

Immunological aspects of acute myelogenous leukemia

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Scientific environment

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Elisabeth Ersvær

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Abbreviations

allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myelogenous leukemia
ALL	Acute lymphoblastic leukemia
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
cMPO	Cytoplasmatic myeloperoxidase
CR	Complete remission
DCs	Dendritic cells
FAB	French-American-British
Flt3	Fms-like tyrosine kinase 3
GVH	Graft-versus-host
GVL	Graft-versus-leukemia
HDAC	High dose cytarabine
HLA	Human leukocyte antigen
IFN γ	Interferon gamma
Ig	Immunoglobulin
ITD	Internal tandem duplications
LAAs	Leukemia associated antigens
mDC	Myeloid derived DC
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
mIg	membrane Ig
MM	Multiple myeloma
NK	Natural killer cells
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
pDC	lymphoid-derived DC
PKC	Protein kinase C
pMHC	peptide-MHC complex
SLE	Systemic lupus erythematosus
SR	Spontaneous remission
TAAAs	Tumor associated antigens
T _C cell	T cytotoxic cell
TCR	T cell receptor
TGF β	Transforming growth factor
T _H cell	T helper cell
T _{reg} cell	Regulatory T cell
VEGF	Vascular endothelial growth factor

Summary

Acute myelogenous leukemia (AML) is an aggressive malignancy characterized by accumulation of immature myeloid cells in the bone marrow. The overall disease-free survival is less than 50% even for the younger patients who can receive the most intensive treatment and hence, there is obviously a need for an improved therapy.

Short time until lymphoid reconstitution after chemotherapy seems to be a good prognostic factor in several malignancies, including AML, and targeting of autologous T cells is considered a possible therapeutic approach. Thus, the major aim of this thesis was to further characterize the immune system of patients with AML.

Disease and therapy induced T lymphocyte defects may contribute to the immune defects in AML. We therefore characterized the T lymphocytes of untreated AML patients as well as the remaining T cells in AML patients receiving intensive chemotherapy. Untreated AML patients were found to have close to normal lymphocyte subset ratios and also the *in vitro* T cell activation response, release of IFN γ , was close to normal after adjustment of cell numbers. In contrast, the released levels of IFN γ and GM-CSF cytokines from *in vitro* activated T cells in whole blood from cytopenia AML patients were lower than for healthy controls. However, the IFN γ and GM-CSF levels could be further increased by the protein kinase C agonist Pep005. The direct effects of the IFN γ on AML blasts included altered *i*) cytokine-dependent AML cell proliferation, *ii*) viability, and *iii*) balance between pro- and antiangiogenic chemokine release.

Several mutated or overexpressed proteins seem to be processed and presented to the immune system as tumor antigens leading to humoral and/or cellular responses. Autoantibodies are thus common in cancer patients but the autoantibody response in AML patients has been included in relatively few previous studies. Our studies report aberrant cyclin B1 expression in primary AML cells and detection of anti-cyclin B1 in a subset of sera from AML patients. In contrast, no autoantibodies against the typically SLE associated antigen P-protein could be detected in sera from untreated and chemotherapy treated AML patients.

To conclude, both the cellular and humoral immune system in AML patients differ from healthy controls. However, our results support the hypothesis that even these immunocompromized patients have remaining immunocompetent cells that can be targeted by immunotherapy.

List of Papers

Paper I

Ersvær E, Hampson P, Wendelbo Ø, Lord J, Gjertsen BT, Bruserud Ø. *Circulating T cells derived from patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex* (2006)

Paper II

Ersvær E, Hampson P, Hatfield K, Ulvestad E, Wendelbo Ø, Lord J, Gjertsen BT, Bruserud Ø. *T cells remaining after intensive chemotherapy for acute myelogenous leukemia show a broad cytokine release profile including high levels of interferon- γ that can be further increased by protein kinase C agonist PEP005.* (2006)

Paper III

Ersvær E, Skavland J, Ulvestad E, Gjertsen BT, Bruserud O. *Effects of interferon gamma on native human acute myelogenous leukaemia cells* (2006) *Cancer Immunol Immunother.* Apr 13; [Epub ahead of print]

Paper IV

Ersvær E, Bertelsen L-T, Espenes LC, Bredholt T, Bøe SO, Iversen BM, Bruserud Ø, Ulvestad E, Gjertsen BT. *Characterization of ribosomal P autoantibodies in relation to cell destruction and autoimmune disease* (2004) *Scand J Immunol.* Jul-Aug;60(1-2):189-98

Paper V

Ersvær E, Zhang JY, McCormack E, Ånensen N, Tan EM, Gjertsen BT, Bruserud Ø. *Cyclin B1 is commonly expressed in the cytoplasm of primary human acute myelogenous leukemia cells and serves as a leukemia associated antigen associated with autoantibody response in a subset of patients* (2006).

INTRODUCTION

1. *Acute myelogenous leukemia*

Acute myelogenous leukemia (AML) is a hematological malignancy characterized by clonal proliferation of immature myeloid precursors and an arrest in the maturation of these cells (1). This results in accumulation of leukemic blasts in the bone marrow (BM) and eventually in the peripheral blood (PB); other tissues are usually not affected. The bone marrow infiltration causes a decrease in the production and thereby a reduction of the peripheral blood levels of mature myeloid cells; platelets (resulting in thrombocytopenia and eventually hemorrhages), erythrocytes (anemia) and neutrophils (granulocytopenia and eventually infections). The initial presentation of patients with AML is with symptoms often related to this pancytopenia (i.e., thrombocytopenia, anemia, and neutropenia) and the symptoms can include weakness, fatigue, infections and/or bleedings (hemorrhage). The incidence of AML in Norway is approximately 125 new cases per year.

1.1. Diagnosis and classification

I. Diagnosis

The definitive diagnosis of AML requires demonstration of three diagnostic components:

- The presence of more than 30% (the older The French-American-British (FAB) standard (2, 3)) or 20% (the newer WHO standard (4)) leukemic blasts in a bone marrow aspirate.
- The detection of myeloid differentiation either by histochemistry or by analysis of myeloid membrane molecules usually by flow cytometry.
- Sub-classification according to (i) the FAB that is based on morphology and histochemistry; or (ii) a more detailed classification based on morphological characteristics together with clinical history (*de novo* or secondary leukemia) and genetic analysis in accordance with the new WHO classification (Table 1).

II. Diagnostic tools

The infiltration of leukemic blasts in the bone marrow is validated cytologically in a bone marrow smear to identify the blasts and highlight their nuclear and cytoplasmic morphology (4).

AML is a heterogeneous disease and the classification into appropriate variant is a part of the diagnosis. According to the morphological FAB classification (2, 3), AML can be divided into eight subtypes (M0-M7). The subtypes differ with respect to the myeloid lineage involved and the degree of leukemic cell differentiation. Recently, the World Health Organization (WHO) modified the FAB classification (4, 5) and grouped AML in four categories; i) AML with recurrent genetic abnormalities, ii) AML with multilineage dysplasia, iii) AML and myelodysplastic syndromes (MDS), therapy related, and iv) AML, not otherwise categorized (reflecting the old FAB classification subtypes M0-M7). The WHO classification is summarized in Table 1. An additional group of acute leukemia of ambiguous lineage is defined, these are rare leukemias and account for less than 4% of all cases of acute leukemia.

Table 1. WHO classification of acute myelogenous leukemia (4, 5).

AML with recurrent genetic abnormalities	<ul style="list-style-type: none"> ○ AML with t(8;21)(q22;q22), (AML1/ETO) ○ AML with abnormal BM eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22), (CBFβ/MYH11) ○ APL with t(15;17)(q22;q12), (PML/RARα) and variants ○ AML with 11q23 (MLL) abnormalities
AML with multilineage dysplasia	<ul style="list-style-type: none"> ○ Following MDS or MDS/MPD ○ Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineage
AML and MDS, therapy related	<ul style="list-style-type: none"> ○ Alkylating agent/radiation – related type ○ Topoisomerase II inhibitor – related type ○ Others
AML, not otherwise categorized	<ul style="list-style-type: none"> AML minimally differentiated AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/acute monocytic leukemia Acute erythroid leukemia (erythroid/leukemia and pure erythroid) Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis Myeloid sarcoma

Cytochemistry and light microscopy (primarily Wright-Giemsa or May-Grünwald-Giemsa staining) is the principle method for the diagnosis and FAB-subclassification of AML (1). To require the diagnosis all cases of AML, except M0, must stain positive for MPO and Sudan Black. Other stains that can be briefly mentioned are non-specific esterase (AML-M2, M4 or M5) and chloroacetate esterase (late myeloblast and early promyelocyte stage AML-M3) (1).

