

Clinical stage is still the most commonly used predictor of survival in CLL, but are not sufficient to estimate the prognosis reliably for patients with early-stage disease (e.g., Binet stage A, Rai stages 0-II) [183]. Therefore, additional parameters have been required to more accurately evaluate the prognosis of patients with CLL. Among the clinical variables, age, gender and performance status are of importance as well as laboratory parameters reflecting the tumor burden or disease activity, such as serum β2-microglobulin, serum lactate dehydrogenase (LDH), bone marrow infiltration pattern, and lymphocyte doubling time (LDT) [186].

The chromosomal aberrations in 11q or 17p, $IgV_H$ mutation, and ZAP-70 expression have proven prognostic value independent of the clinical stage in several studies. Nonetheless, none of these parameters alone is useful to decide when to initiate therapy in patients with CLL.

### 3.6.2 $IgV_H$ mutation status

For a long time CLL B cells was considered to be driven from naive B-cells, however data from new studies over the past recent years revealed that CLL composed of about two equally numbered subsets, half with unmutated $IgV_H$ genes, and half with mutated ones [154, 155]. Mutational status was reported to be strongly correlated with prognosis of the CLL, so that $IgV_H$-mutated CLL patients survive about twice as long as $IgV_H$-unmutated ones [154, 155]. The $IgV_H$ usage status represents the strongest prognostic predictor in CLL [187, 188].

### 3.6.3 ζ-associated protein 70 (ZAP-70)

The cDNA microarray analysis on CLL B cells by Rosenwald et al [161] showed there are some differences between gene expressions of the $IgV_H$ mutated and unmutated groups of which ZAP-70 was the best identifier by overexpression in unmutated patients. Therefore it was suggested as a surrogate marker for distinguishing mutated from unmutated patients.

ZAP-70 is part of signal transduction system in T cells that upon TCR activation participate in transmitting the signal downstream to nucleus. It dose not usually expressed in normal peripheral B cells and its role in CLL cells is not clear although it was experimentally shown that it can enhance the effectiveness of IgM signaling [189] and produce an advantageous migratory and tumor cell survival response [190], thus contributing to aggressive clinical behavior [191].

Flow cytometry based assay for ZAP-70 has not achieved a satisfying correlation with $IgV_H$ mutation status [192-194]. However, ZAP-70 positive, $IgV_H$ mutated patients had a worse survival than those who where ZAP-70 negative, $IgV_H$ unmutated [195]. In a recent study on 156 CLL patients, immunohistochemistry detected ZAP-70 expression showed significant correlation with advanced Binet stage (B-C), diffuse BM infiltration, increased LDH, serum β2-microglobulin levels and LDT <12 months [191]. In addition, immunohistological detection of ZAP-70 on BM biopsies at diagnosis appears a useful methodological approach to identify patients with poor prognosis in CLL [191]. The importance of ZAP-70 expression as a prognostic factor is still under study. Standardized validated techniques for flow cytometry based ZAP-70 evaluation must be awaited until introduced in the routine health care system.

### 3.6.4 Chromosomal aberrations

Several chromosomal aberrations have been associated with CLL. In about 80% of CLL cases recurrent cytogenetic abnormalities has been shown [196]. Aberrations in CLL include del 13q14, trisomy 12, del 11q23, and del 17q13 [187, 197] among which del 11q23 and 17q13 showed prognostic values [183]. Aberrations in 17p and 11q occur almost exclusively in the $IgV_H$ unmutated subgroup and considered as high-risk genomic aberrations [187].

Del 11q23 is classically associated with younger patients with bulky lymphadenopathy and in many series a poor prognosis [187, 197] but this dose not fit with the all reported studies [196].

Del 17p13, which is associated with p53, is the subgroup that has the shortest average survival of 3 years [187]. It has been suggested that at least 20% of cells have to be affected to cause such a poor prognosis [196].

In the study by Krober et al, 17p deletion, 11q deletion, and unmutated $IgV_H$ status found to have the strongest adverse prognostic factors, suggesting that the clinical heterogeneity of CLL might have a biologic basis and genomic aberrations and $IgV_H$ mutation status might have a complementary role in estimating the prognosis of CLL [187].

### 3.6.5 CD38 expression

Although CD38 expression claimed as a clear adverse prognostic factor in CLL, it gives discordant results in $IgV_H$-mutation in about 30% of patients [196]. Its expression changes in about 25% of patients during the course of the disease [196]. Overall, CD38 dose not seem to be a good indicator for prognosis of CLL. Interestingly it has been shown that CD38-expression levels in T in B-CLL patients is

a valuable and independent prognostic factor in male patients, although the mechanisms of the sex-specific role of CD38 T cells in B-CLL is not clear [198].

## 3.7 Treatment of CLL

Treatment for patients with CLL has been improved over the past several years, owing to advances in understanding the biology of the disease. So far no treatment regimen has been shown to be curative in patients with CLL.

### 3.7.1 Which patient needs therapy?

Because no therapy against CLL has been shown to be curative and toxicities associated with the therapeutic regimens are inevitable, the decision to initiate therapy is an important consideration in the clinical management of CLL. It has been demonstrated that immediate therapy with chlorambucil did not prolong survival in patients with early-stage disease [199, 200]. In addition, about one third of patients with early-stage disease do not progress during long-term follow-up and may never need treatment.

