Biological role of the expression of tumor necrosis factor receptor-ligand family molecules on acute lymphoblastic leukemia cells

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Abbreviation

Ag	Antigen
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
APC	Antigen-presenting cell
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
°C	Celsius degree
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CHX	Cycloheximide
cpm	Count per minute
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte Ag-4
DMSO	Dimethylsulfochlorid
DNA	Deoxyribonucleic acid
EDTA	Ethylendiamintetraacetat
ELISA	Enzyme linked immuno sorbent assay
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanat
g	Gram
h	Hour
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
L	Ligand

M	Molar
m	Milli ($1x^{-3}$)
μ	Micro ($1x^{-6}$)
mAb	Monoclonal antibodies
MACS	Magnetic activated cell sorter
MHC	Major histocompatibility complex
min	Minute
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cells
MMC	Mitomycin C
nm	Nanometer ($1x^{-9}$)
NK	Natural killer
NHL	Non Hodgkin's lymphoma
PB	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
PHA	Phytohemagglutinin
PMA	Phorbol myiristic acetate
RNA	Ribonucleic acid
Tab.	Table
TNF	Tumor necrosis factor
%	Percent

1 Introduction

1.1 Leukemia development, biology and classification

Tumors of the immune system are classified as lymphomas or leukemias. In contrast to lymphomas which proliferate as solid tumor within lymphoid tissue, leukemias tend to proliferate as single cells and are detected in the blood, bone marrow and lymph. Leukemia can develop in lymphoid or myeloid lineages. Historically leukemias were classified to acute and chronic according to the clinical progression of the disease. The acute leukemias appeared suddenly and progressed rapidly, whereas the chronic leukemias were much less aggressive and developed slowly as barely symptomatic diseases. Now the maturity of the involved cells is the major distinction between acute and chronic leukemias. Acute leukemias tend to arise in less mature cells, whereas chronic leukemias arise in mature cells. The acute leukemias include acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML). The chronic leukemias include chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML). Acute lymphoblastic leukemia is the most common malignancy in children. It accounts for one forth of all childhood cancers and approximately 75% of all cases of childhood leukemia [1].

Since studies of immunobiology of ALL have confirmed that leukemia transformation and clonal expansion can occur at different stages of maturation in the process of lymphoid differentiation, leukemia cells can be characterized as the neoplastic counterparts of naive, activated, or resting normal cells, expressing unique cell antigens designated by the CD number system. The CD nomenclature has been devised to catalogue cell surface molecules as they are identified and characterized. These molecules are often initially defined on certain cell types by producing monoclonal antibodies which bind to them specifically. CD stands for "cluster of differentiation" referring to the *cluster* of monoclonal antibodies which define a particular *differentiation* molecule. Over 160 CD molecules have been designated so far [2]. Using a panel of monoclonal antibodies associated with various stages of differentiation with information on the presence or absence of cytoplasmic and surface immunoglobulin, ALL were classified into discrete stages according to the degree of differentiation or maturation. Four types of ALL arise from cells that are positive for

B lineage markers. They include early B-precursor ALL (early pre-B-ALL), common ALL (c-ALL), pre-B-ALL (pre-ALL) and B-ALL. About 15% of ALL are designated T-ALL. They arise from early thymocytes and their leukemia cells possess T-cell lineage markers.

During normal lymphoid development, lymphocyte precursors are at higher risk for spontaneous mutation because of the regulated mutagenic activity occurring during the process of gene rearrangement and the high rate of proliferation in these cells [3]. Greaves [4] suggested a model in which two sequential mutations spontaneously occurring in important regulatory genes in a lymphoid cell population undergoing significant proliferative stress could account for most ALL cases. Induction of malignant transformation appears to involve at least two distinct phases: initiation and promotion. Initiation involves changes in the genome but does not, in itself, lead to malignant transformation. After initiation, promoters stimulate cell proliferation and lead to malignant transformation. A number of DNA and RNA viruses have been shown to induce malignant transformation, related to the presence of oncogenes. It was also suggested that oncogenes might not be unique for transforming viruses but might also be found in normal cells as the so-called proto-oncogenes or cellular oncogenes. It is known that most oncogenes (both viral and cellular) are derived from cellular genes that encode various growth-controlling proteins. Proto-oncogenes can be divided into three groups that reflect different activities. One group of proto-oncogenes and their oncogenic counterparts encoded proteins that trigger cellular proliferation [5]. Some of these proteins function as growth factors or as growth factor-receptors. Overactivity of any of these oncogenes may result in unregulated proliferation. A second group includes so-called tumor-suppressor genes that encode proteins that inhibited cellular proliferation [6]. Inactivation of these genes results in unregulated proliferation. The third group of proto-oncogenes regulates programmed cell death (apoptosis). These genes encode proteins that either block or induce apoptosis.

Thus, for leukemia development a series of events is necessary: first, transformation of normal hematopoetic cell into malignant cell, second, immortalisation and proliferation of leukemia cells. Moreover, leukemia can arise only if leukemia cells are able to escape the immune surveillance. The immune surveillance theory suggested that tumor cells frequently arise in the body but are recognized as foreign and eliminated by immune system. Thus, the leukemia arise only if the leukemia cells lose their immunogenicity or if the immune

system is impaired. In spite of this, clinical data provide evidence that immune response can be generated to leukemia cells and that immune system plays an important role in control of leukemia.

Following allogeneic stem cell transplantation, the graft-versus-leukemia effect demonstrates that the immune system contributes to the elimination of residual leukemia blasts [7, 8, 9, 10, 11]. The increased relapse rate in patients having received an autologous or syngeneic transplant has been attributed to the lack of the allogeneic graft versus leukemia effect and efforts have been directed towards enhancing cytotoxic effector mechanisms in this setting by IL-2-mediated stimulation of lymphokine-activated killer cells. Also, identification of leukemia-specific antigens that have been successfully targeted by T-cell responses supports the concept that the immune system plays a prominent role in the control of leukemic disease [12, 13, 14, 15, 16, 17, 18, 19].

Among these leukemia antigens are fusion products of leukemia-specific chromosomal translocations [12, 13, 14, 15, 16] and antigens that are markedly over-expressed in different types of leukemia but are not truly leukemia-specific and so called "shared" antigens that are expressed in different malignancies but not in normal tissue [17, 18, 19]. In addition, cytotoxic T-cell clones specific for minor histocompatibility antigens presented by class I MHC molecules have been shown to lyse lymphoid and myeloid leukemia cells [20, 21, 22, 23].

Yet, while leukemia cells do express specific antigens that can serve as target structures for anti-leukemia immune responses, in the clinical setting the response frequently is not sufficient to prevent leukemia growth. One approach to leukemia treatment therefore is to augment or supplement that anti-leukemia immune response.

1.2 Stimulation of anti-leukemia immune response

For effective T-cell stimulation are required two signals [24, 25]: the first signal is mediated via recognition of the antigen-MHC class I complex by the respective T-cell receptor [24, 25]. The second signal required for an effective T-cell stimulation is delivered by costimulatory molecules that are physiologically expressed on professional antigen-presenting cells [26, 27]. The major costimulatory molecules that have been identified belong to the

B7 family of costimulatory molecules (B7-1/CD80 and B7-2/CD86). In the absence of such secondary signals T-cells are rendered anergic to the presented antigen [24].
Thus deficient immunogenicity of leukemia cells may in principle be due to defective antigen-processing and/or presentation or lack of costimulatory molecules. As leukemia cells generally express MHC class I molecules to high levels, their reduced T-cell stimulatory capacity is largely attributed to deficient costimulation.

1.2.1 Costimulatory molecules CD80 and CD86

Costimulatory molecules of the B7 family deliver important costimulatory signal for T-cells via binding to their receptor CD28 on T-cell [28, 29]. Interaction of both CD80 and CD86 with CD28 receptor, expressed on resting and activated T-cells [30] results in T-cell proliferation, cytotoxic T-cell generation, immunoglobulin (Ig) and cytokine production on B-cells [31, 32, 33, 34, 35]. In addition to CD28, some population of T-cells may also transiently express CTLA-4 (cytotoxic T lymphocyte Ag-4), a second receptor for CD80 and CD86 [36]. CTLA-4 engagement may transduce an "off" signal, disengaging T-cells from further activation and proliferation [34, 37].
Both costimulatory molecules CD80 and CD86 are members of the immunoglobulin superfamily [38, 39]. CD80 a B-cell activation molecule first described in 1981 by Yokochi *et al* [40] was the first ligand to be identified for the CD28 molecule. In addition to its expression on activated B-cells, CD80 was also detected on a variety of antigen-presenting cells (APCs) including dendritic cells, Langerhans cells, activated monocytes and activated T-cells [41]. The functional importance of the CD80 molecule has been demonstrated in a number of studies of T-cell activation. Both anti-CD3 and phorbol myristic acetate (PMA)-induced T-cell proliferation was enhanced by the addition of CD80-transfectants and this activation was blocked by anti-CD28 monoclonal antibodies (mAbs) [42, 38].
The structure of CD86 molecule was found to be very similar to CD80 [39]. Similar to CD80, CD86 transfectants augment T-cell proliferation and IL-2 production to suboptimal stimulation with anti-CD3 or PMA [43, 39]. Either anti-CD86 or anti-CD80 mAbs alone have been shown to be inefficient in their ability to block allogeneic mixed lymphocyte reactions (MLRs) [44, 45]. A combination of both anti-CD80 and anti-CD86 mAbs were the most effective at inhibiting the MLR [46, 47]. The role for both molecules in primary

responses was further supported by the finding that a combination of anti-CD80 and anti-CD86 antibodies can induce anergy [46].

Resting B-cells express undetectable level of CD80 and very low levels of CD86, while both CD80 and CD86 are upregulated following B-cell activation [44]. The regulation of CD80/CD86 expression is controlled via cell-cell interaction and cytokines. The CD40-CD40L pathway plays an important role in controlling CD80 and CD86 expression. Both CD80 and CD86 were induced by signaling through CD40, either through anti-CD40 mAbs or activated T-cells which expressed the CD40 ligand [48]. Also a number of cytokines have been shown to regulate expression of CD80 and CD86. IL-4 is one of the most potent inducers of CD86 [49]. It is also can stimulate to a less extent CD80 on B-cells [49]. IFN-γ increases the expression of CD86 on B-cells [44].

CD28-CD80/CD86-mediated costimulation has provided a new approach to cancer therapy, because the inability of several tumors to stimulate sufficient immune response has been in some cases correlated with deficient CD80/CD86-costimulation. It was shown that leukemia cells are unable to stimulate efficient immune response *in vivo* and that can be explained by the low expression levels of costimulatory molecules. Indeed, it was demonstrated that leukemia cells exhibit low levels of CD80 and heterogeneous pattern of CD86 expression [50, 51]. Therefore, stimulation of CD80 and/or CD86 expression on leukemia cells could convert them into functional APCs. In fact, in leukemias, transgenic expression of the costimulatory surface molecule CD80 enhances the immunogenicity of myeloid and lymphoid leukemia cells [52, 53, 54, 55, 56, 57]. The potency of CD80 as a costimulator is documented in a murine ALL model. Immunization of mice with leukemia cells transduced with CD80 protected the animal from further challenge with non-transfected leukemia cells [57]. *In vitro*, retroviral transduction of murine AML cells with CD80 enhances the expansion of leukemia-reactive CTLs mediating graft-versus-leukemia reactions when infused after syngeneic bone marrow transplantation (BMT) [55].

The function of CD86 as costimulator has been also studied. It has been demonstrated that myeloid leukemia cells transduced with CD86 have improved their reduced immunogenicity, but were unable to stimulate protective anti-leukemia immune response [58]. In other *in vivo* studies, the myeloid leukemia cells transduced to express CD86 have stimulated an anti-leukemia immune response and were able to protect animals against wild-type tumor

challenge and to eradicate minimal residual disease [54, 59].

However, the transfection of CD80 or CD86 molecules into tumor cells has been not uniformly successful in inducting tumor immunity. In some low immunogenic solid tumor models, tumor cells did not induce an immune response even when cotransfected with CD80 and CD86 [60, 61], what suggests that other costimulatory molecules might contribute for sufficient T-cell stimulation. In addition to CD80 and CD86 molecules, members of the tumor necrosis factor(TNF) family and their ligands are known to be involved in the modulation of immunological responses [62]. As the members of this superfamily, CD70 and its receptor CD27 are important costimulatory molecules involved in antigen-dependent T-cell activation [63, 31, 64].

1.2.2 CD27-CD70 costimulatory pathway

CD27 is a type I glycoprotein belonging to the TNF-receptor family and is expressed on the majority of peripheral T-cells and natural killer (NK) cells [32, 31]. CD27 is differentially expressed on B-cells subpopulations depending on their stage of differentiation. CD27 is absent on immature and mature naive B-cells, but is expressed on tonsillary germinal center B-cells and on memory peripheral B-cells [65, 66]. Primed, memory B-cells expressing CD27 differentiate into plasma cells by contact with CD70 on T-cells in cooperation with IL-10. Thus, the CD27-CD70 interaction is involved in the differentiation of B-cells into plasma cells, in which $CD27^+$ rather than $CD27^-$ cells produce Ig [67, 68, 69, 70, 71, 72, 73, 74, 75]. A soluble turnicated form of CD27 (sCD27) was found *in vivo* in human plasma, urine and spinal fluid [76].

Beside CD27 expression on normal cells, CD27 can be detected in many malignancies corresponding to the counterparts of B-cells such as ALL, CLL, mantle cell lymphoma and several non-Hodgkin's lymphomas including follicular lymphoma (FL) [77, 78, 79, 80]. Increased levels of sCD27 in serum were observed in serum and cerebrospinal fluid (CSF) of patients with CLL and low-grade non-Hodgkin's lymphoma (NHL) [80]. It was also described that sCD27 can be useful in the differential diagnosis of CNS involvement in lymphoid malignancies as a tumor marker in patients with either meningeal localization of lymphoid malignancies or primary central nervous system lymphoma [81]. In leukemia B-cells CD27 expression appears to be dysregulated and this dysregulation may have conse-

quences for the behavior of malignant B-cells *in vivo*. Moreover, if membrane-bound CD27 is involved in the functional activity of $CD27^+$ T- and B-cells, the presence of soluble CD27 in plasma might interfere with these function.

CD70, the ligand for CD27, is a transmembran glycoprotein type II, belonging to the TNF family and is expressed by activated T cells, on a small subset (10%) of activated memory peripheral B lymphocytes and on B-cells in the limited number of tonsillary germinal centers [31, 64, 66]. It is well documented that CD27-CD70 interactions provide a proliferative signal for T-cells expressing CD27 that is distinct from that of mediated through CD28 by its counterparts [82, 83]. It has also been described, that the CD27-CD70 interaction enhances NK activity in the presence of IL-2 or IL-12 and is involved in the regulation of cytolytic function of cytotoxic T-cells [84, 85, 86]. CD70 function as costimulatory molecule for both $CD4^+$ and $CD8^+$ T-cell proliferation, enhanced cytokine secretion (TNF-α, IL-10, IL-4, IL-2) and induces the generation of cytotoxic T-cells in mixed lymphocyte culture [87, 31].