Immunophenotyping (i.e. flowcytometric analysis of differentiation-associated molecule expression) analysis plays a central role especially in separating between minimally differentiated acute myeloid leukemia and acute lymphoblastic leukemia (6). Immunophenotyping is generally performed by flow cytometry, but also by immunohistochemistry on slides, and the value of particular markers may differ depending on the technique. All hematopoietic cells express the common leucocyte antigen (CD45) and 40-60% of the cases will express the stem cell marker CD34 (1). Antigens most frequently used for myeloid lineage assessment are the highly specific myeloid antigens CD117 (c-kit) and cytoplasmic myeloperoxidase (cMPO). Other less specific myeloid antigens like CD13, CD33 and CD15, can also occur in acute lymphoblastic leukemia (ALL); and lymphoid markers like CD2 may also be aberrantly expressed in AML (1). In addition to determining myeloid lineage, immunophenotyping can identify monocytic, erythroid, or megakaryocytic differentiation. Markers related to lineage differentiation include CD14 and CD11b in AML-M4 and M5, glycophorin A in AML-M6 and platelet glycoproteins CD41, CD42 and CD61 in AML-M7 (1).

Cytogenetic analysis shows that 50-70% of patients with *de novo* AML have chromosomal abnormalities like translocation (t), deletion (del), inversion (inv) or aneuploidy (i.e. abnormal numbers of specific chromosomes or chromosome sets) (1). Trisomy 8 is the most common numerical cytogenetic abnormalities found in AML. Specific cytogenetic abnormalities (i) can be closely or uniquely associated with morphology and WHO-classification subsets; (ii) may be associated with previous chemotherapy in the WHO-subclasses associated with previous treatment with alkylating agents or topoisomerase II inhibitors; and (iii) can have additional diagnostic (t(8;21) and inv (16) classified as AML independent of the blast count according to the WHO criteria),

prognostic and therapeutic importance. Cytogenetic analysis includes the well-established routine analysis of G-Banded chromosomes, as well as molecular cytogenetics such as fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization (CGH). In addition to cytogenetic analysis the PCR-based techniques are also applied.

III. Prognostic features

If AML remain untreated, most patients will die during days or weeks depending mostly on the levels of blasts in the peripheral blood and bone marrow complications. The median survival for patients only receiving supportive therapy is only 3-4 months, and only exceptional patients survive for more than one year. Complete remission (CR) rates after intensive chemotherapy vary from 10 to 80%, approximately 10-20% of patients will have refractory disease and first line therapies will fail to achieve CR, and 5-20% of those treated will die during induction therapy (see below). This treatment-related mortality depends on the age and the general health condition of the patients and individuals above 60 years of age will usually not receive the most intensive chemotherapy with high-dose cytarabine. A large proportion of those in CR will relapse usually within 1-2 years, and their overall survival is less than 10% (1). It is generally accepted that patients with primary refractory disease AML relapse after intensive chemotherapy cannot be cured by conventional chemotherapy but only by allogeneic stem cell transplantation (7).

Age is a highly significant prognostic value as prognosis worsens with increased age. This is due to the increased treatment-related mortality in elderly patients and an increased frequency of high-risk karyotypic abnormalities. Response to the initial induction therapy and thus the time taken to achieve blast clearance is an important indicator to outcome (1). Failure to clear leukemic blasts by day 16 is a poor prognostic marker as reported by the German AMLCG group (8).

Approximately 60% of AML patients have an abnormal karyotype and this is highly predictive of response (9, 10):

- Approximately 25% of patients have favourable cytogenetics, i.e. t(15;17), inv(16), t(16;16) and t(8;21). These patients have a 5 year disease-free survival of around 65-80%.

- A smaller subset of patients (approximately 10%) will have adverse cytogenetics that include aneuploidy (-7, -5-5q), abnormalities of 3q or a complex karyotype. These patients have a 5 year survival of 10-20%.
- The remaining will have intermediate-risk cytogenetics, many of them with a normal karyotype, and a 5 year survival of 30-40% (1).

In-frame internal tandem duplication (ITD) mutations of the *Fms-like tyrosine kinase 3* (flt-3), exons 14-15, have been found in 15-30% of cases of AML. These mutations result in dimerization and constitutive activation of the receptor (11). ITD mutations have been shown to be an independent poor prognostic factor in several studies (1). In addition, there has been reported a point mutation of codon 835 of flt3 in 7-8% of cases of *de novo* AML (12), resulting in the upregulation of the function of the kinase domain. The prognostic significance of the point mutation remains uncertain although certain investigators have reported an adverse effect (1). On the contrary of the poor prognosis marker ITD-flt3, the mutations of *Nucleophosmin* (NPM), exon-12, occur in 40-50% of AML with normal karyotype and are predictors of favourable prognosis especially for patients without Flt3-ITD (13-16).

Gene expression profiling (GEP) by DNA microarray of AML is becoming more and more established and seem to be valuable, not only for diagnosing different cytogenetic subtypes but also for discovering novel AML subclasses (17). Several studies have emphasized that gene expression signature can be associated with prognosis (17-19).

1.2. The treatment of AML

The initial treatment of AML is remission induction therapy that aims to reduce the leukemic cells to below the cytologically detectable level of approximately 10^9 cells. This treatment is followed by a postinduction or “remission consolidation” therapy, consisting of one or more courses of chemotherapy or stem cell transplantation. The goal of the postinduction therapy is to eradicate

residual leukemia cells and thereby prevent later relapse. The treatment-related mortality is relatively low for consolidation therapy with conventional chemotherapy and autologous stem cell transplantation, whereas allotransplantation has a higher treatment-related mortality. The decision whether or not to recommend an allotransplantation is often based on the following criteria:

- The cytogenetic abnormalities (20) and karyotype (21) of the AML cells and whether they are associated with a high/intermediate/low risk of later relapse.
- The patients age and general health; allotransplantation is usually not recommended for patients above 55-60 years of age.
- Whether an HLA-matched sibling donor is available or an HLA-matched unrelated donor has to be used; the transplantation-related mortality is higher when unrelated donors are used.

I. Remission induction therapy

The most common *induction* remission regime is cytarabine given by continuous intravenous (IV) infusions (100 mg/m² per day) for seven days plus daunorubicin (45 to 60 mg/m² by intravenous short-time infusion) daily for the first three days (the “7+3” regimen). Approximately 60 to 80 percent of patients achieve a CR with this regimen (22-24). Typically, the side effects of this treatment are severe bone marrow suppression (myelosuppression), injury of the mucosal lining of mouth and throat (mucosities), and diarrhea. Alternative intensification regimen with intensification of induction therapy or addition of potentially non-cross-resistant drugs have been explored (25-30), but the relative benefits of these strategies or of long-term maintenance therapy are not firmly clear (31).

Supportive care, like transfusions and antibiotics, has reduced treatment related mortality for both young and elderly patients. Initial uncontrolled trials using GM-CSF or G-CSF suggested decrease in the duration of neutropenia after remission induction chemotherapy (1). However, data from more recent large controlled trials are variable (32, 33) and thus does not firmly support the previous conclusions drawn from the uncontrolled trials. Other cytokines and growth factors to be used as supportive care are under investigation but are not used in routine clinical practice (34).

G-CSF has also been used to sensitize (prime) leukemic blasts and thereby increase the effects of chemotherapy (35, 36), either through enhanced leukemic cell uptake of chemotherapeutic agents (35) and/or by driving resting cells into cell cycle (36). Some of these studies suggest improved overall survival in the subset of patients with intermediate-risk karyotypes (37, 38).

II. Postremission therapy

In general, there are three choices for postremission therapy: conventional consolidation chemotherapy, allogeneic hematopoietic cell transplantation (HCT), or autologous HCT.

Consolidation chemotherapy can be the same chemotherapy regimen used for remission induction or potentially non-cross-resistant drugs. High dose cytarabine (HDAC) seems to provide the best survival, at least for good and intermediate prognosis patients (24). This intensive consolidation therapy results in significantly longer survival than less intensive maintenance therapy alone (true for patients below 60 years of age) and if several courses of consolidation chemotherapy are given, survival at two or three years is 35 to 50 percent for young and middle-aged adults who have achieved CR (22, 24, 39, 40). HDAC cannot be used for patients above 60 years of age due to an unacceptable risk of severe neurological toxicity.

Bone marrow transplantation usually requires that the recipient's own bone marrow is destroyed (myeloblative therapy) or partly destroyed (nonmyeloblative therapy) prior to transplantation. Both peripheral blood mobilized and bone marrow stem cells can be used for transplantation. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) involves related (preferably an human leukocyte antigens (HLA)-identical sibling or an HLA-matched relative) or unrelated donor with the same HLA as the recipient. HLA genes can be categorized into two major types; Type I (e.g. HLA-A, HLA-B and HLA-C) and Type II (e.g. HLA-DR, HLA-DQB1). If there is no complete match, a partially matched donor can be considered. However, this last procedure will increase the risk of graft rejection or severe graft-versus-host (GVH) disease. One beneficial therapeutic component of allo-HCT is that the donor T cells may produce a specific graft-versus-leukemia (GVL) immune response, which may contribute to the eradication of remaining leukemia

cells. This immune response has been correlated with improved disease-free survival. Long-term disease free survival in adult patients receiving allo-HSCT in first CR is approximately 45-65% (41-46). In contrast, survival following allo-HCT in patients with relapsed AML is 35% or less (47). In autologous hematopoietic cell transplantation (auto-HCT) the patients' own hematopoietic stem cells (HSC) are isolated, stored and returned to the body after myeloablative therapy. Treatment-related morbidity and mortality are low ($\leq 5\%$), thereby allowing its use in patients up to 70 years of age, while relapse rates are relatively high (30-50%). Overall outcomes after auto-HCT are not clearly better than for patients who receive conventional chemotherapy (48-50).