The NCI Working Group has provided guidelines for when initiate therapy based on demonstration of active symptomatic and progressive disease [180]. Patients with advanced stage disease usually require treatment already at diagnosis.

### 3.7.2 Single-agent therapies

The most commonly used single agent for CLL treatment was the alkylating agent chlorambucil. With such therapy, the complete remission (CR) rate is however not more than 10%, and the overall response (OR) rate is approximately 50% to 60%, with median survival of 48 to 60 months.

Fludarabine has been the most extensively used purine analogue for the treatment of patients with CLL. The large US Intergroup trial randomized patients among fludarabine, chlorambucil, or the two agents combined [201]. The CR rate for patients in the fludarabine arm was 20% versus 4% in the chlorambucil arm ($p=0.001$). In addition, the OR rates were 63% and 37% for the fludarabine and chlorambucil arms, respectively. Although no survival advantage was confirmed for fludarabine, it may be the most effective single agent in the treatment of patients with CLL [202].

The purine analogue cladribine (2-CdA) and pentostatin (2-deoxycoformyin) also have activity as single agents in treating patients with CLL [203, 204].

In a recent publication of a phase II study, it was demonstrated for the first time that single agent lenalidomide is clinically active in CLL. Lenalidomide is an

immunomodulating drug with antitumor activity. 47% of patients with relapsing or refractory CLL attain a PR or CR to lenalidomide. Antileukemic effects were noted 7 days after the therapy, with incremental responses with extended therapy [205]. Lenalidomide also has T-cell stimulatory effect [96].

Monoclonal antibodies (mAb) are emerging as attractive agents in the treatment of B-CLL [206]. Alemtuzumab is a humanized anti-CD52 mAb that is approved for use in fludarabine-refractory B-CLL [207, 208]. Normal as well as malignant lymphocytes contain about 400,000 CD52 molecules per cell which is considered as high-density expression [209]. In humans CD52 is present on >95% of all lymphocytes, particularly on malignant B- and T-cells in virtually all lymphoid malignancies and on monocytes and macrophage [210]. The precise biological function of the CD52 antigen is unknown. The main side effect when using alemtuzumab is depletion of normal lymphocytes and thereby an increased risk of infections. Due to the expression of CD52 on normal lymphoid cells, long-lasting lymphocytopenia occurs in all patients [207, 211]. The most profound effects are on CD4 T cells, which reach their depths value after about four weeks of therapy, and thereafter show a slow but steady improvement [207, 211]. The rise in CD4 T cells after alemtuzumab therapy may partly be explained by expansion of a small population of pre-existing CD52 negative T cells [211-214]. The function(s) and specificity of these cells are yet unknown. Moreover, following treatment with the alemtuzumab the T-cell repertoire may be severely skewed [213, 215]. Furthermore, a reduced functional competence of T cells has been reported in rheumatoid arthritis patients after alemtuzumab therapy [216].

Not only alemtuzumab but also fludarabine therapy may lead to a decrease of T-cells, including CD4 and CD8 lymphocytes [217, 218]. This cellular immunodeficiency may contribute to an increased risk of opportunistic infections [219, 220]. Fludarabine may also induce dysfunction of T cells [219, 221].

Rituximab is a mAb against CD20 that exist on the surface of malignant B cells, as well as normal B and T cells [202]. The CR and OR rates when used as a single agent at standard doses, and the estimated median progression-free survival were low. The usefulness of maintenance of rituximab in patients with CLL is questionable and not currently supported [202].

### 3.7.3 Combination therapies

There is both laboratory and clinical evidence for synergy between alkylating agents and fludarabine. Fludarabine inhibits excision repair of DNA interstrand cross-links induced by cyclophosphamide, thereby supporting activity of this agent [222]. The combination of fludarabine and cyclophosphamide (FC) was evaluated in phase III

clinical trials versus fludarabine alone and enhanced efficacy in terms of CR rate and time to progressive was seen [223].

Rituximab supports activity of purine analogue-based therapies and has been incorporated a combination regimens. Rituximab sensitizes leukemia cells to fludarabine-induced apoptosis by downmodulating bcl-2 [224]. In addition, fludarabine downmodulates expression of complement-resistance proteins, CD46, CD55, and CD59, on malignant B cells, sensitizes them to rituximab-induced compliment-dependent cytotoxicity [225]. The combination of fludarabine, cyclophosphamide, and rituximab (FCR) has been evaluated in both chemotherapy naive and previously treated patients with CLL [226, 227]. In 224 previously untreated patients with CLL, the CR rate with FCR was 70% and the OR rate was 95%, with most patients having no detectable disease by FACS evaluation of the bone marrow at the end of therapy [226]. More than 40% of complete responders were free of disease in the bone marrow by PCR evaluating. The projected failure-free survival at 4 years was 69%. The efficacy and safety of administration of fludarabine and alemtuzumab combination (FluCam) on 36 patients with relapsed or refractory B-CLL has been evaluated in a phase II trial study by Elter et al [228]. This study showed the FluCam is effective in this group of heavily pretreated/refractory B-CLL patients, with an OR rate of 83% (CR rate, 30%) and a median OS of 35.6 months for all patients [228]. In addition this regimen was shown to be well tolerated and feasible [228].

Regimens combining chemotherapeutic agents with mAb have approached higher CR rates. Ongoing investigations continue to develop more effective regimens as well as new agents with different mechanisms of actions/targeting.