CD70 has been reported to be expressed on CLL cells and lymphoma cells such as hairy lymphoma cells, non-Hodgkins lymphoma cells, diffuse large B-cell lymphoma cells and follicle center lymphoma cells [82, 78, 79]. Until now CD70 expression on ALL cells has not been well studied with exception of one early report [88].

Since the failure of leukemia cells to function as efficient antigen-presenting cells has been attributed not only to low levels of costimulatory molecules CD80 and CD86 on their surface, CD70-CD27 costimulatory pathway is of particular interest. However, only in few reports the importance of the costimulatory CD70-CD27 pathway in leukemia and lymphoma cells has been studied. In one report, CD70 expression on CLL cells has been modulated via CD40-stimulation with reciprocal changes of CD27 expression. It has been demonstrated that these changes in CD70 and CD27 expression contribute to the enhanced antigen-presenting capacity of CLL cells [82]. In another report, it has been demonstrated that the CD27-CD70 pathway plays a role in the reduced immunogenicity of follicular lymphoma cells [89].

In few murine solid *in vivo* tumor models it has already been demonstrated that CD70 expression is able to improve tumor immunogenicity and to induce antitumor immunity at levels similar or even superior to CD80 when tested either in vaccination or therapy models

[90, 91]. Moreover, coexpression of CD70 and CD80 results in additive T-cell responses [92] and is synergistic in inducing an protective antitumor response [91, 93, 94].
To our best knowledge, the role of CD70 expression on ALL cells is presently unknown. Since the low CD70 expression contributes to low immunogenicity of lymphoma and chronic leukemia cells [82, 89], it would be interesting to investigate whether the CD70-CD27 pathway is also involved in reduced immunogenicity and antigen-presenting capacity of ALL cells.

1.2.3 Costimulatory molecule CD40

Another potent pairs of costimulatory surface molecules of TNF receptor-ligand family are the CD40 and its ligand CD40L which is transiently expressed on activated T-cells [95, 96]. CD40 receptor can be found on B-cells, monocytes and dendritic cells [97, 98]. Generally, CD40-CD40L interactions are critical for interaction between T-cells and antigen-presenting cells, in particular for B-cells [99, 100, 101]. Engagement of the CD40 receptor on normal B-cells induces differentiation and survival of mature B-cells promoting Ig class switch, antigen-processing [102, 103], increased MHC expression and up-regulation of costimulatory surface molecules such as CD80 and CD86. Thus CD40L enhances the antigen-presenting capacity of mature B-cells [48, 104].
Since leukemia cells express CD40 [105, 106, 79] it is possible to modify the leukemia cells by cocultivation with CD40L-expressing feeder-cells or with the CD40L-trimer. CD40-induced maturation has been successfully employed in AML [107] enhancing the T-cell stimulatory capacity of myelogenous blasts, induced T-cell-proliferation and cytokine secretion [107].
It has been well documented that CLL and FL cells after CD40-stimulation express costimulatory molecules CD80 and CD86 [48, 108, 109, 110, 111]. CD40-activation turns leukemia cells into efficient antigen presenting cells and this enhanced APC function is mediated in part by the up-regulation of CD80 and CD86 expression on the leukemia cells [48, 108, 109, 111, 112].
It has been shown that after cocultivation of ALL cells with CD40L-trimer or on CD40L-expressing feeder-cells the ALL cells express high levels of MHC class I and II molecules, up-regulate expression of ICAM-1 and LFA-3, and markedly increase expression of CD80 and CD86 [113, 114, 115].

In contrast, expression of CD70 and its receptor CD27 after CD40-stimulation on ALL cells and their costimulatory activity are presently unknown. It would be interesting to study whether CD40-stimulation can modulate CD70 expression on ALL cells and improve therefore their reduced immunogenicity.

1.3 Vaccine strategies

Vaccine strategies aim to compensate the reduced immunogenicity of leukemia cells. In this end, a number of vaccine cells are generated to express leukemia-specific antigens in the context of adequate costimulation. Vaccine cells are then administered via different routes of application in order to stimulate a systemic leukemia-specific immune responses. One of different possible vaccine strategies is to modify the leukemia cells by activation in order to improve their antigen-presenting capacity. This can be achieved by cultivation the leukemia blasts *ex vivo* on CD40L-expressing feeder-cells or in the presence of the CD40L-trimer. These CD40-activated leukemia cells could be used for stimulation of anti-leukemia T-cell response.

Indeed, leukemia-specific cytotoxic T-cell lines has been generated *in vitro* when CD40-stimulated lymphoma or chronic leukemia cells were used for stimulation of allogeneic T-cells [109, 111, 116, 117]. In a clinical phase I study infusion of autologous CD40L-transduced CLL cells results in an increase in the frequency of leukemia-specific T-cells. In some patients a decrease in the absolute leukemia cell count associated with reduction in lymph node size was observed [118].

It has been shown that ALL cells improve their antigen-presenting capacity after CD40 activation and stimulate anti-leukemia cytotoxic T-cell response *in vitro* [119]. The role of CD28-CD80/CD86 interaction in the improved immunogenicity of ALL cells has been described [119], whereas the role of CD27-CD70 costimulation remains unclear. The understanding of the importance of CD27-CD70 costimulation in the generation of anti-leukemia T-cell response, could provide additional strategies for immunotherapy of ALL.

1.4 CD95-mediated programmed cell death

Progression of acute leukemia is characterized by a rapid proliferation and accumulation of malignant hematopoetic cells. Multiple mechanisms may explain why leukemia cells escape the control of the immune system. One of common cause includes the defects in the programmed cell death (apoptosis) pathways.

The best characterized molecule which was initially found as a cell surface molecule directly mediate apoptosis is the CD95 antigen [120, 121, 122]. CD95 is a type I transmembrane glycoprotein, belonging to the TNF receptor superfamily. It is expressed at low level on resting T lymphocytes and is up-regulated on activated T-cells [123, 124]. On B-cells CD95 is expressed at a restricted developmental stage. It can be found on activated B-cells, but not on naive mature B-cells [125, 126].

The ligand for CD95 (CD95L) is a type II membrane protein, which is originally expressed on activated T lymphocytes and NK cells [121, 127]. CD95L can undergo proteolytic cleavage and can be secreted in a soluble form. Both forms have proven to be able to execute a functional death signal on CD95 secreting cells [121].

CD95-CD95L cooperation plays an important role in the peripheral elimination of autoreactive B-cells, regulation and limitation of immune responses by deletion of mature activated T and B-cells. The CD95-mediated apoptosis is also involved in killing targets such as virus-infected cells or cancer cells by cytotoxic T-cells and NK cells.

B-cells are characterized by the dual expression of CD40 and CD95, which can mediate their survival and death, respectively. The balance between the dynamically opposing functions of these two receptors is important for B-cell selection, maturation and homeostasis. It is shown that in a normal immune environment, induction of CD40L on T-cells by B-cell antigen presentation is necessary for B-cells to trigger sensitivity to CD95-mediated apoptosis. Ligation of CD40 inhibits apoptosis and stimulates proliferation of normal B-cells, whereas ligation of CD95 induces apoptosis of activated lymphocytes [128, 129]. Aberrant signaling through the CD40 and CD95 antigens could participate in the pathogenesis of lymphoid malignancies.

It has been well documented, that primary leukemia cells such as ALL, CLL and lymphoma cells express detectable, though low levels of CD95 [124, 130, 131, 132, 133]. In contrast, T

lineage ALL and AML cells express heterogeneous levels of CD95 [134, 135]. On AML cells, CD95 expression was associated with good response to chemotherapy and low expression of CD95 is related to a low complete remission rate after chemotherapy induction [136, 137]. In high grade B-cell lymphomas, loss of CD95 expression or function have been attributed with a more aggressive tumor grade [138].

Whether expression of CD95 on primary ALL cells is also clinical relevant remains unclear. The results of several published investigations are controversial. In one report [139], it has been shown, that CD95 is most frequently expressed in clinically favorable ALL and is associated with increased relapse-free and overall survival. However, these findings were not confirmed in another study [140]. CD95 expression was not found to be correlated with the response to chemotherapy or relapse rate, either for B-cell precursor or T-ALL [140].

Since it was demonstrated that in leukemia cells the CD95 system involved in chemotherapy-induced apoptosis [141, 142], many investigations have been focused on the functional activity of CD95 in leukemias. CLL and B lineage ALL leukemia cells were shown to be resistant toward CD95-mediated apoptosis [130, 143]. In B lineage ALL, their resistance is not caused by a mutation of the CD95 molecule [144]. In primary AML and T-ALL cells, that express heterogeneous level of CD95, apoptosis can be induced through CD95 in some cases [132, 135].

Since CD95-mediated apoptosis is associated with B cell activation, it can be affected by costimulation through other accessory signaling molecules like CD40. As it has been shown that CLL, ALL cells and lymphoma cells [145, 143] up-regulate CD95 after CD40 ligation similar to normal B-cells. The CD40-induced CD95 expression was reported to enhance sensitivity toward CD95-apoptosis only in lymphoma cells [145], but not in cases of CLL [146, 147, 143]. It has been shown in one report that in 5 from 10 ALL samples tested CD40-stimulation sensitized the leukemia cells to CD95-mediated apoptosis, however at low level.

Thus, the importance of CD95 expression on primary and CD40-activated ALL cells remains unclear. It is worth therefore studying the biological role of CD95 expression and its functional activity on primary ALL blasts. It is also interesting to investigate whether CD40 stimulation can sensitize the ALL cells to CD95-mediated apoptosis.

1.5 Aims of the study

The aim of the present work is to study the biological role of the expression of the costimulatory molecules CD70-CD27 and apoptosis-mediating CD95 molecule on ALL cells.

I. In the first part of our work we examine the expression levels of CD70, CD27 as well as CD80 and CD86 on primary ALL blasts. We also analyse the association between expression levels of CD70, CD27, CD80 and CD86 molecules on primary and CD40-stimulated ALL blasts with some prognostic factors. We investigate whether baseline or CD40-induced expression of these molecules is correlate with poor or favorable clinical outcome.

II. In the second part of our work we investigate the modulation of CD70 and CD27 expression on ALL cells after CD40-stimulation.

III. In the third part of our study we determined the role of CD70 expression on CD40-stimulated ALL blasts for their enhanced antigen-presenting capacity. We investigate further the importance of CD70-CD27 costimulation for the expansion and cytotoxic activity of T-cells generated using CD40-stimulated ALL blasts.

IV. In the next part of our work we investigate the baseline expression of CD95 on ALL cells.

V. We also examine whether the expression of CD95 on ALL cells can be enhanced by CD40-stimulation.

VI. Next we determine the sensitivity of primary and CD40-stimulated ALL blasts to CD95-mediated apoptosis. We also analyse whether expression level of CD95 on ALL blasts before and after CD40-stimulation correlate with generally accepted prognostic risk factors.

The results of the present study can help to clear the role of CD70 and CD27 expression on the ALL blasts and in particular their importance for the antigen-presenting capacity of ALL cells. Since the design of efficient leukemia vaccine is based on the enhancement of reduced tumorgenicity of leukemia cells, a deeper understanding of the molecules and mechanisms that are responsible for the induction of the immunogenicity of leukemia cells can have implication for the immunotherapy of ALL.

On the other hand, manipulation of the expression levels and activity of apoptosis-mediated molecules on ALL cells in order to turn them into an efficient target for effector T-cells, can also be have an application for the development of additional therapy strategies of ALL. The results of our study may help to elucidate of the mechanisms underlying resistance of ALL cells to CD95-mediated apoptosis and facilitate the development of novel therapeutic approaches.

1.6 Plan of the study

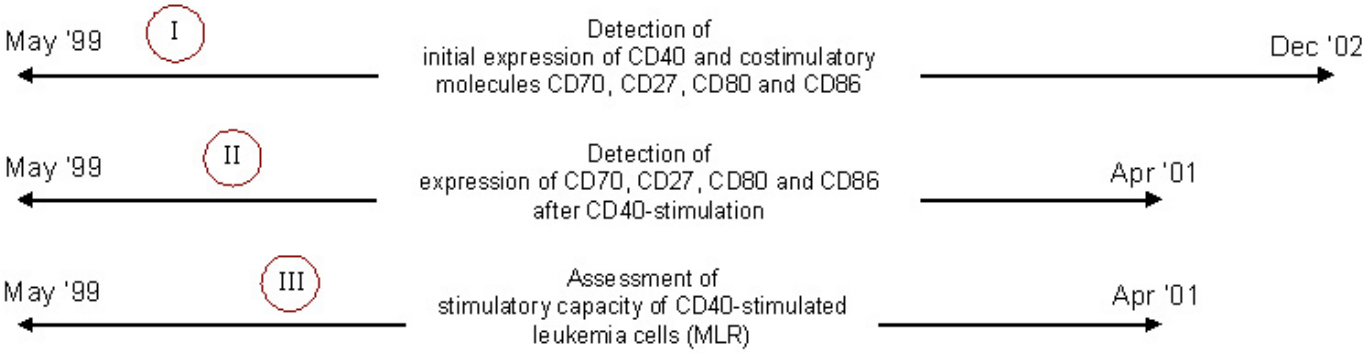
According to aims of the present work the following schema of study was designed as shown on page 15 and 17.