1.3. Additional biological characteristics of the AML cells

Human AML cells are characterized by (i) constitutive cytokine release; (ii) expression of a wide range of cytokine receptors, including several hematopoietic growth factors; (iii) expression of various adhesion molecules involved in the crosstalk between AML cells, extracellular matrix elements and nonleukemic neighbouring cells; (iv) alteration in intracellular signaling pathways, especially in pathways involved in regulation of apoptosis (51-54). These biological characteristics are probably important for leukemogenesis, and these characteristics also seem to be of clinical importance as prognostic parameters:

- Previous studies suggest that autocrine proliferation, i.e. spontaneous *in vitro* proliferation due to autocrine/spontaneous/constitutive release of growth factors, is an adverse prognostic parameter associated with an increased relapse risk and decreased survival (51).
- The constitutive release of angioregulatory mediators seems to have a prognostic impact. A recent publication described that expression of Angiopoietin-2 was associated with a good prognosis (55), whereas VEGF release and high systemic levels of the angioregulatory molecules VEGF and endostatin seem to be associated with an adverse prognosis (56).
- Intracellular signaling events seem to affect prognosis: (i) specific intracellular phosphoresponses to exogenous cytokines seem to be associated with prognosis (52); and (i) the intracellular

balance between pro- and antiapoptotic signaling seem to influence the risk of relapse after intensive chemotherapy (54).

Taken together these observations suggest that leukemogenesis and chemosensitivity depend both on intracellular AML cell characteristics as well as the extracellular interactions between AML cells and matrix molecules or neighbouring nonleukemic cells.

2. The immune system

The immune system (57, 58) may be divided into two major compartments; innate immunity that is antigen non-specific and adaptive immunity that involves the antigen specific humoral and cellular arms of the immune system. However, the innate and adaptive responses are a highly cooperative system, increasing the efficiency of immune responsiveness.

2.1. The innate immune system

Innate immunity (59, 60) constitute the antigen-independent immune mechanism generally involving i) surface barriers including antimicrobial peptides, ii) mononuclear phagocytes (e.g. monocytes and macrophages), iii) polymorphonuclear phagocytes (i.e. neutrophil, eosinophil and basophil), iv) natural killer (NK) cells, v) dendritic cells (DCs), and vi) complement activation. NK cells and DCs are especially important in tumor immunology and immunotherapy.

NK cells are lymphocytes capable of both directly killing of target cells and production of immunoregulatory cytokines (61, 62). Mature NK cells are primarily found in PB (10-15% of total lymphocytes), and typically they express the low affinity receptor for the Fc portion of IgG (CD16), CD56 and CD161 (61, 62). Phenotypically distinct NK cell populations have been suggested to represent independent subsets specialized to primarily mediate one or more NK cell functions based on different levels of spontaneous cytotoxicity or of cytokines produced (61, 62).

DCs are professional antigen presenting cells (APC) and a central player in all immune responses, both innate and adaptive (63). By phagocytosis, endocytosis, pinocytosis, or receptor-mediated uptake, DCs capture antigens for immune presentation. After capture of the foreign material, DCs mature and transport the antigens to lymphoid follicles to deliver it to the B lymphocytes and to present antigenic peptides to the T lymphocytes. As a result, the specific immune responses are induced (63). In PB, there has been described at least two DC subsets; the myeloid-

derived CD11c⁺CD123⁻ DCs (mDCs) and lymphoid-derived CD11c⁻CD123⁺ DCs (pDCs). pDC seem to support the generation of a T_{H2} response, while mDCs predominantly support a T_{H1} response (see below) (64-66).

2.2. The adaptive immune system

Humoral (involving B cells) and cell-mediated (involving T cells) immunity constitute the antigen-dependent immune mechanisms (67).

I. Antigen processing and presentation

The function of the APCs is to present the antigen to the B and T lymphocytes. B cells interact directly with the antigen, via their cell surface B cell receptor (BCR; consisting of membrane immunoglobulin (Ig) and Ig α /Ig β heterodimer), to differentiate into cells that produce antigen-specific antibodies (67). Unlike B cells, antigenic peptides are presented to T cells in complex with HLA molecules from the major histocompatibility complex (MHC) genome area class I or class II. Of the nine classical MHC-genes the HLA-A, B and C genes belong to class I, while six HLA-D genes belong to the class II. Generally, class II MHC presents peptides derived from proteolysis of extracellular antigens in endosomal-type compartments, while class I MHC presents peptides primarily originating from intracellular degradation of proteins in the cytosol (57, 58, 67).

II. T cell maturation

Progenitor double-negative (CD4⁻CD8⁻) T cells from the bone marrow enter the thymus and rearrange the T cell receptor (TCR) genes to become TCR $\alpha\beta$ ⁺ CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells (the majority) or TCR $\gamma\delta$ ⁺ CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells (67). Positive selection in the thymus eliminates T cells unable to recognize self-MHC (i.e. MHC-restriction) and negative selection eliminates thymocytes with high-affinity receptors for self-MHC molecules alone or autoantigen plus

self-MHC (i.e. self-tolerance) (67). Less than 5% of T cells in humans are TCR $\gamma\delta^+$ T cells. In general, $\gamma\delta$ T cells are not MHC restricted, and most do not express the CD4 and CD8 coreceptors. $\gamma\delta$ T cells seem to recognize epitopes and bind to these in much the same way as immunoglobulin receptors of B cells (67).

III. T helper cells

The generation of both humoral immune responses (by B cells) and cell-mediated cytotoxicity (by cytotoxic T (T_C) cells) depends on the activation of CD4⁺ T helper (T_H) cells. Initial T_H cell activation is initiated by the interaction of the TCR-CD3 complex (68) with the processed antigenic peptide bound to a class II MHC molecule on the surface of an APC. TCRs are cell surface heterodimers consisting of either disulfide-linked α - and β - or γ - and δ -chains. Each TCR chain is composed of variable and constant Ig-like extracellular domains, a transmembrane domain and a short cytoplasmic tail. The $\alpha\beta$ TCRs bind peptides-MHC (pMHC) with low affinity (~1-100 μ M) through complementary-determining regions (CDRs) in their variable domains (69). The interaction of the TCR-CD3 complex with the antigenic-peptide MHC molecule is followed by binding of coreceptor CD4 to the invariant regions of the MHC molecule, leading to the assembly of a signaling complex. CD4-associated Lck, a protein tyrosine kinase, phosphorylates the immune-receptor tyrosine-based activation motifs (ITAMs) of CD3 ζ -chain and thus create a docking site for ZAP70 (67). ZAP70 then phosphorylates adaptor molecules that recruit components from several signaling pathways like Pkc-mediated pathways, CA^{2+} - mediated pathways and small G-protein mediated pathways like Ras and Rac. Finally, these events lead to changes in gene expression by several transcription factors (i.e. NFAT, NF-kB, and Elk) (67).

Naive cells require more than the initial interactions, described above, to be fully activated and subsequently proliferate into effector cells. An antigen nonspecific co-stimulatory signal is provided by interactions between CD28 on the T cell and members of the B7 family (B7-1 and B7-2) on the APC. The ligands for B7 are CD28 and CTLA-4 (CD152) which both are expressed on T cells and act antagonistically (67). That is, signaling through CD28 delivers a positive co-stimulatory

signal to the T cell while signaling through CTLA-4 is inhibitory and down-regulates the activation of T cells. If CD28 co-stimulatory signaling occurs, T cells are triggered into G₁ phase of the cell cycle, transcription of the gene for IL-2 and the α -chain of the high-affinity IL-2 receptor (CD25) are induced. The subsequent secretion of IL-2 and its binding to the IL-2 receptor induces the activated naive T cells to proliferate and differentiate into long-lived memory or short-lived effector T cell populations (67). T_H CD4⁺ effector cells form two subpopulations: the T_{H1} subset (IL-12R β 1 β 2⁺) secretes IL-2, IFN γ and TNF- β , and generally aid the activation of cytotoxic T lymphocytes, while the T_{H2} subset (IL-12R β 1⁺) secretes IL-4, IL-5, IL-6 and IL-10, and is generally important as helper cells for B cell activation (57). The cytokines produced by the two subsets also have a cross-regulatory role, i.e. cytokines secreted from activated T_{H2} cells will down-regulate the T_{H1} cells in the neighborhood and vice versa (67).

IV. Cytotoxic T cells

CD8⁺ T cells recognize antigen presented by MHC class I and function as T_C cells (67). Activation of resting T_C involves first of all TCR stimuli and then secondly stimulation with cytokines, especially IL-2, most probably from activated T_H cells. Naive T_C does not express IL-2 receptors on their surface but antigen stimulation increases the expression of the IL-2 receptor and ensures that only the cells recognizing the antigen will become activated (67).