### 3.7.4 Stem cell transplantation

Autologous (ASCT) and allogeneic stem cell transplantation (allo-SCT) is considered as another treatment options for patients with B- CLL. Results of ASCT in CLL are conflicting and the place for this procedure in CLL remains under debate. Initial results of conventional allogeneic transplantation (allo-SCT) showed an unacceptably high mortality, but did show that cure was possible in some patients [229]. A proposal for choosing patients who are preferred for allo-SCT was recently released by an international expert panel [230]. Key elements of the consensus are:

1) allo-SCT is a procedure with evidence-based efficacy in poor-risk CLL;

2) allo-SCT is a reasonable treatment option for younger patients with (i) non-response or early relapse (within 12 months) after purine analogues, (ii) relapse within 24 months after having achieved a response with purine-analogue-based

combination therapy or autologous transplantation, and (iii) patients with p53 abnormalities who requiring treatment;

3) optimum transplant strategies may vary according to distinct clinical situations and should be defined in prospective trials [230].

## 3.8 Microenvironmental support to CLL clone

Signals that are delivered by direct cell contact or soluble factors, which may or may not occur concurrently with BCR engagement, may interfere with the growth of CLL cells. There are many studies on impact of other cells or immunological microenvironment on the CLL diseases. Despite the apparent long life *in vivo*, CLL cells usually undergo spontaneous apoptosis under conditions that support the growth of human B-cell lines *in vitro* [231-233]. This suggests that such *ex vivo* conditions require essential survival factors and that the resistance to apoptosis is not intrinsic to the CLL cells [231-233].

T lymphocytes as well as a variety of stromal cells, appear to have a central role in amplifying a microenvironment able to favor the proliferation and to inhibit the apoptosis of malignant cells [234-237]. It was shown that CLL cells from lymph node and bone marrow, but not peripheral blood, constitutively secret T cell attracting chemokine, CCL22, which attract the $CD4^+CD40L^+$ subpopulation of blood T cells expressing its receptor, CCR4. Following migration, T cells with their CD40L bind to CD40 on the CLL cells that in turn induces proliferation and a strong cytokine/chemokine production by the leukemic clone, including CCL22, which may lead to progressive accumulation of neoplastic cells [235].

Nurselike cells (NLCs) that differentiate from $CD14^+$ cells of blood mononuclear cells of CLL patients can protect CLL cells from apoptosis. NLCs express cytoplasmic vimentin, stromalderived factor 1 (SDF-1), very high levels of B cell-activating factor of the tumor necrosis factor family (BAFF) and a proliferation inducing ligand (APRIL) [237, 238]. $CD14^+$ cells from peripheral blood of healthy donors could differentiate into NLCs when co-cultured in contact with CLL B cells, directly [237] or indirectly [239], but not with normal B cells. So apparently CLL B cells alone could drive $CD14^+$ cells into NLC [239]. A clear link between APRIL signaling and B-cell malignancies came from the observation that mice transgenic for APRIL develop B1 cell associated neoplasm [240]. Multiple studies have reported that APRIL and BAFF levels are elevated in the sera of patients with CLL and both autocrine and paracrine APRIL and BAFF signaling exists in the CLL [240-242].

The effect(s) of BAFF or APRIL on leukemia cell survival appeared additive and distinct from that of SDF-1, which in contrast to BAFF or APRIL induced leukemia cell phosphorylation of p44/42 mitogen-activated protein kinase and AKT [237, 238].

Conversely BAFF and APRIL, but not SDF-1, induced CLL-cell activation of the NFκB and enhanced CLL-cell expression of the antiapoptotic protein Mcl-1 [237, 238]. Apparently BAFF and APRIL from NLCs can function in a paracrine manner to support leukemia cell survival and the distinct mechanisms of BAFF/APRIL from that of SDF-1 indicates that NLCs use multiple distinct non-overlapping survival pathways to support CLL cell survival [237, 238]. In addition, NLCs by providing CD31 to CLL cells support their survival [243]. Binding of CD38 on the CLL cell surface to its ligand CD31 triggers an extensive remodeling of the B-CLL membrane, with relocalization of BCR/CD19 to the CD38/CD31 contact areas, that increases cell survival and proliferation [243].

There is a short report of coexpression of CD38 and CD31 by CLL cells [244]. Therefore the autocrine or B-cell-to-B-cell activation by these two surface molecules can be speculated. CLL B-cells produce and express both ligands and receptors for many prosurvival cytokines, such as IL-2, IL-4, IL-8, TNF-α, INF-α and IFN-γ as well as vascular endothelial growth factor (VEGF), thereby presumably acquiring autocrine pathways that modulate survival [245, 246].

Follicular dendritic cells (FDCs) are closely associated with CLL cells in the early phase of BM involvement as well as in the lymph nodes [247] and they have been concerned in providing signals that stimulate B-cell lymphoma proliferation and survival [248-250]. *In vitro* culture of CLL B-cells with FDCs rescues CLL cells from spontaneous apoptosis [247, 250]. In a model system, where an immortalized FDC line was employed, prolonged CLL cell survival was found to be mediated by direct cell contact dependent on CD44-ligation, and associated with up-regulation of the anti-apoptotic Bcl-2 family protein Mcl-1 [250].