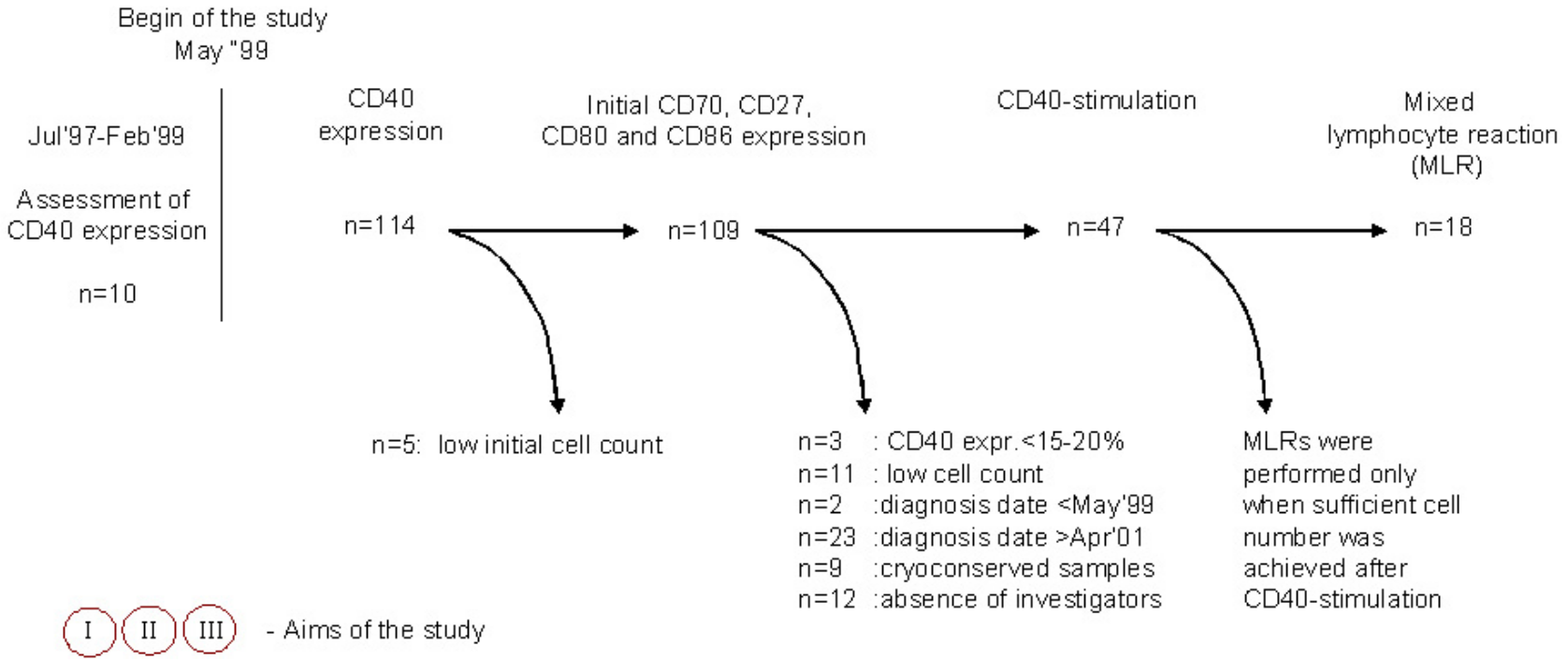
Scheme 1. In preliminary experiments (11.1997-2.1999) we have evaluated the expression of CD40 on limited number of ALL samples (n=10). Further, we have determined initial expression of CD40 and costimulatory molecules CD80, CD86, CD70 and CD27 on the blasts from newly diagnosed patients (n=124) with ALL. In the period of time from May 1999 to December 2002 we have analysed expression of these molecules on primary ALL cells from 73 patients which were diagnosed and treated in our clinic (Dusseldorf). Also the ALL samples from 51 patients with ALL were delivered for our experimental purposes from other clinics (Hamburg and Krefeld).

In the period of time from May 1999 to April 2001 we have investigated the expression of costimulatory molecules and CD70 in particular on the CD40-activated ALL blasts. ALL blasts from selected 47 patients were used for CD40 stimulation. The following selection criteria were used: CD40 expression at level more than 15-20% and sufficient initial cell number. Importantly, to delay any modulation processes by cryoconcervation only fresh isolated ALL cells were used for CD40-stimulation.

After CD40 stimulation ALL blasts from 18 patients were used for mixed lymphocyte reaction in order to investigate the stimulatory capacity of CD40-stimulated ALL blasts. Sufficient number of CD40-stimulated ALL cells was the only selection criterion in these experiments.

Scheme 1: Patients selection and plan of the study





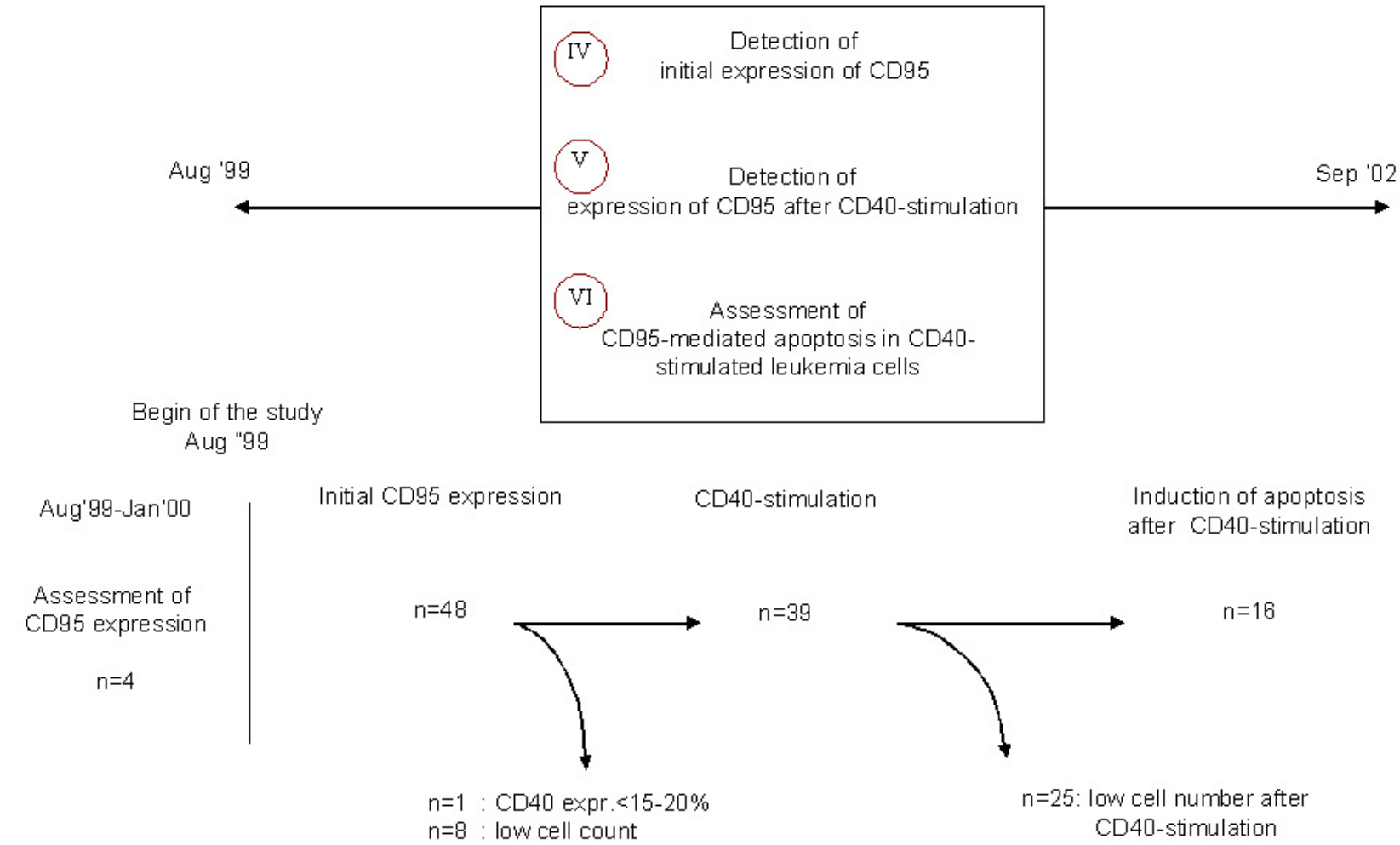
Scheme 2. In the period of time from August 1999 to September 2002 the expression of CD95 on the blasts from newly diagnosed patients with ALL was investigated.

Initial CD95 expression on the primary ALL blasts was assessed in a total 48 patients, whose blasts were also used for assessment of expression of costimulatory molecules.

With aim to study the expression of CD95 on CD40-stimulated ALL blasts, cells from 39 selected patients were stimulated with CD40. The major selection criteria were sufficient cell number and CD40 expression at level more than 15-20%.

Further analysis of CD95-mediated apoptosis on CD40-stimulated ALL cells was performed only in cases when sufficient cell number was obtained after CD40-stimulation (n=16).

Scheme 2: Patients selection and plan of the study



IV V VI - Aims of the study

2 Patients, materials and methods

2.1 Patients selection and characteristic

Prognostic characteristics of childhood leukemia include the initial leukocyte count, age at diagnosis, sex, cytogenetics, immunophenotype, CNS (central nervous system) disease at diagnose and some other factors. In vitro resistance-profile of leukemia blasts against chemotherapeutic drugs - prednison, vincristin and asparaginase - so called PVA score is also one of the new important prognostic factors (used as risk factor from 1997). It is used as additional parameter for stratification regard treatment intensity. On the basis of prognostic factors patients were divided into different risk groups (low/high risk groups) and were treated accordingly. Definition of prognostic risk groups for the patients with ALL is shown below in Table 1.

low risk group	
Leukocyte count at diagnosis $<25000/\mu$l	and
Age $\geq$1 or $<$10 years	and
Immunophenotype (c-ALL or pre-B-ALL)	and
Complete remission on day 29	and
Absence of genetic translocations	and
high risk group	
Leukocyte count at diagnosis $>25000/\mu$l	or
Age $\geq$10 or $<$1years	or
Immunophenotype (early pre-B-ALL, B-ALL or T-ALL)	or
No complete remission on day 29	or
Genetic translocations	or

Table 1:

Definition of risk prognosis groups according coALL protocol.

In our study were used the bone marrow or peripheral blood from patients with acute B lymphoblastic leukemia with initial diagnosis date from 1980 to 2002. Patients were treated according the respective coALL protocols active at the time of diagnosis. All research samples were collected in accordance with ethics committee approval. Patients or parents were informed and gave written consent to give blood or bone marrow for our particular experimental purpose. ALL cells from 73 patients diagnosed and treated in our clinic (Dussleldorf) and 51 patients from other clinics (Hamburg and Krefeld) were used in experiments described in present work. The ALL cells from cryoconserved samples (n=11) were used only to determine the initial expression of tested molecules.

Prognostic characteristics of patients, whose peripheral blood or bone marrow was used in experiments which are described in present work, are summarized in Table 2.

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
1	Ds	c-ALL	m	11	6	111	high	+	-	-	-	-
2	HH	c-ALL	f	3	6	9.4	low	+	+	+	+	-
3	Ds	c-ALL	f	5	6	2	low	-	-	-	-	-
4	Ds	c-ALL	m	13	5	31.2	high	+	-	-	-	-
5	HH	c-ALL	m	11	7	1.9	high	+	+	+	+	-
6	HH	c-ALL	m	1	5	31	high	+	+	+	+	-
7	Ds	c-ALL	m	1	6	28.3	high	+	+	+	+	-
8	Ds	c-ALL	f	3	7	23.8	low	+	+	+	+	+
9	HH	c-ALL	m	3	6	18.7	low	+	-	-	-	-
10	Ds	c-ALL	f	7	5	2.7	low	+	+	+	+	-
11	Ds	c-ALL	m	1	3	24.9	low	+	-	-	-	-

Table 2: Characteristics of patients. m - male; f - female; yrs - years; PVA - *in vitro* resistance-profile of ALL blasts against chemotherapeutic drugs; leuk.count - leukocyte count. Origin of samples: Ds - Dusselforf; HH - Hamburg and Krefeld. • - Tests that were performed, assessment of: costim. - expression of costimulatory molecules; CD95 - expression of the CD95; bs. - basal expression on the primary blasts; st. - expression on the CD40-stimulated/unstimulated blasts; ap. - CD95-mediated apoptosis on the CD40-stimulated blasts.

Patients' characteristics, continued (1).

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
12	HH	c-ALL	m	2	6	30.7	high	+	+	-	-	-
13	Ds	c-ALL	m	5	1	33	high	+	+	+	+	-
14	Ds	c-ALL	m	6	4	21	low	-	-	-	-	-
15	HH	c-ALL	f	2	5	21.6	low	+	-	-	-	-
16	HH	c-ALL	m	5	5	159	high	+	+	+	+	+
17	HH	c-ALL	m	11	9	9.9	high	+	-	-	-	-
18	HH	c-ALL	f	7	5	26.8	high	+	+	+	+	-
19	Ds	c-ALL	m	6	5	103	high	+	+	+	+	-
20	Ds	c-ALL	m	1	8	5	low	-	-	-	-	-
21	Ds	c-ALL	m	5	5	41	high	+	-	-	-	-
22	HH	c-ALL	m	3	3	5.4	low	+	-	-	-	-
23	HH	c-ALL	f	2	3	15.5	low	+	-	-	-	-
24	HH	c-ALL	m	8	8	2.5	low	+	+	-	-	-
25	Ds	c-ALL	f	4	6	45	high	+	-	-	-	-
26	HH	c-ALL	f	4	3	30.7	high	+	+	-	-	-
27*	HH	c-ALL	m	3	-	8.7	low	+	+	+	+	-
28	HH	c-ALL	m	7	5	1.8	low	-	-	-	-	-
29	Ds	c-ALL	f	2	3	14.2	low	+	+	+	+	-
30	Ds	c-ALL	f	4	1	3.3	low	-	-	-	-	-
31	HH	c-ALL	m	1	5	11.5	low	+	-	-	-	-
32	Ds	c-ALL	f	1	3	20.5	low	+	-	-	-	-
33	Ds	c-ALL	f	3	4	22	low	-	-	-	-	-
34	HH	c-ALL	f	3	1	12.5	low	+	+	+	+	+
35 **	HH	c-ALL	m	<1	-	11	high	+	-	+	-	-

Table 2: Characteristics of patients. PVA score not tested due to: * - diagnosis date<1997; ** - patient age (infant).

Patients' characteristics, continued (2).

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
36	Ds	c-ALL	m	2	3	13.5	low	-	-	-	-	-
37	Ds	c-ALL	f	3	6	9.6	low	+	-	-	-	-
38*	Ds	c-ALL	m	2	-	16.2	low	+	-	-	-	-
39	Ds	c-ALL	m	1	1	25	high	-	-	-	-	-
40	HH	c-ALL	m	5	1	42.4	high	+	-	+	+	-
41	Ds	c-ALL	m	4	6	4.6	low	+	+	-	-	-
42	Ds	c-ALL	m	1	6	6.5	low	-	-	-	-	-
43	Ds	c-ALL	m	14	7	6.3	high	+	+	+	+	+
44	HH	c-ALL	m	2	5	3	low	+	+	-	-	-
45	HH	c-ALL	m	5	7	39.9	high	+	+	+	+	-
46	HH	c-ALL	m	4	1	4.7	low	+	-	-	-	-
47	Ds	c-ALL	m	2	4	5.6	low	-	-	-	-	-
48	HH	c-ALL	f	10	4	3.6	low	+	-	-	-	-
49	Ds	c-ALL	m	7	5	6.4	low	+	+	-	-	-
50	Ds	c-ALL	m	9	5	6.1	low	+	-	-	-	-
51	Ds	c-ALL	f	3	5	15.7	low	+	-	+	+	+
52	Ds	c-ALL	f	1	3	8.1	low	+	+	-	-	-
53	Ds	c-ALL	f	4	8	20.6	low	+	-	-	-	-
54	HH	c-ALL	f	1	4	8.7	low	+	-	-	-	-
55	Ds	c-ALL	f	1	4	5.6	low	+	-	-	-	-
56	HH	c-ALL	m	13	1	170	high	+	-	-	-	-
57	HH	c-ALL	f	11	1	2.2	high	+	-	-	-	-
58***	Ds	c-ALL	m	1	-	35.6	low	+	+	+	+	-

Table 2: Characteristics of patients. PVA score not tested due to: * - diagnosis date<1997; *** - reduced cell viability.