Like the CD4⁺ T_{H1} and T_{H2} subsets, effector CD8⁺ T cell subsets can also be identified; T_{C1} and T_{C2} (70). The proximal signal for inducing T_{C1} and T_{C2} seem to be IL-12 and IL-4 cytokines, respectively. The CD8⁺ T cell subsets are suggested to differ in homing as T_{C1} is found to express CC chemokine receptor 5 (CCR5) and T_{C2} express CCR4 (70).

Cytokine-induced killer (CIK) cells are a unique and rare (1-5% in uncultured PBMC) population of cytotoxic T lymphocytes with a characteristic CD3⁺CD56⁺ phenotype. CIK cells are non-MHC-restricted in target cell recognition and killing (71).

V. Regulatory T cells

Tolerance is generally divided into central and peripheral tolerance (72). Central tolerance is the clonal deletion and inactivation of self-reactive immature lymphocytes during differentiation (see above). Peripheral tolerance concerns mature circulating lymphocytes and it is proposed that regulatory T cells are responsible for inducing and maintaining peripheral tolerance. The classical regulatory T cells are the thymus-derived $CD4^+CD25^+FOXP3^+$ T_{reg} cells. However, several phenotypically distinct regulatory T-cell populations of both $CD4^+$ and $CD8^+$ subset have been suggested. Possibly, T_{reg} cells can mediate tolerance by suppressing self-antigen-reactive T cells by various mechanisms, including:

- inducing B7-H4 expression by APCs, which in turn induce T cell cycle arrest through B7-H4;
- directly killing of target cells such as T cells and APCs through perforin- or granzyme B-dependent pathways;
- $CTLA4^+$ T_{reg} cells can induce indoleamine 2,3-dioxygenase (IDO) expression by APCs which in turn suppress T cell activation by reducing tryptophan;
- Release of IL-10 and transforming growth factor ($TGF\beta$) and thereby direct inhibition of T cell activation and suppression of APC function by decreasing their expression of MHC molecules, CD80, CD86, and IL-12 (72).

VI. B cells

Antigen-independent maturation of B cells, involving Ig gene rearrangements, occurs in the bone marrow and generates mature naive B cells expressing membrane IgM (mIgM) and mIgD with a single antigenic specificity (67). During maturation, the self-reactive B cells are eliminated by negative selection. The activation, proliferation and differentiation of naive B cells occur in the periphery and require the presence of antigen. The antigen recognition molecule of the B cell is the surface Ig that associates with the heterodimer $Ig-\alpha/Ig-\beta$, thus forming the BCR. The B cell co-receptor (complex of CD19, CD21 and CD81) provides stimulatory modifying signals that enhance B cell responses. B cell activation through antigen binding to BCR, can occur by two different routes

depending on the nature of antigen, one route is dependent on T_H cells and the other is not. Antigens that can activate B cells in the absence of direct interaction with T_H cells are known as thymus-independent (TI) antigens. TI antigens can be further divided into two groups; (i) TI-1 antigens that are truly T cell independent and give a weaker response with no memory cells formed and IgM as the predominant antibody; and (ii) TI-2 antigens that require T_H cytokines both for efficient B-cell proliferation and for class switch to isotypes other than IgM. The B cell response to thymus-dependent (TD) antigens requires the direct contact with T_H cells. The TD-antigens bind to mIg on B cells and become internalized by receptor-mediated endocytosis, processed into peptides, and presented in MHC class II molecules. Once T_H cells recognize the MHC-peptide, the two cells form a T-B conjugate. This leads to T_H cell cytokine release as well as upregulation of CD40L to provide an essential signal for T cell dependent and antigen-specific B cell activation. Once activated, B cells begin to express membrane receptors for IL-2, IL-4, IL-5 as well as other cytokines. These receptors bind the T_H cytokines and thus induce differentiation with Ig class switching and development of antibody-secreting plasma cells, memory B cells and affinity maturation (67).

3. The immune system in AML patients

It is well known that AML patients are immunocompromized and have an increased risk of infections. These patients often have neutropenia initially due to the disease and later eventually due to intensive chemotherapy. However, as will be seen from the studies reviewed below these patients can also have other disease- or therapy-induced immune dysfunctions. One would in addition expect that there will be differences between patients due to for example differences in chemotherapy regimen, different AML cell phenotypes and age-dependent alterations of the immune system.

3.1. The immune system in untreated AML

I. Cellular innate immunity in AML

Elevated levels of cells with NK phenotype have been found in AML (73). The CD3⁺CD56⁺ NK cells were found significantly increased at diagnosis both in the blood and bone marrow. In contrast, CD16⁺CD2⁺ and CD16⁺CD2⁻ NK cells were only increased in the blood whereas there were normal levels in the bone marrow. When the authors divided the AML cases into two groups according to the absolute number of circulating NK cells, the patients with the highest levels also showed an increased proportion of circulating leukemic blasts. In contrast to elevated NK cell levels, several other studies report of NK cell dysfunction (74-76) or impeded NK cell maturation (77) in patients with cancer and leukemia. In addition, it has been suggested that leukemic cells display a more inhibitory AB killer cell immunoglobulin-like receptor (KIR) phenotype, compared to healthy controls, in favor of escape from NK cell immunity (78). It is difficult to make a firm conclusion

from these studies, but it is justified to state that both qualitative as well as quantitative disturbances in the NK cell system can be detected in cancer patients and probably also in AML patients.

DCs are central in the presentation of tumor antigens to the adaptive arms of the immune system. There are several reports of DC defects in cancer patients (reviewed in (79)). Abnormal frequencies as well as abnormal differentiation and/or maturation possibly caused by tumor derived factors, are some of the dysfunctions described (79). A number of studies throw light on the generation of leukemic DCs in the context of immunotherapy, but there are few studies describing the remaining normal DCs in AML. Mohty et al. (80) reported a quantitative imbalance in circulating blood myeloid DCs (MDCs) and plasmacytoid monocytes (PDCs) in 70% of the AML patients.

II. Cellular immunity in AML

Altered ratios of T_{H1} versus T_{H2} or T_{C1} versus T_{C2} cells have been observed in several malignancies (81-90). However, a general decreased T cell number without such shifts has also been reported (91, 92), as well as close to normal or increased T-cell numbers (93, 94). A possible explanation for this variation is that there are differences between malignancies and possibly also between individual cancer patients.

An abnormal CD4:CD8 ratio has been reported in several malignancies, and this can be due to increased or decreased absolute levels of $CD8^+$ cells (95-97) and/or increased or reduced levels of the $CD4^+$ cells (97-99). In patients with head and neck cancer increased CD4:CD8 ratio was observed in lymph node lymphocytes versus tumor-infiltrating lymphocytes and peripheral blood lymphocytes (PBLs). In these cases the aberrant ratio was attributable to both a significant enrichment in $CD4^+$ T cells as well as a decrease in $CD8^+$ T cells (97). In contrast, analysis of patients with non-small cell lung cancer revealed that tumor infiltrating lymphocytes displayed a lower CD4:CD8 ratio than the PBLs (100). In peripheral blood of patients with multiple myeloma the percentage of $CD3^+$ and $CD8^+$ cells was within the normal range while the percentage of $CD4^+$ cells was slightly reduced for a small subset of patients. On the contrary, in bone marrow of myeloma patients the percentage of $CD4^+$ was profoundly reduced, leading to an altered CD4:CD8 ratio in all multiple myeloma patients (99).

There are some studies suggesting that cancer patients have more memory and less naive T cells applicable for PBL or tumor infiltrating lymphocytes or both (101-104). In hematological malignancies there has also been suggested decreased memory T helper cells (105, 106).

There are a few reports regarding the T lymphocyte subsets in patients with de novo AML. Vidriales and colleagues (73) reported (i) increased T lymphocytes with NK activities ($CD3^+CD56^+$) in PB but normal levels in BM, (ii) normal distribution of $CD4^+CD45RA^+$ (putative naïve) and $CD4^+CD29^+$ (putative memory) cells in the PB, and (iii) increased distribution of the cytotoxic subset $CD8^+CD57^+$ within the $CD8^+$ cells (73). A more recent report (107) outlined that the absolute numbers of $CD8^+$ and $CD8^-$ (putative $CD4^+$) $CD3^+$ T cells in whole blood of 13 patients with AML were similar to those of healthy controls. However, there was a tendency of higher numbers of $CD8^+$ T cells in the patients compared with healthy controls, which was also mirrored in the lower CD4:CD8 ratios (107). These investigators (107) also measured the intracellular cytokine levels of the whole-blood lymphocytes in the absence of *in vitro* stimulation. They did not find any significant changes in the IL-4, IL-10, IL-12 or $IFN\gamma$ levels in the cell subsets derived from AML patients compared with healthy individuals and thus suggesting normal T_{H1} and T_{H2} profile. However, a trend towards higher absolute numbers and percentages of $CD8^+$ and $CD8^-$ lymphocytes with detectable IL-10, IL-12 and $IFN\gamma$ was observed for the AML patients compared with the healthy controls. Again it is difficult to reach a firm conclusion, and this is partly due to the question of patient heterogeneity and the low number of patients in some of the studies.