Environmental antigens or autoantigens could cause/support clonal expansion of CLL cells [142]. CLL cells frequently exhibit polyreactive receptors, which bind multiple antigens, including autoantigens, [251-254] allowing stimulation by both autoantigens and microbial antigens [142]. This mechanism is plausible for unmutated CLL and also for a few cases of mutated CLL, since many unmutated and some mutated $IgV_H$ genes encode such polyreactive receptors [142].

It has been suggested that variations in the environment may be responsible for changes in the clinical course of the disease [142].

## 3.9 T cells in CLL

Elevated total numbers of circulating T cells have frequently been reported in B-CLL patients, which is mainly due to an increase of $CD8^+$ T cells. Total number of $CD4^+$ T cells is also increased. Compared to healthy donors, total T lymphocyte count, $CD8^+$ cells and $CD4^+$ T cells in CLL patients have been reported to be increased about 3

fold [255-258]. A reduced expression of the CD28 co-stimulatory surface molecule correlating with advancing disease stage was noted on both CD4 and CD8 T cells [259, 260]. The reduction in expression of CD28 was more obvious on CD8 compared to CD4 T cells. Surface-bound as well as cytoplasmic CTLA-4 molecules showed a reversed pattern compared to CD28 [261]. The expression level of CTLA-4 was found to be positively correlated to advancing stage of the disease [260]. Within the CD4 T cell population a subset expressing CD57 but not CD28 and containing perforin was detected, suggesting cytolytic potential activity [262].

Spontaneous production of IL-4 and IL-2 has been shown in CD4 T cells, while GM-CSF and TNF-α are produced by both CD4 and CD8 T cells [263]. T cells from patients with progressive disease were more prone to produce cytokines as compared to controls and patients with indolent disease. IL-6 is known to be released by T cells in indolent disease [264, 265]. The reason for the constant cytokine production by the T cells of CLL patients is unclear but these cytokines have been suggested to be growth factors for the leukemic cells [266-269]. CD4 and CD8 T cells of B-CLL patients with indolent disease exhibit a dominance of a type 1 (IFN-γ) over type 2 (IL-4) cytokine production following a short incubation *in vitro*. In contrast, T cells from progressive patients continued to be predominantly type 2 [270]. The impact of type 1 and 2 T cells on the regulation of cancer immunity is still unclear but a shift from a type 1 to a type 2 cytokine pattern has been described in various tumors both in animals and human [261].

The cause for the activation and expansion of various T cell populations in CLL is not clear. A subpopulation of the activated $CD4^+$ T cells in B-CLL is known to express the CD40L (CD154). Ligation of the CD40 receptor on B-CLL cells to its ligand CD40L, induces the secretion of the chemoattractant cytokine CCL22 that in turn increases the migration capability of $CD4^+/CD40L^+$ T cells expressing the receptor for CCL22, CCR4 [235]. The chemoattracted CD4/CD40L T cells may migrate towards CLL cells, bind to CD40 receptor on CLL cells and induce chemokine/cytokine production by the leukemic clone, which may lead to progressive accumulation of the neoplastic cells [235].

CD40 crosslinking might upregulate the expression of anti-apoptotic genes like surviving through which facilitates proliferation of CLL cells [271, 272], thereafter cause the upregulation of surface activation markers like CD80 and CD95 as well as induction of chemokine production such as CCL22 and CCL17 [273-275]. Nevertheless, not all CLL cells respond to *in vitro* CD40 stimulation, indicating the existence of two populations of CLL cells [235, 276]. Activated CD4 T cells secrete several growth factors, which might support the growth of the CLL clone including IL-2, IL-4, TNF-α, GM-CSF, and IL-6 which may also support the growth of normal B cells [263, 264].

In addition to CLL clone, the production of BAFF by different sources may also affect T cell numbers and functions. It is known that activated T cells express one of the receptors for BAFF, called transmembrane activator and CAML interactor (TACI) [277]. *In vitro* studies have indicated that BAFF can stimulate T cell activation and proliferation [278]. BAFF-TACI interactions may thus be one of the possible pathways through which the T cells are activated and maintained in a state of chronic stimulation seen in B-CLL. In an attempt to experimentally verify the supportive role of T cell and stromal cells to CLL clone *in vitro*, short-term support to CLL clone was attributed to T cells while stromal cells showed long-term support [234].

T cell oligoclonal/polyclonal expansion in CLL was reported in several studies [279-281]. The reason for this has not yet been worked out however, it is most likely that polyclonal/oligoclonal expansion of T cells is induced by various factors/Ags released by the malignant cells and/or by surrounding non-tumor cells engaged in the disease process. Involvement of exogenous Ags might also be speculated. These factors also maintain the T cells in a state of chronic activation facilitating in turn the growth of B-CLL cells [248]. These activated T cells may continuously secrete cytokines, which may have an anti-apoptotic effect on the activated T cells and act in an autocrine or paracrine mode resulting in a polyclonal expansion [265, 282]. Clonal re-arrangement of the Vβ chain of TCR was noted in three out of five patients with stage 0 disease, but not in eight patients with advanced disease [281]. This study indicated that the presence of clonal T cells might represent a host response directed against tumor-related Ags or reflects a specific T-cell-to-B-cell interaction [261]. In another study on the analyzing of the usage of 20 T-cell receptor-β chain-variable (TCR-Vβ) subsets in B-CLL (n=10), a statistically significant overexpression of four TCR-Vβ subsets within the CD4 T cell population was found, while only one such subset was detected within the CD8 population. However, an examination of individual patients for overexpression of a particular Vβ family revealed that CD4 T cells of seven out of those ten patients and CD8 T cells of six out of those ten patients demonstrated skewing of the Vβ repertoire [283]. Upon stimulation with autologous leukemic B cells the existence of specific TCR-Vβ subset among the monoclonal/oligoclonal profile of T cells has been shown *in vitro,* therefore presence leukemia cell specific memory T cells *in vivo* in CLL patients has been suggested [279, 284].