Patients' characteristics, continued (3).

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
59	HH	c-ALL	m	3	6	12.3	low	+	-	-	-	-
60	HH	c-ALL	f	3	8	15.6	low	+	-	-	-	-
61	Ds	c-ALL	m	2	7	81	high	+	+	+	+	-
62	HH	c-ALL	m	3	7	4.1	low	+	+	-	-	-
63	HH	c-ALL	m	13	5	64.7	high	+	-	-	-	-
64	Ds	c-ALL	m	5	6	12.1	low	-	-	-	-	-
65	Ds	c-ALL	f	1	5	25.2	high	+	-	-	-	-
66	Ds	c-AlL	m	3	5	7.15	low	+	-	-	-	-
67	HH	c-ALL	m	2	7	108.4	high	+	+	+	+	+
68	Ds	c-ALL	m	2	7	14	low	+	-	-	-	-
69	HH	c-ALL	m	4	4	53.6	high	+	+	+	+	-
70*	HH	c-ALL	m	6	-	72.8	high	+	+	+	+	-
71	HH	c-ALL	m	4	9	200	high	+	-	+	-	-
72*	HH	c-ALL	f	2	-	77.3	high	+	-	+	-	-
73*	Ds	c-ALL	f	3	-	18	low	+	-	+	-	-
74*	Ds	c-ALL	m	14	-	31.2	high	+	-	+	-	-
75*	Ds	c-ALL	f	2	-	29.2	high	+	-	+	-	-
76*	Ds	c-ALL	m	16	-	4.4	low	+	-	+	-	-
77*	Ds	c-ALL	m	3	-	73.9	high	+	-	+	-	-
78*	Ds	c-ALL	f	4	-	21	low	+	-	-	-	-
79*	Ds	c-ALL	f	2	-	87.1	high	+	-	-	-	-
80	Ds	pre-B-ALL	m	1	5	7.5	low	-	-	-	-	-
81	HH	pre-B-ALL	f	1	3	9.3	low	+	-	-	-	-
82	HH	pre-B-ALL	f	3	6	12.6	low	+	-	-	-	-

Table 2: Characteristics of patients. PVA score not tested due to: * - diagnosis date<1997.

Patients' characteristics, continued (4).

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
83	HH	pre-B-ALL	m	4	5	263.7	high	+	+	-	-	-
84	Ds	pre-B-ALL	f	9	5	78.1	high	+	+	-	-	-
85	HH	pre-B-ALL	f	4	7	1.5	low	+	-	-	-	-
86	HH	pre-B-ALL	f	1	5	49	low	+	-	-	-	-
87	Ds	pre-B-ALL	m	6	5	30.5	high	+	+	-	-	-
88	Ds	pre-B-ALL	f	3	1	69.6	high	+	+	+	+	-
89	Ds	pre-B-ALL	m	14	5	32.4	high	+	+	-	-	-
90	Ds	pre-B-ALL	m	3	4	29	high	+	-	+	+	+
91	HH	pre-B-ALL	m	4	5	8.5	low	+	-	+	+	+
92	Ds	pre-B-ALL	m	11	3	0.5	high	+	-	-	-	-
93	Ds	pre-B-ALL	f	6	5	8.3	low	+	+	-	-	-
94	Ds	pre-B-ALL	f	7	3	18.4	low	-	-	-	-	-
95	Ds	pre-B-ALL	m	4	0	71	high	+	-	-	-	-
96	HH	pre-B-ALL	m	6	1	4.6	low	+	-	+	+	+
97	Ds	pre-B-ALL	m	8	7	6.2	low	+	+	-	-	-
98	Ds	pre-B-ALL	m	7	7	12	low	+	+	-	-	-
99	HH	pre-B-ALL	f	11	4	4.9	high	+	-	-	-	-
100	HH	pre-B-ALL	m	2	4	124	high	+	-	+	+	-
101	Ds	pre-B-ALL	m	5	5	10.7	low	+	+	+	+	-
102	HH	pre-B-ALL	m	10	7	3.6	high	+	+	+	+	+
103	Ds	pre-B-ALL	m	1	3	36	high	+	+	+	+	-
104**	Ds	pre-B-ALL	f	<1	-	324.1	high	+	-	-	-	-

Table 2: Characteristics of patients. PVA score not tested due to: ** - patient age (infant).

Patients' characteristics, end (5).

patient number, origin		**leukemia subtype**	**sex**	**risk factors**			**risk groups**	**Tests•**				
				age (yrs)	PVA score	leuk. count		**costim.**		**CD95**		
								bs.	st.	bs.	st.	ap.
105	Ds	pre-B-ALL	f	9	5	72.2	high	-	-	-	-	-
106	Ds	pre-B-ALL	f	3	3	8.1	low	+	-	+	+	+
107	HH	pre-B-ALL	m	1	5	88.1	high	+	-	-	-	-
108	Ds	pre-B-ALL	m	7	8	6	high	+	-	+	-	+
109***	Ds	pre-B-ALL	m	9	-	19.9	low	+	-	-	-	-
110**	Ds	pre-B-ALL	f	<1	-	10.9	low	-	-	-	-	-
111*	Ds	pre-B-ALL	m	7	-	141	low	+	+	-	-	-
112	Ds	pre-B-ALL	m	2	5	10.2	low	+	-	+	+	+
113*	Ds	pre-B-ALL	m	7	-	2.4	low	+	+	-	-	-
114***	Ds	pre-B-ALL	m	5	-	1.6	low	+	-	-	-	-
115	Ds	pre-B-ALL	f	4	7	10.7	low	+	-	-	-	-
116*	Ds	pre-B-ALL	m	16	-	44	high	+	+	+	+	-
117	HH	pre-B-ALL	f	6	0	27.5	high	+	-	+	-	-
118	HH	early pre-B-ALL	m	2	5	165.3	high	+	-	+	+	+
119	HH	early pre-B-ALL	f	<1	-	222	high	+	-	+	+	+
120**	HH	early pre-B-ALL	m	<1	-	547	high	+	+	+	+	-
121**	Ds	early pre-B-ALL	f	4	9	104.4	high	+	+	+	+	-
122	Ds	early pre-B-ALL	m	1	1	32.8	high	+	+	-	-	-
123	HH	early pre-B-ALL	f	8	1	6.3	high	+	+	+	+	-
124	HH	B-ALL	f	5	1	1.1	high	+	-	-	-	-

Table 2: **Characteristics of patients.** PVA score not tested due to: * - diagnosis date<1997; ** - patient age (infant); *** - reduced cell viability.

2.2 Chemicals

Albumin bovine	Sigma
Aqua ad injectabilia	Braun
Ammoniumchlorid (%)	Apotheke Heinrich-Heine University
Cycloheximide (CHX)	Sigma
Dimethylsulfochloxid (DMSO)	Sigma
Ethylendiamintetraacetat (EDTA)	Biochrom
Ethanol, absolute	Merck
Ficoll-Paque^{TM} Plus	Amersham
Fetal calf serum (FCS)	Gibco BRL
Human serum AB off the Clot	PAA
Isopropanol	Merck
L-glutamin	Gibco
Phosphate buffered saline (PBS)	Serag Wiessner
Penicillin/streptomycin	Gibco
Propidium iodide	Santa Cruz
RPMI-1640 medium	Gibco BRL
Sheep erythrocytes	Behring
Trypan blue	Sigma
Trypsin/EDTA	Gibco
Tween80	Merck

2.3 Enzymes

Neuraminidase	Dade Behring

2.4 Antibodies and Cytokines

Anti-CD3-PE/FITC	Immunotech
Anti-CD19-PE/FITC	Immunotech
Anti-CD10-FITC	Immunotech
Anti-CD70	Pharmingen
Anti-CD70-FITC	Pharmingen
Anti-CD80	R&D Systems
Anti-CD80-FITC	Immunotech
Anti-CD86	Immunotech
Anti-CD86-FITC	R&D Systems
Anti-CD40-PE	Immunotech
Anti-CD27-PE	Immunotech
Anti-CD95(CH-11)	Immunotech
Anti-CD95-PE	Becton Dickinson
Anti-MHC I-FITC	Pharmingen
Anti-HLA-DR-FITC	Becton Dickinson
Anti-CD4-PE	Immunotech
Anti-CD4-FITC	Becton Dickinson
Anti-CD8-PE	Becton Dickinson
Anti-CD8-FITC	Immunotech
Anti-CD69-PE	Becton Dickinson
Anti-CD25-PE	Immunotech
Anti-CD56-PE/FITC	Immunotech
Proleukin (IL-2)	CHIRON
IL-4	R&D Systems
IL-7	R&D Systems

2.5 Apoptosis-detection kit

Annexin V-FITC/Propidium iodide kit	Immunotech

2.6 *Enzyme Linked Immuno Sorbent Assay* (ELISA)

IL-10	Immunotech
IFN-γ	Immunotech

2.7 Cell lines

murine L-fibroblasts	ATCC catalog No.56-X
murine CD40L-L-fibroblasts	kindly provided from M.Roskrow, München
K562	ATCC catalog No.CCL-243

2.8 Media

RPMI-1640 medium supplemented with FCS, penicillin, streptomycin and L-glutamin as indicated below, was used as cell medium for cell culture.

10% (v/v)	FCS
10 U/ml	Penicillin
10 U/ml	Streptomycin
2 mM	L-Glutamin

2.9 Isolation of mononuclear cells and leukemia cells

ALL cells were obtained from fresh or cryoconcerved bone marrow (BM) or peripheral blood (PB) of patients with more than 80% marrow or peripheral blood involvement. Mononuclear cells were isolated from buffy-coat preparation from peripheral blood from healthy donors or patients by density centrifugation over Ficoll-Hypaque. The cell suspension was diluted 1:2 with PBS, layed on to Ficoll using a 1:1 ratio of Ficoll:cell suspension and centrifuged at 1100 xg at room temperature (RT) for 15 min. The MNC's band at the interface between the Ficoll and the remaining plasma was collected in a steril Falcon-tube (Falcon) and washed with PBS (666 xg, 5 min, RT). The still remaining red cells were lysed by incubation with ammonium chloride (5 min on the ice) and then eliminated via 2 times washing with PBS. Isolated mononuclear cells were then diluted in RPMI-1640 supplemented with FCS, L-glutamine and penicillin/streptomycin (further R-10 medium).

Cell viability was determined by trypan blue staining. Due to the impaired membrane integrity dead cells take up the trupan blue dye and can be assessed by counting under a microscope.

Methodically the sufficient number of leukemia cells is rapidly to obtain from bone marrow or peripheral blood of patients, whereas the maintenance of leukemia cells in culture remains the major technical problem. We have observed that culture of leukemia cells isolated from patients PB or BM that have been pre-incubated over 24 hours at room temperature shown lower viability when compared to culture of fresh isolated leukemia cells. The ALL cells were therefore isolated as soon as possible from fresh obtained BM or PB samples.

2.10 Isolation of T lymphocytes

2.10.1 Production of neuraminidase-treated sheep erythrocytes

10ml sheep erythrocytes (Behring) were centrifuged at 1185 xg for 9 min and cell-free supernatant was removed. The erythrocytes were then washed 2 times with PBS (1185 xg for 9 min, RT). The erythrocytes were diluted in 25ml PBS and cocultivated with 0.5ml neuraminidase for 2 hours at 37 °C. The erythrocytes were then washed 2 times with PBS. The pelleted erythrocytes were diluted in 25ml of R-10 medium and stored before use at 4 °C.

2.10.2 T lymphocytes isolation

For the isolation of T lymphocytes mononuclear cells were isolated as described (see subsection 2.9, page 27) and diluted at concentration of 25 x 10^6 cells in 10ml of PBS. After addition of neuraminidase-treated sheep erythrocytes (2ml) cells were pelleted by centrifugation (132 xg, 6 min, RT) and incubated on ice for 1 hour. The cells were then resuspended and subjected to a second Ficoll-density centrifugation. The T lymphocytes were then sedimentated through Ficoll, due to their bounding with sheep erythrocytes. Erythrocytes were lysed using ammonium chloride. The remaining T lymphocytes were washed 3 times in PBS. Using trypan blue staining, viability was checked to be 95%. The purity of the T-cell population was determined by staining with antibodies directed against

CD3 and following flow cytometry (see subsection 2.12, page 31). One typical example of a purity-analysis is shown on Fig.1, page 29. The T lymphocytes were used only in cases when more then 90% CD3 positive cells were isolated.

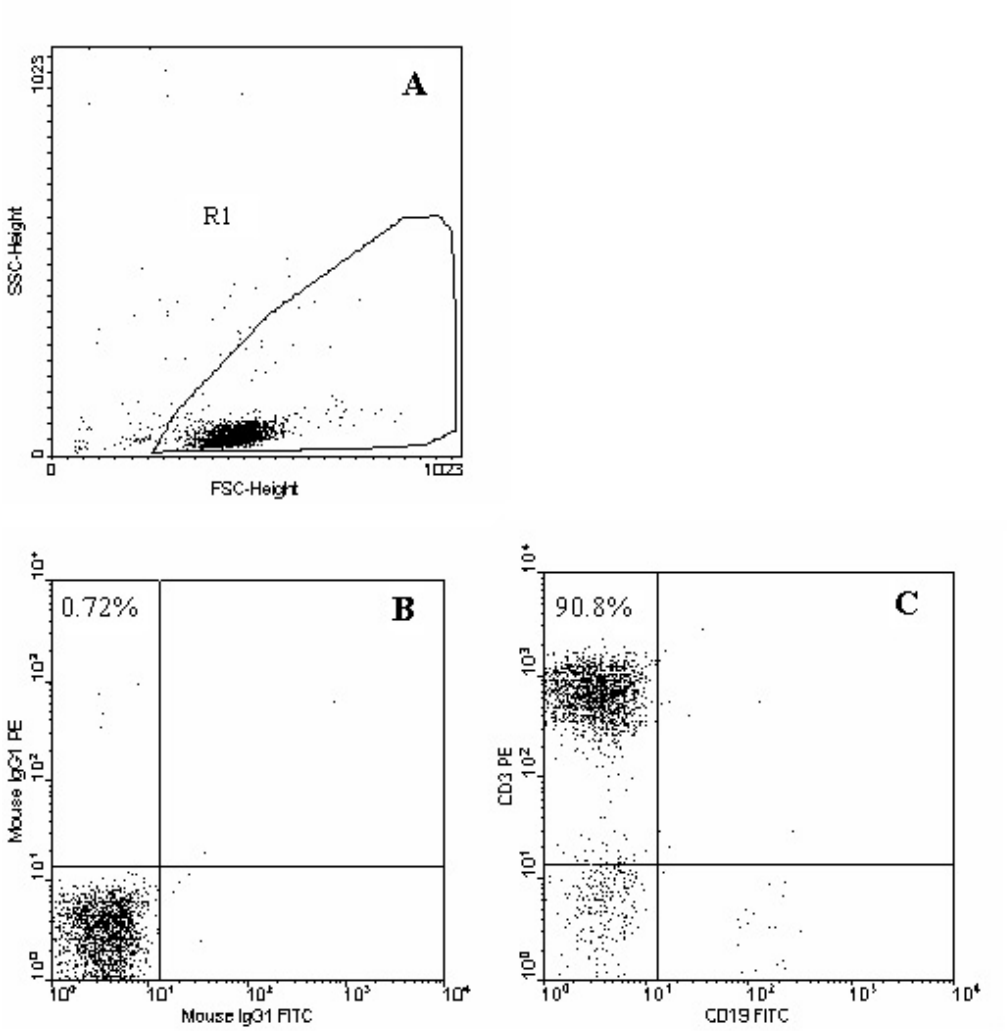


Figure 1:

T-cell purity. T-cells were isolated via T-cell rosetting. The purity was checked using FACS-analysis. **A** - one typical example of lymphocytes gate R1. **B** - the isotype control staining. **C** - the cells stained with T-cell specific marker CD3. In this example 90.8% of cells from gate R1 are positve for CD3.