T_{reg} cells control autoimmune T cell reactivity in the periphery and may also suppress immune responses against cancer cells (72). An increased number of T_{reg} cells has been reported for patients with ovarian cancer, lung cancer, breast cancer, gastrointestinal malignancies and lymphoma (108-114). Wang et al. reported significantly higher proportions of $CD4^+CD25^{high}$ T_{reg} cells in AML patients compared with healthy controls (115). These cells were (i) $CD45-RA^-$, $CD69^-$, $CD45-RO^+$, $CD95^+$, intercellular $CTLA-4^+$; (ii) secreted low levels of $TNF-\alpha$ and IL-10 and did not release IL-2, IL-4, IL-5 and or $IFN\gamma$; and (iii) behaved as T_{reg} cells by inhibiting $CD4^+CD25^-$ T cell proliferation and cytokine production during *in vitro* activation (115).

III. Humoral immunity in AML

There are sporadic reports of spontaneous remission (SR) of cancer, including leukemia and myelodysplasia (116, 117), often but not necessarily following bacterial infections or blood transfusions (118). Some authors have suggested that the mechanism leading to SR is an underlying humoral immunologic response (116), although there are also other possibilities (119, 120). However, spontaneous remission in cancer is rare, despite the presence of naturally serum antibodies against tumor-associated antigens (TAAs) in 14-31 per cent of patients (121). It is not known why these antibodies against cancer are not more effective. On the other hand, the use of antibody therapy in cancer treatment suggests that cancer-reactive antibodies can mediate strong anti-cancer effects (122). However, despite the encouraging experience with antibody therapy in certain malignancies it is at present hard to believe that humoral immune responses alone can be responsible for spontaneous remissions in cancer patients.

There is an expanding list of autoantibodies against tumor-associated antigens (TAAs) that can be detected in cancer patients, including anti-oncoprotein (HER-2/neu), anti-tumor suppression antigen (P53), anti-proliferation associated antigens (cyclin A, cyclin B1, and CDKs), anti-onconeural antigens (Hu, and Yo), and anti-cancer/testis antigens (NY-ESO-1 and MAGE-1), and others (see (123) for review). The identification of a cancer autoantibody signature may become useful as a diagnostic and prognostic specimen (124-126), but their possible role in the immune defence against cancer need to be further investigated.

The number of autoantibodies known to occur in AML patients is few compared to other malignancies. However, autoantibodies against the following antigens have been detected at an increased frequency in AML patients compared with healthy controls: Wilms tumor gene product (WT1) (127, 128), single-stranded DNA (129), anticardiolipin antibodies (ACA) (130), the M-phase phosphoprotein 11 (MPP11) (131), receptor for hyaluronan acid-mediated motility (RHAMM) (132), MAZ (133), PASD1 (134), and Rhamm-like protein (135). Such antibodies have been reported for 20

to 100 per cent of the AML patients examined. Some of these autoantibodies have been detected in either conditions associated with tissue destruction such as systemic lupus erythematosus (SLE) (ssDNA) (129) or infections (ACA) (130). Some studies have in addition reported the disappearance of autoantibodies in AML patients when they reach hematological remission (128, 136), possibly as a consequence of a lower AML cell burden and decreased antigenic stimulation leading to decreased autoantibody production.

IV. Leukemia derived factors affecting the immune system

Numerous immunosuppressive factors like TGF- β , IL-10, vascular endothelial growth factor (VEGF) and sFas-L have been detected in tumor microenvironments (137). Such tumor-derived factors can for instance induce apoptosis in T cells (138-140) or inhibit a Th1 response (141) and thereby contribute to local immunosuppression. The AML microenvironment has also been shown to inhibit T cell cytotoxic activity (142, 143), and AML culture supernatants show a TGF- β , IL-10 and VEGF independent inhibition of T cell activation, T cell proliferation and Th1 cytokine production (143). AML supernatants have also been shown to affect major signaling pathways involved in T cell activation and proliferation;

- reduced nuclear translocation of NFATc and NF- κ B;
- delayed activation of c-Jun N-terminal kinase 1/2;
- no phosphorylation of pRb, cyclin-dependent kinase 6/4-cyclin D, and of p130 was found;
- no induction of c-Myc, cyclin D3, and p107.

However, calcium mobilization, extracellular signal-regulated kinase 1/2, p38, and STAT5 remained unaffected (143).

Misoguchi et al. (144) were the first to suggest that immune dysfunction in cancer patients was due to an altered composition of the T-cell receptor signaling complex. Today, decreased expression of CD3- ζ (CD3- zeta) in T cells have been demonstrated in several malignancies (145-148). Reduced CD3- ζ expression in tumor infiltrating lymphocytes even seems to be an independent

prognostic factor for patients with oral carcinoma (149). Furthermore, Buggins et al. (150) reported abnormal expression of CD3- ζ in 64 % out of 46 myeloid leukemia patients examined (of which 11 were acute) and successful remission induction was associated with recovery of CD3- ζ expression. Moreover, the CD3- ζ associated protein tyrosine kinases (p56^{lck}, p59^{lyn}, and ZAP-70) showed variable but often reduced expression in these patients (150). Finally, in a murine AML model (151) there were seen reduced responses (proliferation and IL-2 secretion) to mitogenic anti-CD3- ϵ but not to PMA/ionomycin (which is TCR independent) as early as one week following the injection of leukemic cells, whereas loss of CD3- ζ protein expression and signaling abnormalities (calcium mobilization and tyrosine kinase activity) were detected only in advanced disease (4 weeks after injection). It was also shown *in vitro* that leukemia-derived factor(s) stimulated splenic macrophages to secrete a second soluble factor(s) that caused the loss of CD3- ζ (151). Thus, altered intracellular signaling during T cell activation probably contributes to the immunodeficiency in AML.

3.2. The immune system in AML patients after chemotherapy

I. Effects of conventional chemotherapy on the cellular immune system

Patients receiving conventional intensive chemotherapy or myeloblastic treatment prior to auto-SCT, encounter a post-treatment period of severe cytopenia, including T lymphopenia (152, 153). Hematopoietic reconstitution (neutrophils, monocytes, platelets) often occurs relatively early after chemotherapy compared with lymphoid reconstitution (153).

For patients receiving conventional chemotherapy most peripheral leukocytes are T lymphocytes, whereas B lymphocytes and monocytes show a wide variation among patients and usually represent less than 10% of the cells (154). Most of the circulating T cells in these patients express the activation markers HLA-DR as well as CD25 and CD69 (153). Following chemotherapy there seems to be an absence of CD45RA⁺CD4⁺ T cells, all the remaining CD4⁺ T cells expressed the CD45RO⁺ isoform (152, 153).

There has also been some reports regarding functional evaluation of T cells, these have included the examination of cytokine release and cytokine responsiveness of the *in vitro* expanded clonogenic T cell minority derived from AML patients with therapy-induced cytopenia (155-159). Circulating CD4⁺ and CD8⁺ TCRαβ⁺ T cells include a minor subset of clonogenic cells (155) that secrete a range of immunoregulatory cytokines (156). The frequency of these clonogenic T cells is often reduced compared to healthy individuals (155).

Reports of functional analysis of peripheral T cells other than the clonogenic minority are scarce. However, there are a few studies regarding the proliferative response of T cell derived from patients with leucopenia evaluated in a whole blood assay (154, 160). In this assay T cells were found to have proliferative responsiveness equal to healthy controls in the presence of optimal costimulation with anti-CD3 + anti-CD28, but responses were significantly reduced in the AML patients with anti-CD3 stimulation only. Furthermore, the responses were significantly lower for ALL than for the AML patients (154).

Patients receiving chemotherapy for malignant disorders seem to develop a CD4⁺ T lymphopenia together with high serum level of the pleiotropic cytokine IL-7 (161, 162). In contrast, patients with untreated AML and severe chemotherapy-induced leucopenia with CD4⁺ T lymphopenia showed decreased IL-7 serum levels, and the detection of circulating IL-7-responsive T cells indicated that variations in systemic IL-7 levels are functionally important and may contribute to an additional qualitative T cell defect in T lymphopenic AML patients (163). Other studies have demonstrated that administration of recombinant interleukin-7 to humans could selectively increase total CD4⁺ and CD8⁺ T lymphocytes together with decrease in the percentage of CD4⁺ T_{reg} cells (164).

Very few studies have examined the T cell system in AML patients after hematopoietic reconstitution. Long-lasting T cell defects can occur after intensive chemotherapy for other malignancies, especially in adult patients (152). One would expect that similar effects may occur after AML therapy.