# 4 AIMS OF THE THESIS

- To investigate the correlation between the expression of signal transduction molecules as well as cytokine production in CD4 and CD8 T cells from MM and B-CLL patients and their tumor burden.

- To evaluate long-term effects of fludarabine and alemtuzumab treatment on T-cell signaling molecule expression and cytokine production in B-CLL patients.

- To uncover the genes by means of which T cells might be involved in the regulation of the B-CLL clone.

- To uncover the genes that may be responsible for expansion of T cells in CLL.

# 5 PATIENTS AND METHODS

## Patients

The patients included in paper I had either stage I MM (n=11) all of which were non-progressive and in a stable phase (range 51-86 yr) or had stage III MM (n=11), all of which had symptomatic progressive disease requiring therapy (range 47-83 yr). Ten aged-matched healthy individuals were also included as controls. None of the patients had received prior chemotherapy before the experiment.

In the paper II, ten patients (mean age 68 yr; range 62–80 yr) with CLL in a progressive phase and 10 patients (mean age 69 yr; range 61–78 yr) with CLL in an indolent or plateau phase were included. Ten aged-matched healthy individuals were also included.

In paper III, nine patients who had been treated with fludarabine as most recent therapy were included as well as ten patients who had received alemtuzumab treatment. All patients were in long-lasting unmaintained partial remission and plateau phase following cessation of last therapy at the time of sampling. The median time from cessation of treatment to sampling was 24 months in the fludarabine group and 25 months in the alemtuzumab group. Ten patients with previously untreated indolent B-CLL and ten age-matched healthy control individuals were included as controls.

In paper IV, five B-CLL patient with indolent Rai stage 0-I disease and five patients with asymptomatic MM stage I were sampled for the microarray analysis. Five normal individuals were also included in the study as healthy control. Samples for subsequent experiments performed to confirm the results of the microarray were collected from 14 CLL patients, 6 MM patients, and 10 healthy donors.

The studies were approved by the institution’s Ethics Committee and informed consent was obtained from each patient.

## T-cell purification

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by separation on Ficoll/Isopaque gradient centrifugation. The B-CLL cells that formed the majority fraction of the PBMC were depleted by filtration through a nylon wool column (paper III and IV). Further enrichment of T cells (paper IV) was carried out by immunomagnetic depletion of B cells, NK cells, and monocytes using MidiMACS columns with anti-CD19, -CD56, and -CD14 MACS MicroBeads. For the MM patients and healthy donors, T-cells were purified using selection with immunomagnetic beads. The purity of T-cells was >95% as determined by flow cytometry using anti-CD3 monoclonal antibody.

For the QRT-PCR assay (paper IV), $CD4^+$ and $CD8^+$ T cells from healthy donors and MM patients were purified from PBMC by immunomagnetic selection using two runs of anti-CD4, -CD8 MACS MicroBeads, respectively. $CD4^+$ and $CD8^+$ T cells from CLL patients were purified with the same strategy starting with enriched T-cells passed through nylon wool columns. The purity of immunomagnetically purified CD4 and CD8 T cells were >95% as determined by flow cytometry using anti-CD3, -CD4, -CD8, -CD19, -CD14, and -CD56 mAb. The contamination of monocytes, B cells, and NK cells was <1%.

## Cellular staining and flow cytometry

Fluorescence-activated cell sorting (FACS) was performed to determine the purity of separated T cells (paper III and IV) and to characterize internal and surface markers (paper I-III). Surface markers were determined by staining with fluorochrome-conjugated mAbs. Appropriate concentration of antibodies were added to the cells in 100 µL staining buffer (PBS, 1% FCS, 0.1% azide) and incubated for 30 min at 4 °C in dark. For intracellular markers (paper I, II, and III), the cells were first fixed with 2% paraformaldehyde on ice for 10 min in the dark, then permeabilized with 0.1% saponin in PBS and after washing, incubated with the appropriate antibodies for 30 min at room temperature in the dark. After washing the cells were used for surface staining. Analyses were done using a FACSCalibur flow cytometer and the CellQuest® software. A minimum of 30,000 lymphocyte-gated events, as determined by forward and side scatter, were acquired and analyzed on $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD14^+$, and $CD56^+$ cells. Criteria for positive staining were set at fluorescent intensities displayed by <1% of the cells stained with the appropriate fluorochrome-conjugated isotype control mAbs.

## Proliferation assay

T cell proliferation (paper III) was measured by incorporation of [$^3$H]-thymidine. $2x10^5$ T cells from patients and healthy controls were stimulated with 5 µg/ml phytohemagglutinin (PHA), or 2.5 µg/ml tuberculin purified protein derivative (PPD) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat inactivated pooled human $AB^+$ serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in 96-well U-bottomed tissue culture plates and incubated in humidified $CO_2$ 5% at 37°C. After 126 hr of stimulation, 1 µCi [methyl-$^3$H]-thymidine was added to each well followed by further incubation for 18 hr. Cells were then harvested and incorporated thymidine was measured in a beta scintillation counter.