2.11 Isolation of ultrapure B-lymphocytes using positive *Magnetic Activated Cell Sorter* (MACS)-selection

Cell rosetting with ferric oxide beads containing maghemite (magnetic beads or microbeads), which can be attracted by a magnet, allows for the quick and efficient recovery of cell sub-

population. Selective binding of beads occurs if they have a specific antibodies coupled to them (direct method). For MACS separation, cells are magnetically labeled with microbeads and separated on a column which is placed in the magnetic field of a MACS separator. The magnetically labeled cells are retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, magnetically retained cells can be eluted as positively selected cell fraction.

2.11.1 Cell staining with antibodies-coupling magnetic beads

Mononuclear cells were isolated as described (see subsection 2.9, page 27) and pelleted via centrifugation (666 xg, 6 min, RT). The supernatant was removed and the cell pellet was resuspended in 80μl of MACS buffer (2mM EDTA, 0.5% BSA in PBS) and 20μl of anti-CD19 microbeads per 10^7 total cells were added. After incubation for 15 min at 4 °C cells were washed and diluted in 500μl MACS-buffer .

2.11.2 Magnetic cell separation

For MACS separation, cells are magnetically labeled with CD19 microbeads and separated on a column which is placed in the magnetic field of a MACS separator. The magnetically labeled $CD19^+$ cells are retained in the column while the unlabeled $CD19^-$ cells run through. After removal of the column from the magnetic field, the magnetically retained $CD19^+$ cells can be eluted as positively selected cell fraction. The positive selection columns type MS^+/MR^+ for 10^4-10^8 positive cells were chosen. The columns were first washed with 1ml MACS-buffer. The cell suspension in appropriate buffer amount was then applied into the column. The magnetically labeled $CD19^+$ cells are retained in the column while the unlabeled $CD19^-$ cells run through. The column was washed with appropriate amount (1-2ml) of buffer. The purity of positively selected cells was determined by immunoflourescence staining and FACS-analysis (see 2.12, page 31). The B-lymphocytes were used only in cases when more then 90% CD19 positive cells were isolated.

2.12 Surface and intracellular marker analysis by flow cytometry using a *Fluorescence Activated Cell Sorter* (FACScan)

Flow cytometry measures optical and fluorescence characteristics of single cells. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by side-angle scatter) can resolve certain cell populations. Antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed through a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their stable states, the fluorochromes emit light energy at certain wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously. Commonly used dyes include propidium iodide (PI), phycoerythrin and fluorescein, although many other dyes are available. A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector (FSC), a side-scatter detector (SSC), and multiple fluorescence emission detectors: green (525 nm; FL1-detector), red (578 nm; FL2-detector) and dark-red (680 nm; FL3-Detector). The signals are amplified and converted to digital form for analysis and display on a computer screen. The FSC parameter is the cell size measure, while the intracellular granularity can be assessed by SSC. Using dot-plot-picture the dead cells could be separated from viable cells. PI-staining can also be used to determine dead cells.

2.12.1 Staining of surface molecules

0.5-0.1x10^6 cells were pelleted in polypropylene-tubes (Falcon) and the supernatant was eliminated. The cells were then incubated with 15μl direct-conjugated antibodies 15 min in dark at RT (direct staining). After washing with PBS (666 xg, 6 min, RT) cells were diluted in 200μl of PBS. When non-conjugated antibodies were used (indirect staining) the cells were stained in two steps. Cells stained as described above with non-conjugated antibodies were then incubated in darkness with mouse-immunoglobulin-specific, fluorescence-labeled secondary antibodies for 15 min at RT. Cells were washed twice with PBS (666 xg, 6 min, RT), diluted in 200μl of PBS and stored at 4 °C before analysis. Nonspecific staining was

determined using FITC/PE-conjugated Ig control mAbs.

Determination of CD40, CD70, CD80, CD86, CD27 expression on ALL blasts was performed as part of the routine laboratory leukemia immunophenotyping.

2.13 Detection of cytokine secretion by using *Enzyme Linked Immuno Sorbent Assay* (ELISA)

The secretion of cytokines was determined using commercial ELISA kit (Immunotech). Mitomycin C-treated (0.01mg/ml) unstimulated or CD40-stimulated leukemia cells were cocultured in 24-well flat-bottom plates at concentration of 0.5 x 10^6 cells/well in a final volume of 1ml with allogeneic T-cells, obtained from buffy coat (1 x 10^6 cells/well), in presence or absence of blocking antibodies as indicated in the text. After 5 days of incubation at 37 °C in 5% CO_2 supernatants were collected and stored at -80 °C till usage. The 96-well plates coated with antibodies against IFN-γ or IL-10 were used for IFN-γ and IL-10 determination, respectively. The 50μl of examined supernatants or human IFN-γ/IL-10 as standard were placed into each well. Plates were incubated for 2 hours at RT. Plates were further washed 3 times with washing buffer (0.05% Tween 80 in PBS). Next 50μl of biotinylated antibodies to IFN-γ/IL-10 and 100μl of horseradish peroxidase conjugated with streptavidin were added to each well. Plates were incubated for 30 min at RT and washed 3 times. After development with colorimetric reagent (100μl/well) for horseradish peroxidase (tetramethylbensidin) at RT in the dark for 15 min, 50μl of stop solution (H_2SO_4) was added to each well. Cytokine concentration in each well was determined by absorbance reading with ELISA reader (TECAN) at the wavelength of 450nm.

2.14 Cultivation of cells

2.14.1 Cultivation of cell lines

The used cell lines (see subsection 2.7, page 27) were cultured in cell culture flasks (Corning Costar) with R-10 medium (see subsection 2.8, page 27) at 37 °C in a 5% CO_2, 95% air humidified atmosphere. The cell lines were diluted with fresh R-10 medium one or two times per week depending on cell-proliferation. In case of adhesive cell lines the medium

was removed and cells were then incubated with Trypsin/EDTA-solution (0.05%/0.02% w/v; PAA) in the incubator at 37 °C for 2-5 min. These detached cells were diluted in 10ml medium and pelleted by centrifugation (666 xg, 5 min, RT). The pellet was dispensed in new culture flasks in ratio from 1:3 to 1:10 (dependent on cell number).

2.14.2 Cryoconserving and thawing of cells

For cryoconservation cells were removed from flask, pelleted in 10ml medium (1400 rpm, 5 min, RT). Pellet was resuspended in 1.5ml FCS with 10% DMSO (Sigma). These cryoconserved cells were stored in liquid nitrogen till usage. To defrost the cell lines, deeply frozen cells were incubated for 2-5 min at 37 °C in water bath and resuspended in 15ml of R-10 medium. Cells were then washed by centrifugation (666 xg, 5 min, RT) and transfered into culture flasks.

2.14.3 Stimulation of leukemia cells by CD40 cross-linking

Leukemia cells is difficult to culture because their high spontaneous death *in vitro*. The number of dead cells may be increased in particular when using thawed cryoconserved or not freshly isolated ALL cells. It is therefore important to isolate them carefuly, rapidly and place them immediately in culture media and control the cell culture each day.

CD40L-expressing mouse fibroblasts and CD40L-negative mouse fibroblasts were treated with mitomycin C (0.01 mg/ml) at 37 °C in 90 min and plated at concentration of 5 x 10^4 cells/well in 24-well plates in R-10 medium. Mitomycin C-treated fibroblasts were incubated in plates at 37 °C in 5% CO_2 for a minimum of 2 hours. Before use, plates were washed twice with R-10 medium. the leukemia cells were placed in the plates on the mitomycin C-treated fibroblasts at concentration of 1 x 10^6/ml in R-10. On day 3-4, CD40-stimulated leukemia cells and unstimulated leukeima cells were harvested, washed and used for phenotypic and functional analysis. Cell-surface expression of different molecules was determined by standard immunofluorencence staining with FITC/PE-conjugated mAbs and by flow cytometry analysis with FACScan.

2.15 Primary *Mixed Lymphocytes Reaction* (MLR)

Mitomycin C-treated unstimulated or CD40-stimulated leukemia cells were used as stimulator cells to induce the T-cell proliferation. Leukemia cells were placed at concentration of 5 x 10^4 cells/well in a final volume of 200μl with allogeneic T-cells, obtained from buffy coat (1 x 10^5 cells/well) in 96-well round-bottom plates. The cells were incubated together for 5 days at 37 °C in 5% CO_2. The culture medium used was R-10 medium. All microculture were performed in triplicate. The blocking anti-CD80/ anti-CD86 and anti-CD70 antibodies was added at the concentrations of 2 and 4μg/ml, respectively. Cells were pulsed with 1μ Ci of [H^3 - Thymidine (Amersham Bioscience) for the last 18 hours of the culture period. Cells were harvested onto glass fiber filters (Dunn Labortechnik GmbH) and the [H^3]-incorporation (cpm) was measured by liquid scintillation spectrophotometry (Beckman). Stimulation index (SI) was calculated for each individual experiment as follows:

$$\mathrm{SI} = \frac{\mathrm{cpm(T-cells + ALL\ cells)}}{\mathrm{cpm(T-cells)}}\,.$$

2.16 Generation of anti-leukemia-specific T-cell lines

The generation and expansion of autologous or allogeneic anti-leukemia-specific T-cell lines was performed using methodology depicted in Fig. 2. As effector cells were used allogeneic or autologous PB cells or PB stem cells. The cells were first stimulated in presence of IL-7 (10ng/ml) with CD40-stimulated or unstimulated leukemia cells in the absence of IL-2. Cells were then cultured at a concentration of 2x10^6/ml in 24-well plates in RPMI supplemented with 5% human AB serum (further RPMI-HS5), glutamine, penicillin/streptomycin at 37 °C. On day 3, fresh RPMI-HS5 media and IL-2 (50U/ml) were added. On day 10 cells were harvested, washed and restimulated with CD40-stimulated or unstimulated leukemia cells. The same sequence was repeated on day 20, as described in Fig. 2.

Because the long-term culture always carries the risk of contamination, generation of CTLs need more attention as short-term culture. Cells were placed in the middle on the plate, and the other wells were filled with sterile medium. This have not only warned our culture of contamination, but have also humidified the immediate area around the cells due to the

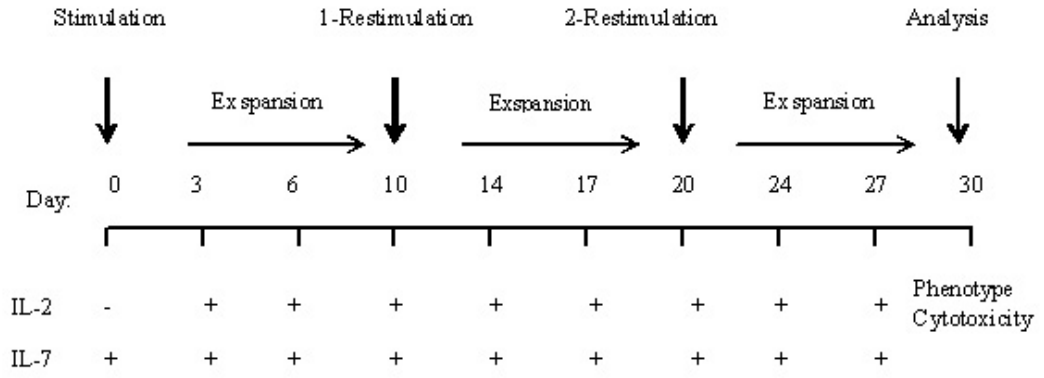


Figure 2:

Protocol for the generation anti-leukemia-specific T-cell lines. Effector cells were stimulated with CD40-stimulated or unstimulated leukemia cells in the presence of IL-7 (10ng/ml) and expanded by restimulation with CD40-stimulated or unstimulated leukemia cells and IL-2 (50U/ml).

decreasing evaporation. For optimal cell proliferation cultured cells need certain cell density pro well. The cell cultures were therefore controlled each day and dulited if necessary in fresh RPMI-HS5 medium.

2.17 Cytotoxic assay

T-cell-mediated toxicity was determined using a standard ^{51}Cr-release assay [148]. CD40-stimulated and unstimulated leukemia cells, as well as autologous PHA-blasts and NK-sensitive K562 cells were used as targets. 10^6 of target cells were placed in 50μl of FCS and incubated with 0.1mCi ^{51} Cr for 1 hours at 37 °C. Cells were then washed 2 times in R-10 medium. 100μl of labeled target cells were then plated in 96-well U-bottom plates at 5000 cells/well. 100μl/well of effector cells were plated at different effector:target ratios (from 6.2:1 to 100:1). All of the experiment were performed in duplicates. The plates were centrifuged for 3 minutes at 132 xg and incubated for 4 hours at 37 °C. After the incubation period, the 150μl of supernatants were harvested and radioactivity was measured in an automatic gamma counter (Beckman) or beta counter (Packard). Specific lysis was determined for each individual experiment as:

$$\text{specific lysis}(\%) = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \text{ x } 100\%$$

Maximum release was determined by the addition of 100μl of IPEGAL (Sigma, diluted 1:100 in Aqua dest) for the target cells. For detection of spontaneous release of ^{51} Cr from target cells were incubated with 100μl of R-10 medium.

2.18 Apoptosis

2.18.1 Induction of apoptosis

Detection of apoptosis in culture of leukemia cells is particular difficult because of high spontaneous death of leukemia cells *in vitro*. We have used only fresh isolated ALL cells for apoptosis detection. After CD40-stimulation ALL blasts were very rapidly washed and replaced in fresh medium for apoptosis induction.