II. The immune system after autologous and allogeneic stem cell transplantation

The recirculation pattern of leukocyte subpopulations during the first 24h after auto-HSCT differs between leukocyte subsets (165). The number of CD3⁺ T lymphocytes increased during this period, whereas CD56⁺ NK cells decreased rapidly and remained low throughout the observation period. B lymphocyte levels were also low during the observation period (165). The early reconstitution of T lymphocyte response after both auto- and allo-BMT is mainly due to the peripheral expansion of mature T cells transferred with the graft (166, 167). The recovery of polyclonal T lymphocytes occurs gradually, and complete reconstitution of humoral and cellular immunity may take more than one year (168). Similar to the observations in patients only receiving intensive chemotherapy, there seem to be an absence of CD45RA⁺CD4⁺ T cells early after auto-transplantation with a remaining population of CD4⁺CD45RO⁺ circulating T lymphocytes (152, 153, 169). One study has suggested that BM-resident memory T cells are resistant to both pretransplant chemotherapy and ex vivo pharmacological purging and thus may contribute to the immune reconstitution after auto-BMT (170).

The studies in this thesis did not include allografted patients and thus a detailed review of reconstitution after allografting is therefore not included in this presentation. Anyhow, recently, the CD4⁺ helper T cell recovery after allo-HSCT have been characterized for patients with various hematological malignancies (171). In short, the early recovery of CD4⁺ T cells at 3 months was a favorable prognostic factor together with higher CD34⁺ cell transplant dose in terms of overall survival and non-relapse mortality (171). Also, early immune recovery has been suggested enhanced following blood stem cell allografting compared with BM allografting (172).

Aims of the thesis

New and less toxic therapeutic strategies are needed to improve survival after chemotherapy and also as an alternative to chemotherapy for elderly patients, and for this reason immunotherapy is considered in AML. A characterization of the immune system in AML patients is therefore important as a basis for the design of clinical therapeutic approaches.

The aims of the studies were:

- to characterize the circulating T cells in patients with untreated AML
- to further characterize the remaining T cells in AML patients receiving intensive chemotherapy, including the T cell release of IFN γ as well as other cytokines known to affect primary human AML cells
- to characterize in detail the functional effects of the T cell derived cytokine IFN γ on primary human AML cells for a large group of consecutive patients
- to investigate the humoral immune response against autoantigens in AML patients, including a more detailed investigation of whether cyclin B1 should be regarded as a leukemia-associated antigen in AML

Methods and subjects

Unless otherwise stated we included unselected patients in our studies, i.e. either randomly selected patients or consecutive patients. For our studies of AML cells we investigated cells that were stored frozen in liquid nitrogen. The advantage of this approach are that (i) cells from the same patient can be available for several experiments; (ii) an extensive biological characterization is possible; and (iii) large groups of patients can be investigated within a reasonable time and in the same experimental setup. However, two major disadvantages are present: the cells have a decreased viability after storage and even the viable cells have alterations especially in the membrane molecule expression (173-176)

Our *in vitro* cultures were usually prepared in serum-free medium (177). However, we regard the serumfree Stem Span medium as suboptimal for fibroblasts, osteoblasts and bone marrow stromal cells (178) and for this reason cocultures of AML cells and these nonleukemic cells were prepared in serum-containing Stem Span as described in these studies.

When investigating T cell responses for patients with chemotherapy-induced cytopenia we used a whole blood assay that has been characterized in detail in previous studies (154, 160). The cells were then activated by using well-characterized T cell stimulatory signals. The major disadvantage of this assay is that the number of T cells per culture well is not determined. The major advantage is that relatively large experiments can be designed even when only small blood samples can be collected. It can be argued that the use of a whole blood assay is a disadvantage because the culture conditions will differ between patients, e.g. autologous serum is included in the cultures and the number of other cells in peripheral blood will differ. On the other hand, it can also be regarded as an advantage that the immunocompetent cells are investigated in their natural immunoregulatory network by using diluted whole blood.

Summary of the results

Paper I

Ersvaer E, Hampson P, Wendelbo O, Lord J, Gjertsen BT, Bruserud O. *Circulating T cells derived from patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex* (2006)

Objectives. T lymphocyte defects may contribute to the immune defects in acute myelogenous leukemia (AML). We characterized the T cell system for a large group of untreated AML patients.

Methods. T lymphocyte subsets were analyzed by flow cytometry for 45 patients. The *in vitro* interferon (IFN) γ release in response to stimulation with anti-CD3 + anti-CD28 in the presence of autologous AML cells was examined for 32 consecutive patients.

Results. The majority of circulating lymphocytes were CD3⁺ T cell, and CD19⁺ B cells usually constituted <10% of the lymphocytes. Most T cells expressed the $\alpha\beta$ T cell receptor (TCR $\alpha\beta$ ⁺), and only a minority of the cells was TCR $\gamma\delta$ ⁺. Both CD4⁺ and CD8⁺ T cells were detected, the CD4:CD8 ratio showed a wide variation but was usually >1.0. The majority of CD4⁺ T cells were CD45RA⁺, whereas most CD8⁺ T cells were CD45R0⁺. The T cells could be stimulated to release interferon- γ (IFN γ) in response to anti-CD3 + anti-CD28 even in the presence of excess autologous AML blasts, and for a subset (15 of 45) of patients these IFN γ levels could be further increased by the protein kinase C (PKC) δ agonist Pep005.

Conclusions. Circulating T cells in patients with untreated AML are mainly CD4⁺ or CD8⁺ TCR $\alpha\beta$ ⁺; both CD45RA⁺ and CD45R0⁺ can be detected, and these cells can be activated through the CD3/TCR complex even in the presence of excess AML cells. For a subset of patients the T cell responsiveness can be further increased by targeting PKC.

Paper II

Ersvaer E, Hampson P, Hatfield K, Ulvestad E, Wendelbo O, Lord J, Gjertsen BT, Bruserud O. *T cells remaining after intensive chemotherapy for acute myelogenous leukemia show a broad cytokine release profile including high levels of interferon- γ that can be further increased by protein kinase C agonist PEP005.* (2006)

Objectives. Several cytokines are released during T cell activation, including the potentially antileukemic interferon- γ (IFN γ) and the hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) that enhance proliferation and inhibit apoptosis of acute myelogenous leukemia (AML) cells. In the present study we investigated the release of IFN γ and GM-CSF by circulating T cells in AML patients with chemotherapy-induced cytopenia.

Methods. T cells were activated with anti-CD3 plus eventually anti-CD28, and the cells were investigated in a whole blood assay in the presence of their natural cytokine network. We examined 63 samples derived from 16 AML patients during 28 chemotherapy cycles. Activation potential of protein kinase C agonist Pep005 was also explored.

Results. Activated T cells showed a broad cytokine release profile, but IFN γ and GM-CSF levels showed a significant correlation and were generally higher than the other cytokine levels. IFN γ and GM-CSF levels were associated with high CD4:CD8 ratio, low age and no ongoing chemotherapy, whereas duration of neutropenia or kind of chemotherapy were not similarly associated. The levels of both cytokines were lower than for healthy controls but higher than for multiple myeloma patients with cytopenia following autotransplantation. The cytokine levels could be further increased by the protein kinase C agonist Pep005.

Conclusions. We conclude that remaining T cells after intensive AML therapy show a broad cytokine release profile including high and significantly correlated levels of potentially antileukemic IFN γ and the AML growth factor GM-CSF. The final effect of an AML-initiated T cell cytokine response will then depend on the functional characteristics of the AML cells because the distribution of IFN γ and GM-CSF receptors differ between AML cells of different patients.

Paper III

Ersvaer E, Skavland J, Ulvestad E, Gjertsen BT, Bruserud O. *Effects of interferon gamma on native human acute myelogenous leukaemia cells* (2006) *Cancer Immunol Immunother.* Apr 13; [Epub ahead of print]

Objectives. T cell targeting immunotherapy is now considered as a possible strategy in acute myelogenous leukemia (AML), and IFN γ release may then contribute to the antileukemic effects. We investigated effects of IFN γ on native human AML cells.

Methods. The expression of IFN γ receptor α chain and intracellular Stat and Erk1/2 phosphorylation after IFN γ stimulation were analyzed by flow cytometry. Exogenous IFN γ was added to *in vitro* cultures of AML blasts, and the effect on proliferation (^3H -thymidine incorporation), apoptosis (Annexin-V and PI) and cytokine release (ELISA) was measured

Results. Normal T cells could be activated to release IFN γ in the presence of AML cells. Furthermore, high levels of CD119 (IFN γ receptor α chain) expression were observed for all 39 patients examined. Receptor expression was decreased after exposure to exogenous IFN γ , and receptor ligation caused Stat1 phosphorylation but no phosphorylation of the alternative messengers Erk1/2. The effect of exogenous IFN γ on AML blast proliferation was dependent on the local cytokine network and IFN γ (i) inhibited proliferation in the presence of exogenous IL1 β , GM-CSF, G-CSF and SCF; (ii) had divergent effects in the presence of IL3 and Flt3 (65 patients examined); (iii) inhibited proliferation in the presence of endothelial cells but had divergent effects in the presence of fibroblasts, osteoblasts and normal stromal cells (65 patients examined). IFN γ increased stress-induced (spontaneous) *in vitro* apoptosis as well as cytarabine-induced apoptosis only for a subset of patients. Furthermore, IFN γ decreased the release of proangiogenic CXCL8 and increased the release of antiangiogenic CXCL9-11.