## Isolation of total RNA

Total RNA was extracted (paper IV) from freshly purified T cells using TRIzol reagent followed by further decontamination using the RNeasy mini kit to eliminate

the possible trace of genomic DNA contamination. The purity and quality of all extracted RNA samples were confirmed by measuring A260/A280 ratio and separation on agarose gel to ensure RNA integrity prior to microarray and QRT-PCR analyses.

### Microarray experiments and data analysis

In paper IV, first and subsequently second strand cDNA was synthesized from 5 μg high quality purified total RNA using a T7-$(dT)_{24}$ primer and SuperScript Choice system kit. Biotin-labeled cRNA was then synthesized. After fragmenting, the labeled cRNA was hybridized to HG-U133 oligonucleotide array chips. The arrays were then washed and stained with Streptavidin-Phycoerythrin (SAPE) in an Affymetrix fluidics station. The arrays were then scanned in an Affymetrix scanner and the expression values for each probe set were estimated using the Affymetrix Microarray Suite Software (MAS v5.0). 3′/5′ ratios for GAPDH and β-actin were confirmed to be within acceptable limits (<three-fold), and BioB spike controls were found to be present on all chips. In addition, the internal controls BioC, BioD and CreX were present in increasing intensity.

The signal values were then imported into the GeneSpring 7.0 software tool to find out genes with a significantly differential expression. In order to identify genes that show significant changes, genes were examined for significant up- or down-regulation of two fold and more. Filtering was done using the ANOVA p-value <0.05, indicating significant deviation from the control value or from ratio of 1.

Unsupervised hierarchical clustering analysis of the present probe sets was performed using GeneSpring 7.0 software with the minimum distance set to 0.001 and the separation ratio set to 0.95. Assessment of the overrepresentation of functional groups, according to Gene Ontology was carried out using the publicly available tool EASE (v2.0).

### Quantitative real-time RT-PCR (QRT-PCR)

Total RNA was extracted from purified T-cells of CLL patients, MM patients, and healthy donors. Random-hexamer primed 1$^{st}$ strand cDNA was then synthesized using SuperScript™ II Reverse Transcriptase. The QRT-PCR was performed using the iQ$^{TM}$ SYBR Green Supermix with the iCycler iQ$^{TM}$ Multi-Color detection system. All the QRT-PCR reactions were performed on 25 μL volume and duplicated.

Analysis of the sequences of interest was performed by comparative Ct method of relative quantification using β-actin as endogenous control and PBMC of a normal donor as calibrator. $2^{-\Delta\Delta Ct}$ gives the amount of target, normalized to the endogenous reference and relative to the calibrator.

## Statistical analysis

The non-parametric Mann-Whitney U-test was used to analyze differences between independent groups and the non-parametric Wilcoxon signed rank test for dependent groups was used to calculate statistical significance of the QRT-PCR results. The p-values $<0.05$ were considered significant. The statistical analysis for the gene profiling analysis is described in the preceding section on microarray experiments and data analysis.

# 6 RESULTS AND DISCUSSION

## Paper I

**Signaling molecules and cytokine production in T cells of multiple myeloma-increased abnormalities with advancing stage.** (British Journal of Haematology 2004;124:315–324)

There have been some previous studies on aberrant T cell-functions and -characteristics in patients with MM, such as abnormal expression of surface molecules and cytokine secretion profile [285-287]. T-cell immune dysfunction in patients with malignant tumors has been mainly attributed to the altered expression of components of the TCR/CD3 complex and their associated intracellular protein tyrosine kinases; the TCR signaling pathway. In this study, four-color flow cytometry was applied to study the surface bound molecules TCRαβ, CD28, CD152 and CD154 involved in T-cell signaling and the signal transduction molecules CD3ζ, Lck, Fyn, ZAP-70 and phosphatidyl-inositol-3 kinase (PI3-kinase) as well as the intracellular cytokines IFN-γ, IL-4 and IL-2 as a functional read-out of non-stimulated and superantigen (staphylococcus enterotoxins B)-stimulated blood T cells of MM patients at different stages of the disease. Chemotherapy naïve MM patients with stage I [80] (n=11), and stage III (n=11) disease or age-matched healthy individuals (n=10) were included in the study.

The results of this study shows multiple abnormalities particularly in those patients with a high tumor burden (stage III), both in freshly isolated T cells and following *in vitro* activation. Most surface expressed structures and down-stream intracellular signal transduction molecules appeared to be significantly downregulated, a finding that was particularly pronounced in patients with advanced disease. Although T lymphocytes in patients with MM had a normal expression of the TCRαβ heterodimer, i.e. the structure binding the antigenic peptide initiating 'signal 1' for T-cell activation, the co-stimulatory molecules mediating 'signal 2' (as CD28) were downregulated. This may hamper the interaction between CD28 on T cells and CD80/86 on normal as well as malignant B cells and DCs. Notably, surface markers such as CD152 and CD28 that 'down-tune' the immune response [288, 289], also appeared to be downregulated.

The signaling molecules CD3ζ-chain, Lck, Fyn and ZAP-70 were all generally downregulated and did not respond normally to an TCR activating signal. In addition, these abnormalities became more pronounced with advancing stage. Reduced levels of the protein kinases Lck and Fyn in T cells have been shown before in various malignancies [290-292].