Primary, CD40-stimulated or unstimulated ALL cells were harvested, washed, resuspended in R-10 medium and plated in 96-well (flat-bottom, tissue non-treated) plates at concentration 0.1x10^6 cells per well. The cells were incubated for indicated times with 1 μg/ml anti-CD95 (CH-11) antibodies in presence or absence of 0.1 μg/ml cycloheximide (CHX). Anti-CD95 and CHX were added at the initiation of culture at 50μl/well to reach final volume of 100μl per well.

2.18.2 Apoptosis detection

For detection of apoptosis, cells were collected in polypropylene tubes and washed with ice-cold PBS (666 xg, 3 min, 4 °C). Supernatant was discarded, the cells were then resuspended in ice-cold binding buffer (Immunotech) to 1x10^6 cells/ml. 1μl of the annexin V-FITC solution (Immunotech) and 5μl PI were added to the cells with subsequently incubation for 10 minutes in the dark. The incubation with annexin V-FITC and PI has been carried out on ice in order to arrest further apoptosis of the cells. The percentage of viable and apoptotic cells was analysed by flow cytometry. One typical example of apoptosis detection using flow cytometry is shown in Fig.3.

The biparametric presentation shows three distinct populations; the viable cells which have low FITC and low PI signal, the apoptotic cells, which have high FITC and low PI signal and the secondary necrotic cells which have high FITC and high PI signal (Fig.3).

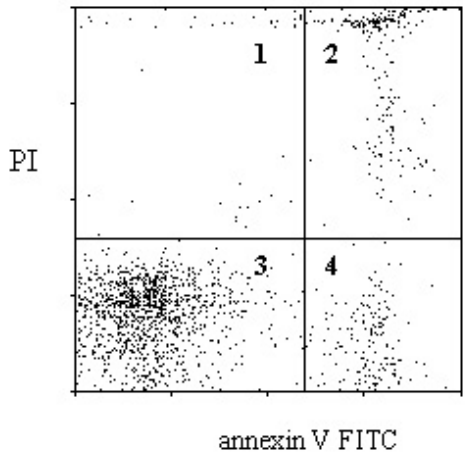


Figure 3:

Determination of apoptotic cells via annexin V-FITC/PI-staining. 200μl of ALL cells were placed in polypropylene tubes at a concentration of 1x10^6 cells/ml. 1μl of the annexin V-FITC solution (Immunotech) and 5μl PI (Immunotech) were added. Samples were incubated for 10 minutes in the dark at 4 °C and analysed by flow cytometry. Quadrant 2: 11% (secondary necrotic cells). Quadrant 3: 81% (viable cells). Quadrant 4: 8% (apoptotic cells).

The proportion of cells undergoing CD95-mediated apoptosis was calculated as follows:

$$\text{specific apoptosis}\,(\%) = \frac{\text{experimental apoptosis}\,(\%) - \text{spontaneous apoptosis}\,(\%)}{100\% - \text{spontaneous apoptosis}\,(\%)}\,\text{x}100\%$$

We have present the results of experiments only if spontaneous apoptosis was $< 5\%$.

2.19 Statistical analysis

The statistical significance between the analysed groups was determined using two-sample t-test. Differences with $p < 0.05$ were considered significant. For calculations we used the Microsoft Excel.

The expression of costimulatory molecules or CD95 on ALL cells from patients from different risk groups was analysed using the Chi-quadrat and Mann-Witney tests with reliability index p. When p was < 0.05 the difference between compared groups was considered significant.

3 Results

3.1 CD40 expression on ALL blasts

Interaction between CD40 and CD40L provides the signals required for B-cell activation and differentiation as well as a costimulatory signal for T-cell activation. Since CD40-stimulation can modulate expression of costimulatory molecules on mature B-cells that are necessary for efficient T-cell stimulation, we first analysed the CD40 expression on leukemia blasts. CD40 expression was assessed in a total of 117 patients with c-ALL (n=79) and pre-B-ALL (n=38), 6 patients with early pre-B-ALL and in one case with B-ALL (see patients' characteristics Tab. 6, page 49).

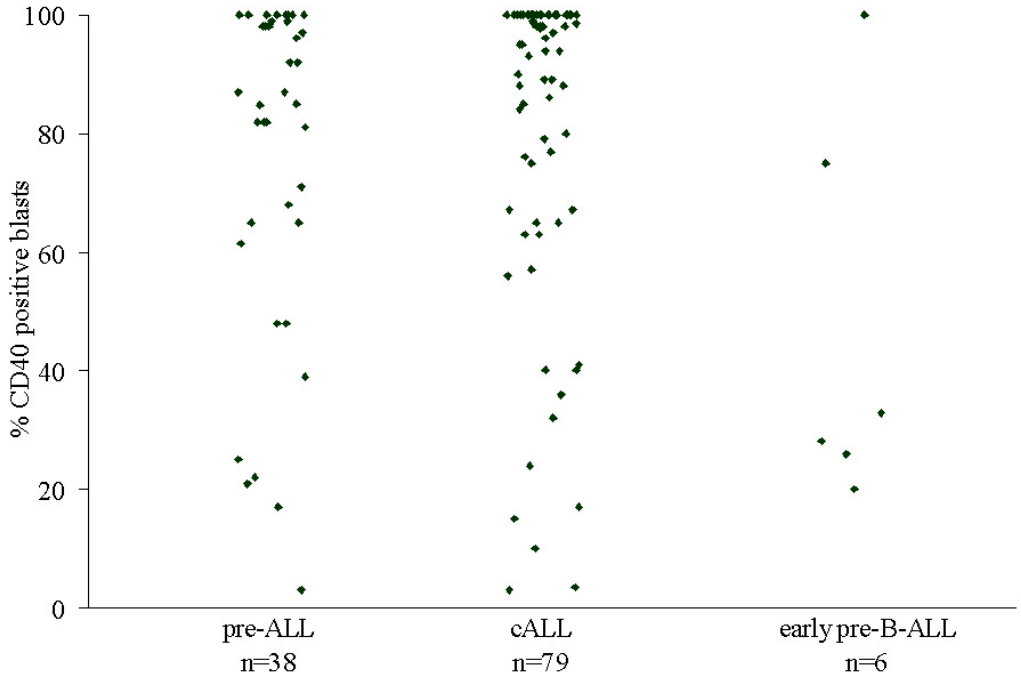


Figure 4:

Expression of CD40 molecule on primary ALL blasts. ALL blasts were obtained from bone marrow or peripheral blood of patients with more than 80% blast cell count and isolated by Ficoll-Hypaque density centrifugation. $CD19^+$ cells were stained with antibodies directed against CD40 and subjected to flow cytometry analysis.

We found that in more than 80% of cases tested, pre-B- and c-ALLs express CD40 (Fig.4). In 96 of this 117 patients, blasts express CD40 at high level (>60%), while in 7 patients no significant CD40 expression was detectable (<20%). In the remaining 14 patients CD40

expression varied between 20% and 60%. CD40 expression level on leukemia blasts did not differ between patients with c-ALL (n=79) versus pre-B-ALL (n=38). Only in 2 of 6 patients, blasts from patients with early pre-B-ALL CD40 expression level was greater than 60%. In the case of one B-ALL patients CD40 expression was high (98%).

3.2 Expression of costimulatory molecules and CD27 on ALL blasts

The major costimulatory signals for augmenting and sustaining a T-cell response are provided the costimulatory molecules CD80 and CD86. CD70-CD27 interaction is another costimulatory pathway important for T-cell activation. Therefore, we next investigated the baseline expression of these costimulatory molecules (CD80, CD86 and CD70) on freshly isolated or cryoconserved leukemia blasts as well as expression of CD70 receptor - CD27 molecule. The expression of costimulatory molecules was examined on pre-B-ALL (n=34) and c-ALL (n=68) blasts as shown in Fig. 5A and 5B respectively. Characteristic of patients whose blasts were used for detection of expression of costimulatory molecules is shown in Table 6, page 49.

All but one patient tested (CD80 expression=29%) showed low levels of CD80 expression (0-15%) independent of diagnosis (n=100). In contrast, the CD86 expression (n=100) was more variable, but also independent of diagnosis. Blasts from few patient samples (15 of 100) demonstrated high levels of CD86 expression (>50%) and the other group include samples (85 of 100) that demonstrated intermediate or low level of CD86 expression (<50%). Low level of CD70 expression (0-15%) was detected in 89 of 101 samples tested. The ALL blasts in only 12 patients expressed the CD70 molecule between 15% and 30%. With respect to the CD27 expression the patients could be divided into two groups. While 34 of 101 of patients blasts expressed CD27 at low and intermediate level (<50%), 67 of 101 patients leukemia cells showed high level of CD27 expression (> 50%).

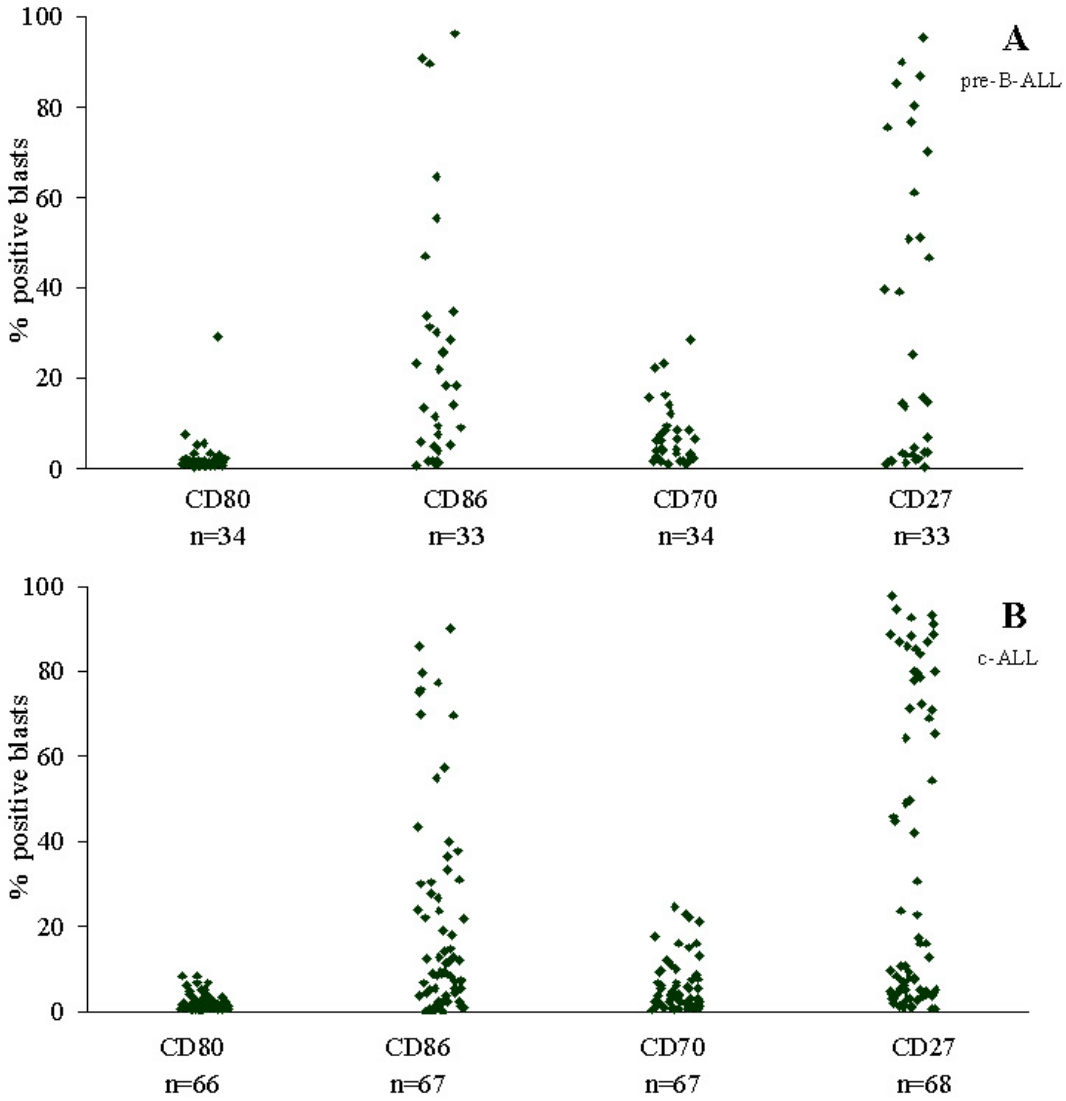


Figure 5:

Expression of costimulatory molecules and CD27 molecule on ALL blasts. ALL blasts were obtained from bone marrow or peripheral blood of patients with pre-B-ALL (**A**) and c-ALL (**B**) with more than 80% blast cell count and isolated by Ficoll-Hypaque density centrifugation. $CD19^+$ cells were stained with antibodies directed against CD80, CD86, CD70 and CD27 and subjected to flow cytometry analysis.

In all but one cases tested, primary ALL blasts from patients with leukemia subtypes of early pre-B-ALL and B-ALL expressed very low level of costimulatory molecules (Table 3, page 41). CD86 and CD27 which are heterogeneously distributed on pre-B-ALL and c-ALL blasts, were expressed at lower level on blasts with early pre-B- and B-ALL.

leukemia subtype	**ALL blasts**			
	$CD80^+$ median (range)%	$CD86^+$ median (range)%	$CD70^+$ median (range)%	$CD27^+$ median (range)%
B-ALL n=1	12.2	0.9	2.0	1.9
early pre-B-ALL n=6	2.1 (0-9.7)	7.5 (3.2-92.2)	5.7 (0.5-19.8)	3.7 (1-31)
pre-B-ALL n=34	1.6 (0.3-29)	20.3 (0.7-96.2)	5.3 (0.8-28.6)	14.7 (0.4-95.3)
c-ALL n=68	1.5 (0.3-8.4)	11.2 (0.1-90)	3.7 (0.3-21.9)	16.8 (0.5-97.7)

Table 3:

Median and range of expression level of costimulatory molecules and CD27 on B-, early pre-B-, pre-B- and c-ALL blasts. ALL blasts were obtained from peripheral blood or bone marrow from patients with early pre-B-ALL and B-ALL. $CD19^+$ blasts were stained with antibodies directed against CD80, CD86, CD70 and CD27 and subjected for flow cytometry analysis.

We have analysed whether ALL blasts from cryoconserved samples express costimulatory molecules at lower or higher level when compared to fresh obtained ALL blasts. It was observed no significant differences between expression of costimulatory molecules on ALL blasts obtained from fresh (n=109) and cryoconserved (n=15) samples.