Conclusions. We conclude that IFN γ can be released in the presence of native human AML cells and affect AML cell proliferation, regulation of apoptosis and the balance between pro- and antiangiogenic chemokine release.

Paper IV

Ersvaer E, Bertelsen LT, Espenes LC, Bredholt T, Boe SO, Iversen BM, Bruserud O, Ulvestad E, Gjertsen BT. *Characterization of ribosomal P autoantibodies in relation to cell destruction and autoimmune disease* (2004) Scand J Immunol. Jul-Aug;60(1-2):189-98

Objectives. Autoantibodies against the ribosomal P proteins are related to cell death and tissue destruction and are frequently exhibited in patients with systemic lupus erythematosus (SLE). We attempted to explore the effect of tissue destruction on the induction of anti-P autoantibodies and analysed antibody levels for patients with autoimmune disorders and acute myelogenous leukemia patients.

Methods. We searched for anti-P autoantibodies by enzyme-linked immunosorbent assay in 201 antinuclear antibody (ANA)-positive individuals, in 10 patients with treated kidney SLE and in 45 untreated acute leukaemia patients. The autoantibody reactivity was further characterized using one- and two-dimensional immunoblot analysis and immunofluorescence.

Results. Anti-P were detected in 5.5% (11/201) of ANA-positive individuals, but not in kidney-affected SLE patients or in patients with leukaemia. Seven of 11 anti-P-positive patients had SLE (3/11), primary Sjogrens's syndrome (1/11) and other autoimmune diseases (3/11). An association between disease activity and anti-P was suggested by follow-up examinations in one SLE patient, supported by the absence of anti-P autoantibodies in the 10 treated kidney SLE patients. Anti-P autoantibodies were detected by immunoblot in one patient with SLE indicating anti-P2 predominance and in the patient with Sjogrens's syndrome indicating anti-P1 predominance. Diverging humoral responses in these ANA- and anti-P-positive patients were further illustrated by immunofluorescence, elucidating varying nuclear reactivity and anti-P pattern. Anti-P could not be detected for any leukemia patients before therapy; a smaller patient group was also investigated following intensive chemotherapy but anti-P could not be detected for these patients either.

Conclusion. The observation of anti-P in individuals with active autoimmune disease, but not in patients with chemotherapy-induced cell damage, suggests that anti-P antibodies are part of a specific disease process, and not elicited as a response to cell destruction per se.

Paper V

Ersvaer E, Zhang JY, McCormack E, Anensen N, Tan EM, Gjertsen BT, Bruserud O. *Cyclin B1 is commonly expressed in the cytoplasm of primary human acute myelogenous leukemia cells and serves as a leukemia associated antigen associated with autoantibody response in a subset of patients* (2006).

Objectives. Cyclin B1 is involved in cell cycle regulation of both normal and malignant cells. Cytoplasmic expression of cyclin B1 in epithelial malignancies is associated with a specific T cell response and presumably also a humoral immune response. We therefore investigated the protein expression of cyclin B1 in native human acute myelogenous leukemia (AML) cells and the occurrence of cyclin B1 specific antibodies in AML patients.

Methods. Level of intracellular cyclin B1 expression was measured by flow cytometry, while confocal laser microscopy indicated the cyclin B1 localization within the naive and dendritic AML cells. Serum antibodies were analyzed by ELISA.

Results. AML cell expression of cyclin B1 was detected for all 42 patients investigated by flow cytometry; but the percentage of cyclin B1 positive cells showed a wide intra patient variation. Confocal laser microscopy demonstrated that most of these patients (32/42-76%) showed abnormal cytoplasmic expression. Furthermore, both cytoplasmic and nuclear expression was maintained after 14 days of in vitro culture and differentiation of the AML cells in the direction of a dendritic cell phenotype. AML engraftment in NOD/LtSz-Prkdc^{scid}/B2m^{-/-} mice showed no clear correlation with cyclin B1 expression. Cyclin B1 specific serum antibodies could be detected for 8 out of 66 consecutive patients with untreated AML. Fourteen of these patients of these patients were in addition tested later following intensive chemotherapy; three of them had detectable antibody levels that decreased following treatment.

Conclusions. Our studies demonstrate that primary human AML cells show aberrant cytoplasmic expression of cyclin B1 for a majority of patients; this expression pattern has been associated with induction of specific immune responses in patients with epithelial malignancies and a specific humoral immune response was also detected for a subset of patients with untreated AML.

General discussion

The bone marrow as an immunological compartment

AML is a bone marrow disease, and the bone marrow is a part of the lymphocyte recirculation network. Recently the bone marrow was described in mice to function as a secondary lymphoid organ, providing a microenvironment for priming of T cell responses (179). Naive T cells can thus home to the bone marrow as a part of their normal recirculatory pathway and become primed by CD11c⁺ dendritic cells in the stroma to generate primary and memory T cell responses (179). These observations are further supported by a study describing a secondary in vivo bone marrow T cell response induced by a tumor cell vaccine of mice (180). By using peptide/MHC-tetrameric complexes clonal expansion of antigen-specific T cells could be detected during the primary response in BM and during the secondary response in the peritoneal cavity and BM (180). Feuerer et al. have also shown that BM stroma in mice constitutively expressed ICAM-1, VCAM-1, MadCAM-1, and P-selectin adhesion molecules relevant for homing of blood T lymphocytes to the bone marrow, and also bone marrow expression of the T cell co-stimulatory molecule CD80 (181). In the same study, CD3⁺ T cells were detected together with BM resident CD11c⁺ DCs, often enriched in follicle-like structures in the BM parenchyma. Interactions between transferred antigen specific transgenic CD4⁺ T cells and antigen loaded BM-DCs then formed multicellular clusters in situ in BM, generated lymphoblasts and lead to clonal T cell expansion within such clusters. The great majority of BM-CD4⁺ T cells had a memory phenotype suggesting that the BM microenvironment facilitates maintenance of CD4⁺ memory (181).

As the bone marrow of patients with AML will be severely compromised by the presence of large numbers of AML blasts, it is possible that reduced T cell responsiveness in this environment could contribute to the immunodeficiency that accompanies AML.

IFN- γ in patients with AML

IFN γ (182) is a lymphokine released especially by activated T_{H1}, T_{C1} and NK cells. Binding of IFN γ to the IFN γ receptor complex on target cell transmits signals to the cytoplasm and nucleus through the Jak-Stat1 mechanism as well as Stat1 independent pathways (183, 184). In a recent study it was demonstrated that intracellular signaling events after IFN γ receptor ligation could be used in prognostic classification of AML patients (52). In the *in vitro* culture experiments exploring the effects of IFN γ on AML blasts (paper III), we showed that exogenous IFN γ could affect AML cell proliferation, regulation of apoptosis and the balance between pro- and antiangiogenic chemokine release. We would emphasize that these alterations probably are caused by direct effects of IFN γ effects on the AML cells because we used highly enriched leukemic cell populations in our studies.

IFN γ is supposed to induce the expression of MHC class II on the surfaces of various cell types and thereby probably converting them to nonprofessional APCs. The presentation of leukemia-associated antigens to T cells may then be improved, but it has also been suggested that MHC II restricted antigen presentation by this excess of non-professional APCs may result in antigen-specific T cell anergy or induction of T_{reg} cells (182). Also, a recently study concluded that IFN γ appeared to have a negative regulatory role of the *in vivo* DC function (185). Thus, further investigations have to clarify whether local IFN γ release will enhance or downregulate antileukemic T cell reactivity.

The *in vivo* effects of IFN γ on malignant cells seem to differ and both suppression of tumorigenicity (182, 186) and enhancement of tumor growth or metastatic potential (182, 187) has been described. These differences can be caused by differences in the direct effects of IFN γ on the malignant cells, but indirect effects mediated via stromal cells may also be important. We characterized the direct effects of IFN γ on AML cells, but a recent experimental study in mice suggested that the crosstalk between leukemic and immunocompetent may be more important than the direct effects (188). In this model Stat-1, which is the major intracellular target for IFN γ receptor ligation in AML, is regarded as a tumor suppressor and considered as a key regulator of the surveillance of developing malignancies. In contrast to these previous studies, these authors described that Stat-1 acted as a tumor promoter for leukemia development, and absence of Stat-1 was

then associated with decreased MHC class I expression and thereby more efficient recognition of leukemia cells by NK cells. These experimental observations illustrate the importance of investigating potential antileukemic immune reactivity within the natural immunoregulatory network.