The frequency of freshly isolated PBMC producing the IFN-γ and the IL-4 was increased in stage I MM patients but these T cells did not respond normally to a TCR

stimulating signal. Whether this might represent a chronic *in vivo* activation, induced by malignant B cells, is currently not yet known. The major finding in this study is that the expression of most signaling molecules in both CD4 and CD8 T cells declined with advancing stage.

These data may be of particular importance in relation to immune-based emerging therapeutic principles such as vaccination, which may then be explored particularly in early stage MM i.e. before T cell functions become severely impaired. Attempts should also be made to rectify the diminished T-cell compartments in patients. Treatment with the combination of new agents for T cell stimulation, with a through characterization of the functional status of T cells in individual patients may enhance the probability of achieving antitumor immunity in vaccine trials. Added benefits that may be potentially achieved are boosting the immune defense mechanisms and minimizing the risk of opportunistic infections during immunosuppressive therapy in patients with MM and B-CLL.

# Paper II

**Signaling Molecules and Cytokine Production in T Cells of Patients with B-Cell Chronic Lymphocytic Leukemia (B-CLL); Comparison of Indolent and Progressive Disease.** (Medical Oncology 2005;22:291–302)

Most studies in B-CLL focus on the intrinsic abnormalities of the malignant B cells. However, there was increasing evidence that aberrant T-cell signaling may abrogate immune surveillance against the leukemia or even foster survival and progression of B-CLL cells through the secretion of soluble factors [293].

This study was undertaken to examine signaling compartment as well as cytokine production in T cells of CLL patients with indolent and progressive stages. Ten patients in a progressive phase and ten patients in an indolent stage/plateau phase were included in the study. Ten aged-matched healthy individuals were also included. Four-color flow cytometry was utilized to determine the expression of the intracellular signaling molecules (CD3ζ chain, Lck, Fyn, ZAP-70 and PI3-kinase) as well as the T-cell regulatory cytokines (IFN-γ and IL-4).

Although there were no major statistically significant differences in the absolute number of IL-4 and IFN-γ-producing T cells *in vivo* between healthy donors and patients, there were major differences in intensity of these cytokines as assessed by mean fluorescent intensity (MFI). The reason for a high spontaneous secretion of IL-4 and IFN-γ in both CD4 and CD8 T cells in B-CLL patients is not clear but, is interesting to note that several T-cell derived cytokines including IL-4, IFN-α, and IFN-γ may inhibit apoptosis of B-CLL cells, particularly by upregulating bcl-2 [248].

The absolute numbers of T cells that expressed signaling molecules were generally similar in indolent patients, progressive patients, and healthy controls. However, the B-CLL patients showed significantly higher intensity of CD3-ζ-chain expression as compared to healthy donors, but there was no difference between indolent and progressive patients. Regarding the intensity of ZAP-70 in T cells, indolent patients showed significantly higher expression than healthy donors and progressive patients.

Cumulatively, the data of this study suggest that several but not all T cell signaling molecules may be normal or even overexpressed in B-CLL patients in relation to normal control donor's T cells and especially in patients with indolent CLL. In addition, the expression of CD3-ζ-chain and ZAP-70, which are key molecules in the initiation of intracellular TCR signaling pathways, as well as IFN-γ and IL-4, were more overexpressed in indolent patients than in progressive patients. The biological meaning of these findings remains unclear but it might be speculated that interaction of T cells and B cells during the indolent phase may be involved in the progression of the disease. Another possibility could be that as the disease progress, malignant B cells exert greater negative feedback on T cell functions,

despite the increasing number of T cells that accompany the expanding number of B-CLL cells as disease progression.

# Paper III

**Signaling molecules and cytokine production in T cells of patients with B-cell Chronic Lymphocytic Leukemia: long-term effects of fludarabine and alemtuzumab treatment.** (Leukemia & Lymphoma 2006;47:1229–1238)

Fludarabine and alemtuzumab are routinely used for treatment of B-CLL. Purine analogues such as fludarabine were able to produce significant improvements in remission rates and durations, however, alone they did not lead to improved survival [201]. Alemtuzumab is a humanized anti-CD52 mAb, which is approved for use in fludarabine-refractory B-CLL [207, 294]. CD52 is present on >95% of all lymphocytes and particularly on malignant B- and T-cells in virtually all lymphoid malignancies and on monocytes and macrophages. A small proportion (1-5%) of normal T cells are CD52 negative [211, 213, 214]. The main side effect of alemtuzumab is depletion of normal lymphocytes and thereby an increased risk of infections. Not only alemtuzumab but also fludarabine therapy may lead to a decrease of T-cells, including CD4 and CD8 lymphocytes and long-lasting reductions and suppressions of CD4 lymphocytes were observed following treatment with fludarabine [217, 218].

The aim of the present study was to compare the expression of signaling molecules and cytokine production by T-cells of B-CLL patients in long-term unmaintained remission/plateau phase following fludarabine or alemtuzumab treatment with that of indolent/untreated B-CLL patients and healthy donors. The frequency and intensity of TCR-CD3ζ chain, Lck, Fyn, ZAP-70, PI3-Kinase as well as IFN-γ and IL-4 production in CD4 and CD8 T cells was examined by flow cytometry. T-cell function was assessed by stimulation with tuberculin purified protein derivative (PPD) and phytohemagglutinin (PHA). Despite reduction in cell numbers, expression of IFN-γ and IL-4 in T-cells in the treated patients was significantly higher than in healthy donors. Intensity of most signaling molecules in treated patients was relatively unaffected versus healthy donors but lower than in untreated-indolent patients. The total numbers of T cells which expressed each of the signaling molecules however, were decreased in the patients with no difference between fludarabine and alemtuzumab treated patients. The T-cell response to PHA but not PPD was reduced in treated patients. The results suggest that despite some alterations in signaling molecules and a marked reduction in T-cell number, overall T-cell functions may be relatively well preserved after treatment with fludarabine or alemtuzumab.