3.3 Stimulation of ALL blasts by CD40-cross-linking

Since most of the examined leukemia blasts express CD40 (Fig.4, page 38), we investigated whether CD40-stimulation modulates the expression of the costimulatory molecules CD80, CD86, CD70 and CD27. Due to low survival of leukemia cells cultured in the absence of feeder cells, murine fibroblasts were used as feeder cells in our experimental system. To investigate the modulation of costimulatory molecules, ALL blasts were cocultivated with mitomycin C-treated CD40L-expressing mouse fibroblasts ($CD40L^+$-fibroblasts) for 3-4 days. As a control, non-transfected CD40L-negative fibroblasts ($CD40L^-$-fibroblasts) were used for coculture. In subsequent experiments, ALL blasts cocultured in the presence of $CD40L^+$-fibroblasts are reffered as CD40-stimulated ALL blasts, ALL cells cocultured in the presence of $CD40L^-$-fibroblasts are designated as unstimulated ALL cells.

ALL blasts from 43 patients with pre-B-ALL (n=15) and c-ALL (n=29) and 4 patient with early pre-B-ALL (see patients characteristic, Tab. 8, page 51) were included in the experiments. Expression of costimulatory molecules was not assessed in cases when cell viability was low (<20% of viable cells) after 3-4 days of culture. In few cases it was not possible to assess the expression of each of the molecules tested (CD80, CD86, CD70 and CD27) after CD40-stimulation due to a low initial cell number. Results were included for analysis only in cases when determination of the tested molecules was available on primary blasts and following CD40 stimulation. An assessment following culture on $CD40L^-$-fibroblasts was not possible in individual cases due to low viability under this culture condition.

As shown in Fig.6A CD40-stimulated ALL blasts significantly up-regulate expression of CD80 from 1.9±0.3% to 41±4% ($p<0.05$).

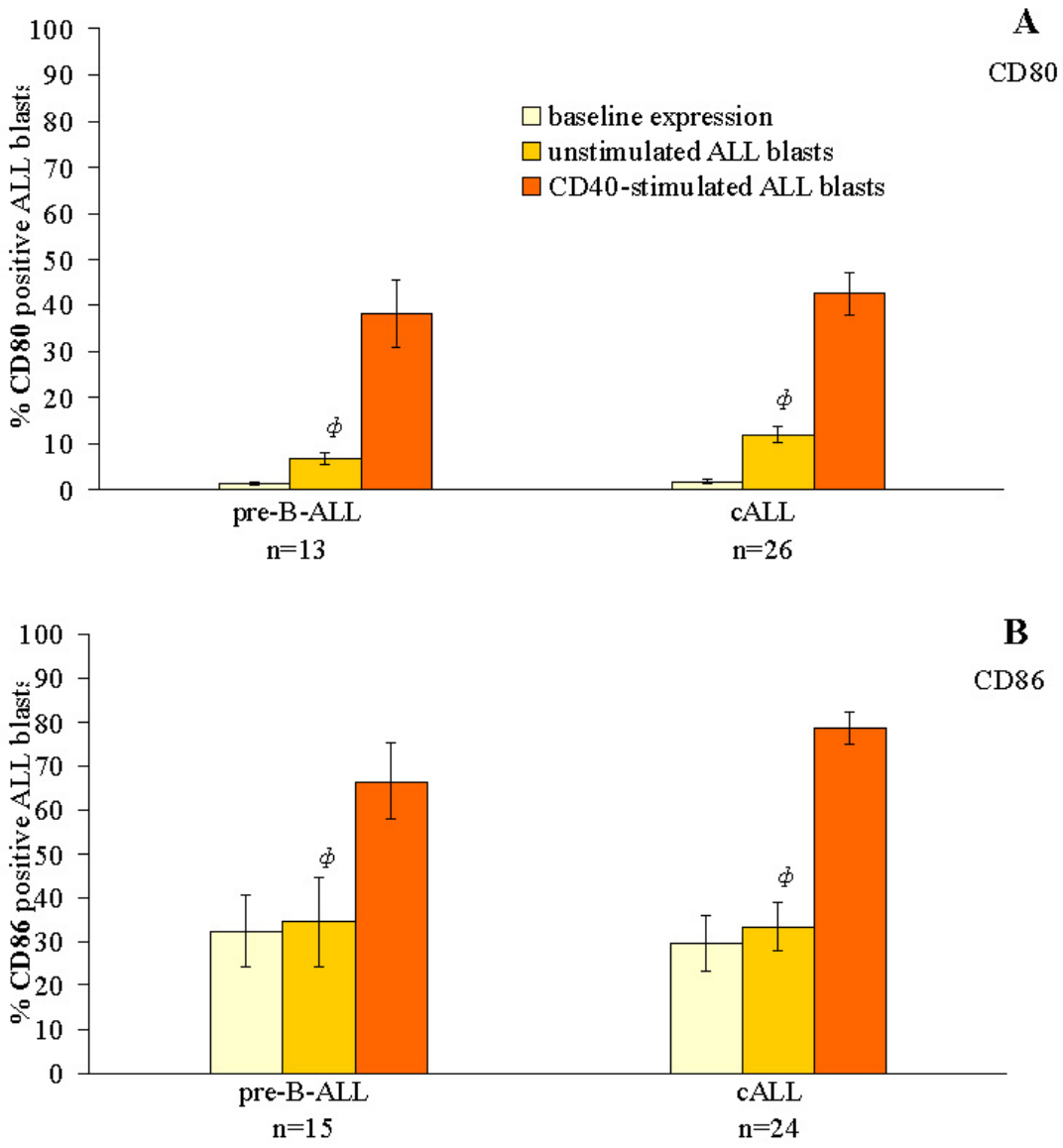


Figure 6:

Expression of CD80 and CD86 on the CD40-stimulated ALL blasts. ALL blasts were cocultivated for 3-4 days with mitomycin C-treated $CD40L^+/CD40L^-$- fibroblasts. **A**: $CD19^+$ blasts were double-stained with antibodies directed against CD80. $^{\phi}$ - expression was tested in n=9 (pre-B-ALL) and n=23 (c-ALL). **B**: $CD19^+$ blasts were double-stained with antibodies directed against CD86. $^{\phi}$ - expression was tested in n=10 (pre-B-ALL) and n=22 (c-ALL). Results are expressed as the mean percentage of double-positive cells±SE.

Following activation via CD40 receptor expression of CD86 was significantly enhanced from 31±5% up to 74±4% ($p<0.05$) in all ALL blasts tested (Fig.6B). This up-regulation

is clearly demonstrated on ALL blasts with initial low expression of CD86 (Fig.7A). Also, in blasts with initial high CD86 expression level a tendency to increase the CD86 expression following CD40-stimulation was observed (Fig.7B). CD40-induced up-regulation of both CD80 and CD86 did not differ between different subtype of leukemia (pre-B- and c-ALL).

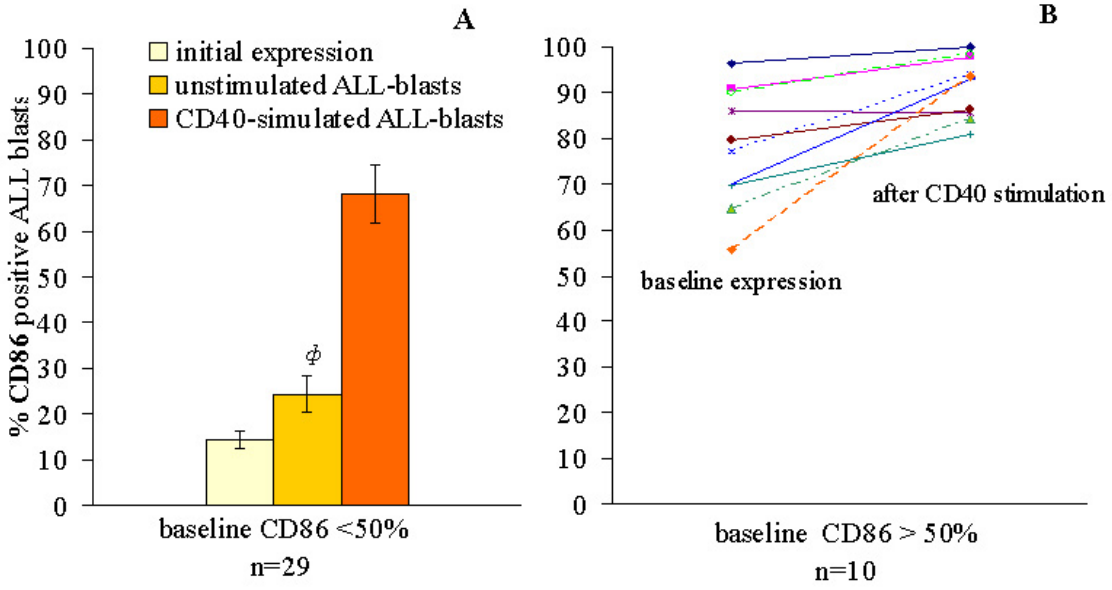


Figure 7:

Up-regulation of CD86 expression on ALL blasts after CD40-stimulation. ALL blasts were cocultivated for 3-4 days with mitomycin C-treated $CD40L^+/CD40L^-$- fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD86. $^\phi$ - expression of CD86 was tested in n=25. Results are expressed as the mean percentage of double-positive cells±SE.

Markedly, CD80 expression was already up-regulated at a low level when ALL blasts were cultured on $CD40L^-$- fibroblasts ($p<0.05$). In the group of patients with initial low CD86 expression on the blasts, cultivation of ALL blasts on $CD40L^-$- fibroblasts induced up-regulation of this molecule, however at lower level when compared to CD40-stimulated ALL blasts. This could be partly due to the presence of some adhesive molecules on the fibroblasts or secretion of soluble factors by the fibroblasts. These factors increase the survival of ALL blasts and could stimulate ALL blasts, however with a low intensity.

CD70 expression on CD40-stimulated ALL blasts was enhanced up to 63±4%, compared to native ALL blasts (6±1%) as shown in Fig.8A ($p<0.05$). As seen in Fig.8B up-regulation of CD70 expression was accompanied by reciprocal changes in CD27 expression (from 35±6% to 14±3%; $p<0.05$).

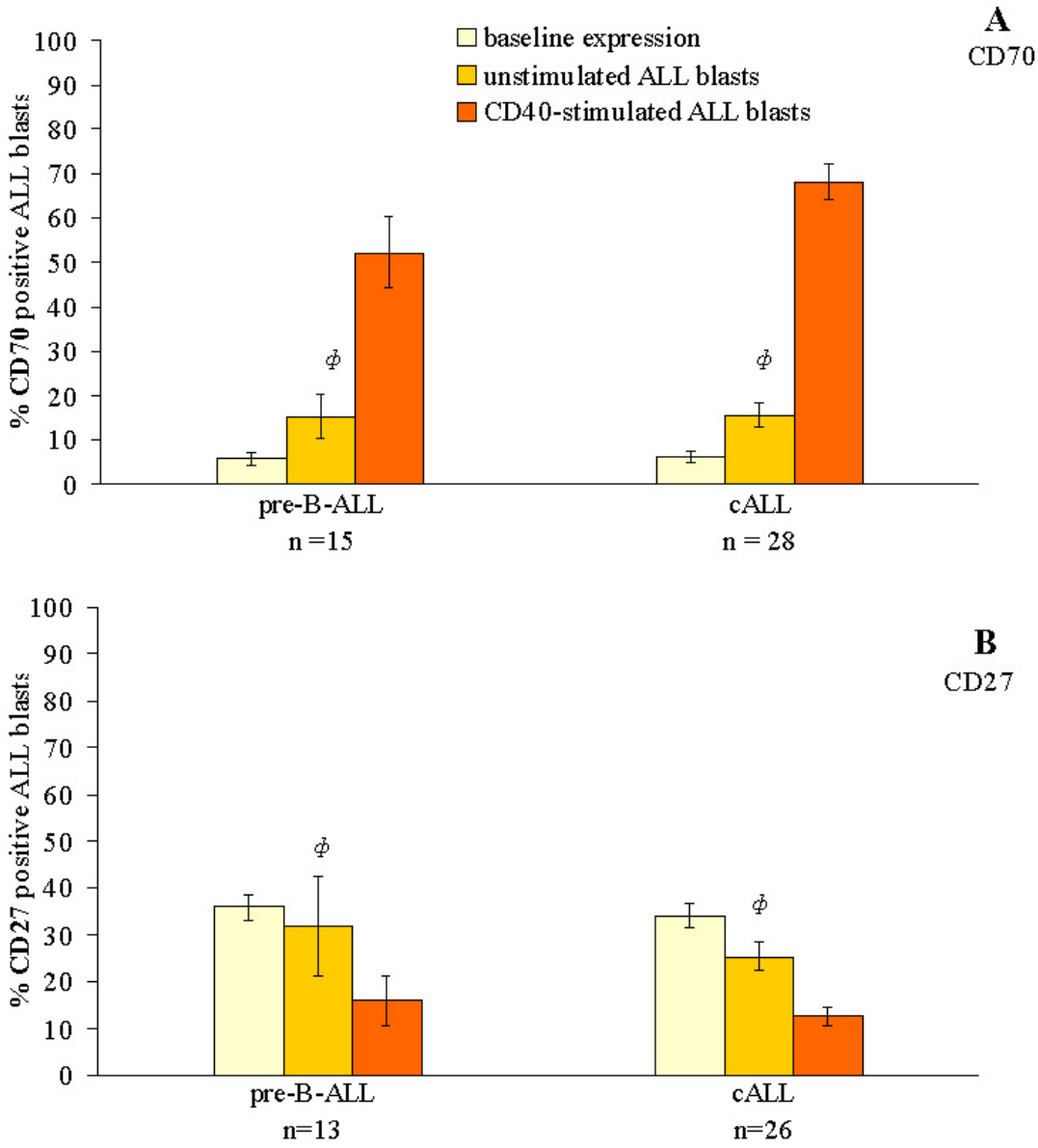


Figure 8:

Expression of CD70 and CD27 on the CD40-stimulated ALL blasts. ALL blasts were cocultivated 3-4 days with mitomycin C-treated $CD40L^+/CD40L^-$- fibroblasts. **A**: $CD19^+$ blasts were double-stained with antibodies directed against CD70. $^\phi$ - expression was tested in n=12 (pre-B-ALL) and n=25 (c-ALL). **B**: $CD19^+$ blasts were double-stained with antibodies directed against CD27. $^\phi$ - expression was tested in n=11 (pre-B-ALL) and n=23 (c-ALL). Results are expressed as the mean percentage of double-positive cells±SE.

The expression of CD27 was strongly down-regulated independent of its initial expression level (Fig.9).