Naive versus memory T cells

Several studies (189-191) suggest that naive and memory T cells cannot be estimated by using only the typically CD45RA and CD45RO markers, respectively, and for an accurate estimation one has to use additional markers (i.e. CD62L, CD11a, CD27). However, de Rosa et al. report that when a third marker was included to identify naive cells, a population of cells were found to be discordant. Functional studies and analysis of the V β repertoire suggest that the discordant cells appeared to be non-naive (190). Sallusto et al. (191) described that CCR7 identifies a subset of CD8⁺ memory cells that coexpress CD62L and de Rosa et al. (190) described that truly naive CD8⁺ cells had to be identified by three additional markers similar to CD4⁺ naive cells. Thus, the frequency of naive T cells among CD8⁺ and CD4⁺ CD45RA⁺ cells show a wide variation, and would be expected to vary even more in patients than for healthy individuals. From our study regarding the T cell expression of CD45RA and CD45RO in untreated AML patients (paper I), there can only be concluded that the AML patients are heterogeneous with regard to circulating CD45RA/RO cells, and this heterogeneity in membrane molecule expression probably reflects heterogeneity with regard to frequencies of memory and naive T cells. However, a proper examination of this question would be to combine CD4/CD8 with at least 3 additional markers to eliminate discordant cells, and then to investigate the V β repertoire of these cells. The CD45RA⁺ and CD45RO⁺ populations are heterogeneous in healthy individuals, and there is no reason to expect a less complex situation in patients.

The possibly use of PEP005 in AML

A recent study suggested that the active agent in the sap of *Euphorbia peplus*, ingenol 3-angelate (PEP005), had antileukemic activity that was mediated through the delta isoform of PKC (192). PEP005 induced apoptosis in AML cell lines as well as in primary AML cells, whereas normal hematopoietic progenitors were resistant to PEP005-induced apoptosis. However, the study included

primary AML cells only from a relatively small number of patients, and it will be important to investigate these effects in a larger group of well-characterized unselected patients. Preliminary studies of AML xenografts using the NB4 cell line in NOD/SCID/ $\beta 2m^{-/-}$ mice show no prolonged survival for mice receiving PEP005. These animals die due to leukemic infiltrates in the brain but the tumor burden in abdominal lymph nodes and ovaries seem to be reduced (personal communication with Dr. E. Mc Cormack and Dr. KQ. Wang). A possible explanation for these observations could be that the antileukemic effect is not observed in the central nervous system due to the blood-brain barrier and thereby lower drug concentrations in this compartment.

The antileukemic action of PEP005 was proposed to be mediated through the activation of PKC- δ (192). PKC is a family of 11 isoenzymes of which 8 show *in vitro* responsiveness to PEP005 (classical α , $\beta 1$, βII , γ and novel δ , ϵ , η , θ PKCs) (193). PKC seem to have emerging and diverse roles in immune cell signaling (194), however the effect of PEP005 on T cell functions have not been examined previously. Our results suggest that PEP005 enhances the *in vitro* activation of T cells derived from patients with chemotherapy induced cytopenia (paper II), but an enhancement was observed only for a minority of patients when testing T cells derived from patients with untreated AML. This difference could be caused either by (i) the presence of IL2, this was added to the cultures only when testing cytopenic patients, or (ii) different culture conditions and especially the presence of peripheral blood components when testing cytopenic patients with the whole blood assay. The presence of AML cells probably does not have any major influence on the overall results because the effect of Pep005 was similar when we compared healthy controls and patients with untreated AML. However, differences in the AML cell phenotype, especially the constitutive release of immunoregulatory cytokines, may be important for certain patients and contribute to the variation between patients.

Taken together these studies suggest that Pep005 should be tried in cancer therapy because (i) the drug seems to have direct antileukemic effects; (ii) the drug seems to enhance T cell reactivity and may therefore also enhance antileukemic immune reactivity. However, it remains to be clarified whether Pep005 will affect chemotaxis of immunocompetent cells and thereby their local recruitment

to the cancer cell compartments. These future studies have to include the effect of this drug on the AML cells release of T cell chemotactic chemokines.

Disease-induced alterations in the cellular immune system in AML and multiple myeloma patients

MM is a clonal B-cell malignancy characterised by an excess of mature plasma cells in the BM, and the large majority of patients has monoclonal immunoglobulin protein (complete molecules or only light chains) in serum and/or urine and usually develop decreased normal immunoglobulin (Ig) levels. The cellular immune system of patients with multiple myeloma has been examined previously, and several differences were then observed compared with our and others observation in AML patients:

- a decreased CD4+/CD8+ ratio is usually observed in circulating T lymphocytes of patients with MM (95, 195), whereas in AML patients the CD4/CD8 ratio close to normal ((107) and paper I);
- myeloma patients seem to have elevated TH1/TH2 ratio (88), whereas Panoskaltis et al. (107) described T_{H1} and T_{H2} cytokine profiles close to normal in AML patients.

In our comparison of myeloma and AML patients (paper II) we describe a difference in T cell cytokine release between these patients. Treatment-induced alterations probably contribute to this difference because our AML patients receive intensive chemotherapy alone, whereas the myeloma patients receive a different chemotherapy regimen in combination with autologous stem cell support.

Autoantibodies in patients with malignant disorders

It is well-established that autoimmunity can be associated with cancer, and one of the forms of its expression is the development of autoantibodies and eventually autoimmune disease (196). Several mutated or overexpressed proteins seem to be processed and presented to the immune system as tumor antigens leading to humoral or cellular responses (125). One of the most extensively studied cancer-associated antigens are p53 (197), but more recent studies have demonstrated that

autoantibodies against a wide range of autoantigens can be detected in patients with malignant disorders (125, 198). Zhang et al. investigated a group of 174 cancer patients for autoantibodies against 7 known autoantigens, each autoantibody could then be detected in 10-20% of the patients but approximately half of the patients had at least one autoantibody (125). Thus, cancer-associated humoral autoimmunity is a common phenomenon, but autoimmune disease is uncommon (196).

Phage display is a technique in which bacteriophages are engineered to fuse a foreign peptide or protein with their capsid proteins and hence expose or display it on their surface. The combination of phage display technology and immunoscreening can also be used to identify antibody-binding peptides (199, 200). Examination of peptide phage libraries allows the screening for autoantibodies without knowledge about the parental antigen (201), and it is then possible to profile specific immune responses in patient sera and identify new autoantigens. A recent study described the identification of peptides and cDNA encoded proteins that are recognized by breast cancer IgG antibodies but not by antibodies from healthy controls, and the reactivity of some of these antibodies seemed to be linked to survival (202).

The detection of autoantibodies may become important in future clinical medicine:

- Detection of autoantibodies or autoimmune disease may be the first sign of cancer (196).
- Analysis of autoantibodies may become useful in the screening for cancer and possibly cancer-specific autoantibody profiles can be identified.
- Although the clinical importance of autoantibody detection is uncertain, at least some studies suggest that detection of autoantibodies may have a prognostic impact. Detection of p53 autoantibodies in breast cancer patients seem to be associated with high-grade tumors and poor survival (197), whereas a small clinical study suggested that antibodies against a specific peptide was associated with long survival (202).
- Antibody “fingerprints” to TAAs are proposed as a future biomarker for early cancer diagnosis and may thereafter function as guidance for individualized therapeutic interventions and to predict response to therapy or risk of relapse (203).

We examined AML patient sera for antibodies against cyclin B1 and the ribosomal p protein. We have described the novel findings of abnormal cytoplasmatic distribution related to constitutive expression of cyclin B1 in AML blasts, as well as the detection of a specific humoral immune response against cyclin B1 in sera from a minor subset of AML patients (paper V). In contrast, we did not detect autoantibodies against the SLE associated ribosomal P proteins in sera from untreated AML patients nor in sera from AML patients with chemotherapy-induced cell damage (paper IV). p53 antibodies in breast cancer seem to be associated with aggressive disease and poor survival. AML is one of the most aggressive malignancies with a median survival of only 3-4 months for patients only receiving supportive therapy. Even though other autoantibodies seem to be associated with aggressive disease, the frequency of AML patients with anti-cyclin B1 was similar to the frequency previously observed for less aggressive malignancies like prostate, gastric, breast, colorectal, lung, and hepatocellular cancers (198). This low frequency was observed even though a major part of AML patients show cytoplasmic cyclin B1 expression that has been associated with autoimmunity in other malignancies (204, 205). Furthermore, our analysis of AML patients receiving intensive chemotherapy suggest that neither the development of cyclin B1 nor ribosomal p protein antibodies is dependent of extensive tissue damage, at least not for immunocompromized AML patients receiving intensive chemotherapy. It seems more likely that at least the presence of anti-P antibodies are a part of a specific autoimmune disease process rather than a response to cell destruction, and this is also supported by proposals of SLE as a disease with an apoptotic clearance deficiency (206) and association of anti-P with organ-specific kidney (nephritis) disease in SLE (207).

Concluding remarks

The overall disease-free survival for patients with AML is less than 50% even for the younger patients who can receive the most intensive therapy, and there is obviously a need for a more effective therapy. Immunotherapy (e.g. targeting of autologous T cells) is considered as a possible therapeutic approach. Short time until lymphoid reconstitution after chemotherapy seems to be a

good prognostic factor in several malignancies, including AML. Both the cellular and humoral immune system in AML patients differ from healthy controls. The main aim of this thesis was therefore to characterize the immune system of patients with AML. Our results substantiate the hypothesis that even these immunocompromized patients have remaining immunocompetent cells that can be targeted by immunotherapy.

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