# Paper IV

**Gene expression profiling of peripheral T cells in patients with indolent B-CLL.** (Manuscript)

Despite the apparent long life *in vivo*, CLL cells usually undergo spontaneous apoptosis under conditions that support the growth of human B-cell lines *in vitro* [231-233]. This suggests that such *ex vivo* conditions require essential survival factors and that the resistance to apoptosis is not intrinsic to the CLL cells [232, 233, 295].

There is substantial evidence that T cell functions are dysregulated in B-CLL patients and it has been speculated that T cells may contribute to the survival and growth of the leukemic clone [261]. In this paper, we have compared Affymetrix-platform's global gene expression profiles of purified T cells from the peripheral blood of untreated, indolent B-CLL patients with healthy donors and, as control, non-progressive multiple myeloma (MM) stage I patients in an attempt to delineate T cell factors that may have an impact on supporting the malignant B-CLL cells. We have also attempted to mark out genes whose deregulation may be related to the underlying the cause of the T cell expansion and aberrant functions noted in B-CLL.

The results of this study demonstrate that expression of a large number (356) of genes that are involved in different cellular pathways and activities including signaling, proliferation control, apoptosis, metabolism, immune response, and cytoskeleton formation are deregulated in comparison to healthy donors and patients with MM. The results of gene expression profiling was verified using quantitative real time PCR (QRT-PCR) on highly purified CD4 and CD8 T cells of 14 patients with B-CLL in indolent stage, 6 patients with MM stage I, and 10 healthy donors. The purification of T cells was carried out by immunomagnetic depletion of B cells, NK cells, and monocytes.

Three genes that demonstrated the greatest upregulation were the chemokines XCL1, XCL2, and the cytokine IFN-γ. CCL4 and CCL5 are two other important chemokines that also were found to be specifically upregulated in T cells of B-CLL patients, as well as the transcription factor KLF6. KLF6 increases the production of inducible nitric oxide synthase (iNOS), which in turn enhances the production of intracellular nitric oxide (NO). It was previously shown that NO inhibits the apoptosis of B-CLL cells [296-298]. Moreover, the immunosuppressive effects of NO, could partly explain the impairment in T cell function noted in CLL patients [298, 299]. TRAF1 is another factor that was observed to be upregulated in the T cells and could exert the antiapoptotic effect on T cells, contributing in expansion of T cells in CLL.

It might be assumed that these highly upregulated molecules, may have an effect on the survival of neoplastic cells. Further studies are needed to examine this and to get better understanding of B-CLL pathobiology.

# 7 FUTURE PROSPECTS

The annual statistical data reported by the American Cancer Society reveals that the rate of mortality from cancer has not changed over the past 50 years [300]. So far our knowledge about cancer appeared to have only minor effects on efforts to clinically control the cancer. The reason might be, at least in part, because of targeted cancer therapies and cancer biomarkers are in the middle of its way from lab/reports to clinical reality [300]. Following advances in the molecular diagnosis, especially by microarray, a new molecular signature of different types of cancers will be available which hopefully lead us to discover specific and more effective form of cancer therapy.

The cooperative impact of microenvironment or non-malignant cells to the malignant cells is very well known. In this study we have shown that in MM and CLL along with the malignant cells, T-cells are also abnormal in phenotype and function. We also have shown that in CLL, T cells release factors that may support the disease. Therefore in any therapeutically approach for restoring the T-cells, functionally and numerically, or specifically targeting the aberrant T cells/T-cells' destructive products in patients are of great importance. One interesting approach could be to restore and/or expand spontaneously occurring leukemia specific T cells (which exists in a very low number [261]) by new emerging techniques such as treatment with IL-2 [301, 302], *ex vivo* production of Xcellerated T cells using anti-CD3 and -CD28 coated beads [303, 304], treatment with CD28 superagonists [305], or perhaps lenalidomide [96]. If successful, such effort may lead to development of more effective immune-based therapy of MM and B-CLL. Some studies showed that activated and expanded T cells sustain a broad T-cell repertoire [306, 307], which is critical for raising an effective immune response to infection, cancer, and vaccination [308].

Many scientists are trying different approaches of immunotherapy to target MM and CLL. Vaccination, especially DC based, may be attractive. Since T cells, both CD4 and CD8, are central part of the adaptive immune response and since T cells in MM and CLL appears to be dysfunctional, such effort to restore T cells may be central.

Selecting the appropriate patient group seems to be another essential factor for clinical success. Therefore, it is very important to take the action against the disease as early as possible especially in MM patients who showed the correlation between T cell abnormalities and advancing disease. Patients with a pre-existing anti-tumor activity, although very weak, seem to be the candidates most likely to respond to vaccine therapy. It could be easier to boost an existing immunity than to induce de novo immunity against weak Ags [309, 310]. It has become clear that high number of

T cells attacking the tumor is of major importance [309]. The best approach should generate highest possible number of effector cells.

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