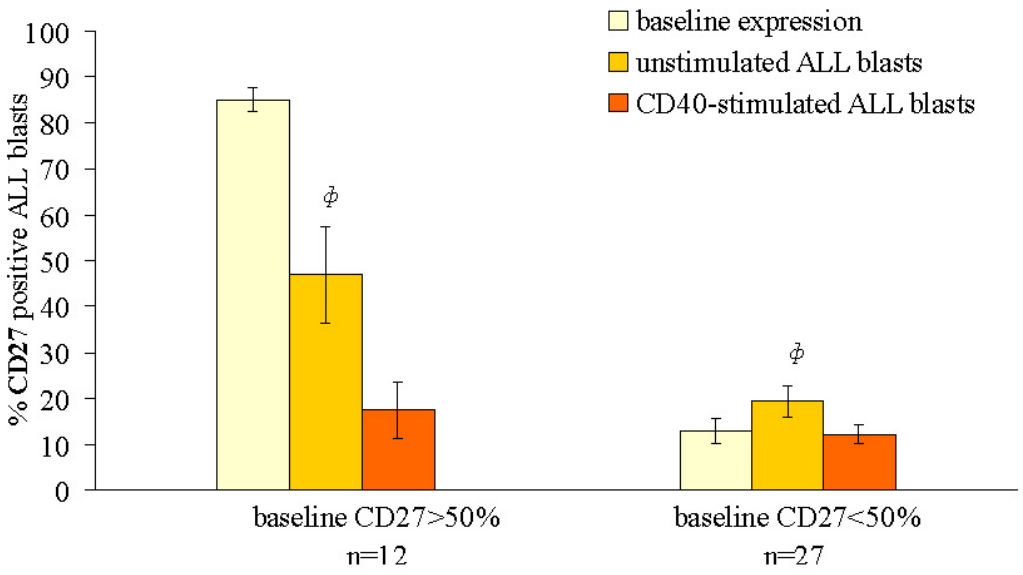


Figure 9:

Up-regulation of CD27 expression on the CD40-stimulated ALL blasts. ALL blasts were cocultivated 3-4 days with mitomycin C-treated $CD40L^+/CD40L^-$- fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD27. $^\phi$ - expression of CD27 molecules was not tested due to low cell viability in 3 cases. Results are expressed as the mean percentage of double-positive cells±SE.

patient number	**baseline expression**				**CD40-stimulated early pre-B-ALL blasts**			
	$CD80^+$	$CD86^+$	$CD70^+$	$CD27^+$	$CD80^+$	$CD86^+$	$CD70^+$	$CD27^+$
pt.120	9.7	3.9	9.8	1.1	20.4	15	36.3	1.6
pt.121	3.6	35.9	7.8	31	26.1	79.8	38.6	8.9
pt.122	0	3.2	5.1	5.3	88.8	76.1	94.2	21.6

Table 4:

Expression of costimulatory molecules and CD27 before and after CD40-stimulation on early pre-B-ALL blasts. ALL blasts from patients with early pre-B- and B-ALL were cocultivated 3-4 days with mitomycin C-treated $CD40L^+$- fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD80, CD86, CD27 and CD70 and subjected for flow cytometry analysis. Results are expressed as the mean percentage of double-positive cells.

ALL blasts from 3 patients with early pre-B-ALL also up-regulate costimulatory molecules after CD40-stimulation (see Table 4, page 46). Blasts from the 4^{th} patient with early pre-B-ALL did not survive after cocultivation on $CD40L^-$- as well as on $CD40L^+$-fibroblasts and therefore did not included in the analysis.

In order to compare the capacity of fresh and defrosted ALL blasts to express costimulatory molecules in response to CD40 stimulation ALL blasts from cryoconserved samples were used for stimulation. As seen in Tab. 5 in two from 3 samples tested expression of CD80 and CD70 on CD40-stimulated ALL blasts from cryoconserved probes was lower when compared to expression of these molecules on CD40-stimulated fresh isolated ALL blasts.

patient number	**CD40-stimulated ALL blasts**							
	$CD80^+$		$CD86^+$		$CD70^+$		$CD27^+$	
	fr.	df.	fr.	df.	fr.	df.	fr.	df.
pt.19	23	23±8	88	88±2	40	40±6	51	21±4
pt.43	15	25±3	33	38±3	40	28±2	14	2±0
pt.95	n.t	n.t	n.t	n.t	11	11±2	n.t	n.t
pt.121	57	18±1	73	82±1	68	31±2	4	10±1

Table 5:

Expression of costimulatory molecules and CD27 before and after CD40-stimulation on ALL blasts from fresh and cryoconserved samples. Fresh (fr.) and defrosted (df.) ALL blasts were cocultivated 3-4 days with mitomycin C-treated $CD40L^+$- fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD80, CD86, CD27 and CD70 and subjected for flow cytometry analysis. Results are expressed as the mean percentage of double-positive cells.

3.4 Analysis of the correlation between the expression of costimulatory molecules and the biological course of leukemia

3.4.1 Patients' characteristics

Expression of costimulatory molecules on ALL blasts is important for the antigen-presenting capacity of leukemia cells and therefore could be important for the stimulation of an anti-leukemia immune response *in vivo*. In order to answer the question whether high baseline expression of any costimulatory molecule could be associated with poor or favorable prognosis we have analysed the correlation between initial expression of costimulatory molecules and selected prognostic factors.

As shown in Tab. 1, page 18 patients are assign to different risk groups (low or high risk group) on the basis of prognostic factors. Patients with initial leukocyte count greater than

25000 cells/μl are universally recognized as having a particularly poor prognosis. Patients who are young at diagnosis (<2years) and older patients (≥10years) have a relatively poor prognosis compared with children in the intermediate age group. Infants (i.e., younger than 12 months of age) with ALL have an extremely poor prognosis and stratified separately for treatment purpose. Immunophenotype also appears to correlate with prognosis. Patients with B-lineage ALL have the worst prognosis, T-lineage ALL has been associated with a poor prognosis. *In vitro* resistance-profile of ALL blasts against chemotherapeutic drugs - prednison, vincristin and asparaginase - the so called PVA score is also one of the important prognostic factors that is used within the framework of coALL protocol as a risk factor since 1997. The chemotherapeutic resistant blasts show a PVA score between 7- 9.
In our study we used bone marrow or peripheral blood from 13 patients with B-lineage ALL with initial diagnosis date from 1980 to 1997 and from 111 patients with B-lineage ALL with initial diagnosis date from 1997 to 2002. Patients were treated according coALL protocol.
Among the risk parameters that define the risk groups in our patient cohort leukocyte count at diagnose and PVA score are the main determinators for group allocation. 86% of patients belong to the high risk group due to the high leukocyte count at diagnosis (≥ 25000/μl). 19% of children possess the risk factor - age (<1year or ≥10year). For 3 children the age was the only risk factor for assigning to the high risk group.

Characteristics of patients whose blasts were used in experiments which are described in present work include some important prognostic factors that are summarized in Table 6 (page 49). Characteristics of each of the patients whose blasts were used in experiments described in present work are shown in Table 2 (subsection "Patients' characteristics", page 19- 24).

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age (yrs); (range)	PVA score ≥ 7	leuk. count $\geq 25000/\mu l$	low	high
c-ALL n=79*	50	29	3; (0-16) n=11(≥10yrs); n=1(<1yrs)	14	29	46	33
pre-B-ALL n=38**	23	15	5; (0-16) n=5(≥10yrs); n=2(<1yrs)	6	16	20	18
early pre-B-ALL n=6***	3	3	1.5; (0-8) n=0(≥10yrs); n=2(<1yrs)	1	5	0	6
B-ALL n=1	0	1	5	1	0	0	1

Table 6:

Characteristics of ALL patients whose blasts were used in experiments described in present work. m - male; f - female; yrs - years; PVA - *in vitro* resistance-profile of ALL blasts against chemotherapeutic drugs; leuk.count - leukocyte count. PVA score was not tested: * - in 13 cases (11pts with diagnosis date<1997; 1pt - infant, in 1pt was not tested); ** - in 7 cases (1pt with diagnosis date<1997; 2pts - infant; in 4pts was not tested); *** - in 2 cases (infants).

Expression of CD40 molecule was determined on the all 124 patients, whereas expression of costimulatory molecules CD80, CD86, CD70 and CD27 was not tested in n=11 patients with c-ALL (pts numbers: 3, 14, 20, 28, 30, 33, 36, 39, 42, 47 and 64) and in n=4 patients with pre-B-ALL (pts numbers: 80, 94, 105 and 110).

Among 124 patients whose blasts were used in experiments described in present work in 8 patients CNS disease was detected at diagnosis and in one patient CNS disease was detected at relapse (see table 7).

patients number	**leukemia subtype**	**sex**	**age, yrs**	**PVA score**	**leuk. count x$10^6/\mu$l**	**risk groups (low/high)**
pt.71	c-ALL	m	4	9	200	h
pt.61	c-ALL	m	2	7	81	h
pt.72	c-ALL	m	2	n.t.**	77.3	h
pt.70	c-ALL	m	2	n.t.**	72.8	h
pt.35	c-ALL	m	0	n.t.*	11	h
pt.87 •	pre-B-ALL	m	6	5	30.5	h
pt.117	pre-B-ALL	f	0	27.5	6	h
pt.120	early pre-B-ALL	m	0	n.t.*	547	h
pt.119	early pre-B-ALL	f	0	n.t.*	222	h

Table 7:

Characteristics of ALL patients with CNS disease. • - CNS disease detected at relapse. PVA score was not tested due to * - age (infant); ** - diagnose date <1997.

ALL blasts from 15 patients with pre-B-ALL (pts numbers: 83-84, 87-89, 93, 97-98, 101-103, 111, 113, 116) and from 28 patients with c-ALL (pts numbers: 2, 5-8, 10, 12-13, 16, 18-19, 24, 26-27, 29, 34, 41, 43-45, 49, 52, 58, 61-62, 67, 69-70) and 4 patient with early pre-B-ALL (pts numbers: 120-123) were stimulated with CD40. Patients' characteristics are shown in Tab. 8, page 51.

3.4.2 Prognostic factors and expression of costimulatory molecules on primary ALL blasts

Since surface expression of costimulatory molecules provides the necessary 2^{nd} costimulatory signal required for induction of an efficient immune response, we have analysed the correlation between expression levels of costimulatory molecules on ALL blasts and clinical parameters. First we have calculated the general probability to belong to low risk versus high risk prognosis group of patients in the specific cohort of our study. Next, the probability to fall into low versus high risk group was calculated among patients whose blasts express more/less than the defined level of costimulatory molecule. These both

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age(yrs); range	PVA score ≥ 7	leuk. count $\geq 25000/\mu$l	low	high
c-ALL n=29 *	21	8	3; (1-14) n=2($\geq$10yrs); n=0(<1yrs)	8	14	14	15
pre-B-ALL n=15**	12	3	7; (1-16) n=3($\geq$10yrs); n=0(<1yrs)	3	9	6	9
early pre-B-ALL n=4***	2	2	2.5; (0-8) n=0($\geq$10yrs); n=1(<1yrs)	1	3	0	4

Table 8:

Characteristics of patients those ALL blasts were used for CD40-stimulation. - PVA score was not tested: * -in three cases (2 pts with diagnosis <1997; in 1pt was not tested); ** - in three cases (3 pts with diagnosis <1997); *** - in 1 case (infant).

probabilities were compared.

We have compared the expression level of costimulatory molecules (CD80, CD86, CD70 and CD27) and prognostic factors (PVA score and leukocyte count at diagnosis), risk groups definition and relapse frequency.

For the analysis of probability to get a relapse, only patients with diagnosis date> august 2001 were selected, i.e. patients who received the complete therapy.

3.4.3 Association between expression of CD80 and CD86 on primary ALL blasts and prognostic factors

We have found that the patients whose cells expressed more then 3% of CD80 have a tendency to lower probability to get relapse when compared to all patients tested or to patients whose blasts originally expressed less as 3% of CD80 (p =0.076) (Fig.10, page 52). It is worth to underline that baseline CD80 expression on ALL blasts is slightly above the background level and some methodical mistakes could influence our statistical calculations. However, as we discuss later, that even minor up-regulation of CD80 expression over minimum level (3%-5%) can be important for stimulatory capacity of ALL blasts.

We did not found any significant correlation between CD80 expression levels on primary ALL blasts and clinical prognostic factors tested (PVA score and leukocyte count at diagnosis).

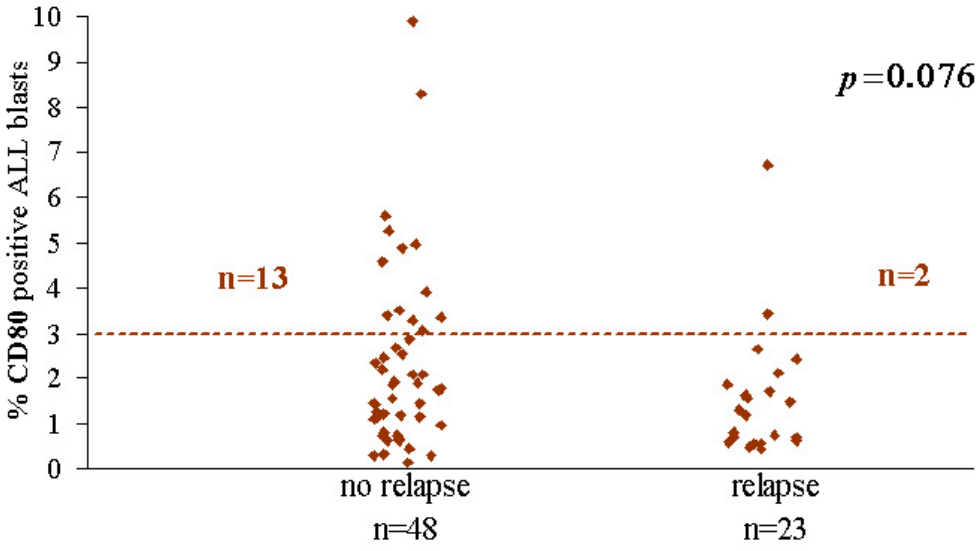


Figure 10:

CD80 expression and probability to get relapse. Among patients whose blasts express more than 3% of CD80 the probability to have a relapse was calculated and compared with probability to have a relapse for patients whose ALL blasts express less than 3% CD80. Probabilities were compared using a Chi-quadrat test with reliability index p.

Despite the fact that CD86 was heterogeneously expressed on primary ALL blasts, we did not found any significant correlation between the baseline expression of CD86 and prognosis factors tested (PVA score and leukocyte count at diagnosis) and relapse frequency.

3.4.4 Association between expression of CD27 and CD70 on primary ALL blasts and prognostic factors

High expression of CD27 - receptor for the CD70 - on primary ALL blasts has found to be associate with low leukocyte count at diagnosis.

As seen in Fig.11A (page 54) the probability to belong to the group with low initial leukocyte count among patients whose blasts expressed CD27 at level >50% is a significantly higher when compared with the general probability for all patients to fall in this group and significantly higher when compared with probability to fall in this group for patients with CD27 expression <50% (p <0.01). Also, CD27 expression on the patients blasts at level more than 50% is the statistic significant criteria to belong to the patients group with low initial leukocyte count (<25000/μl).

As seen in Fig.11B (page 54) patients whose ALL blasts originally expressed >50% CD27 also have higher probability to belong to the low-risk group when compared to all patients tested and when comapred to patients with the CD27 expression lower as 50% (p <0.05).

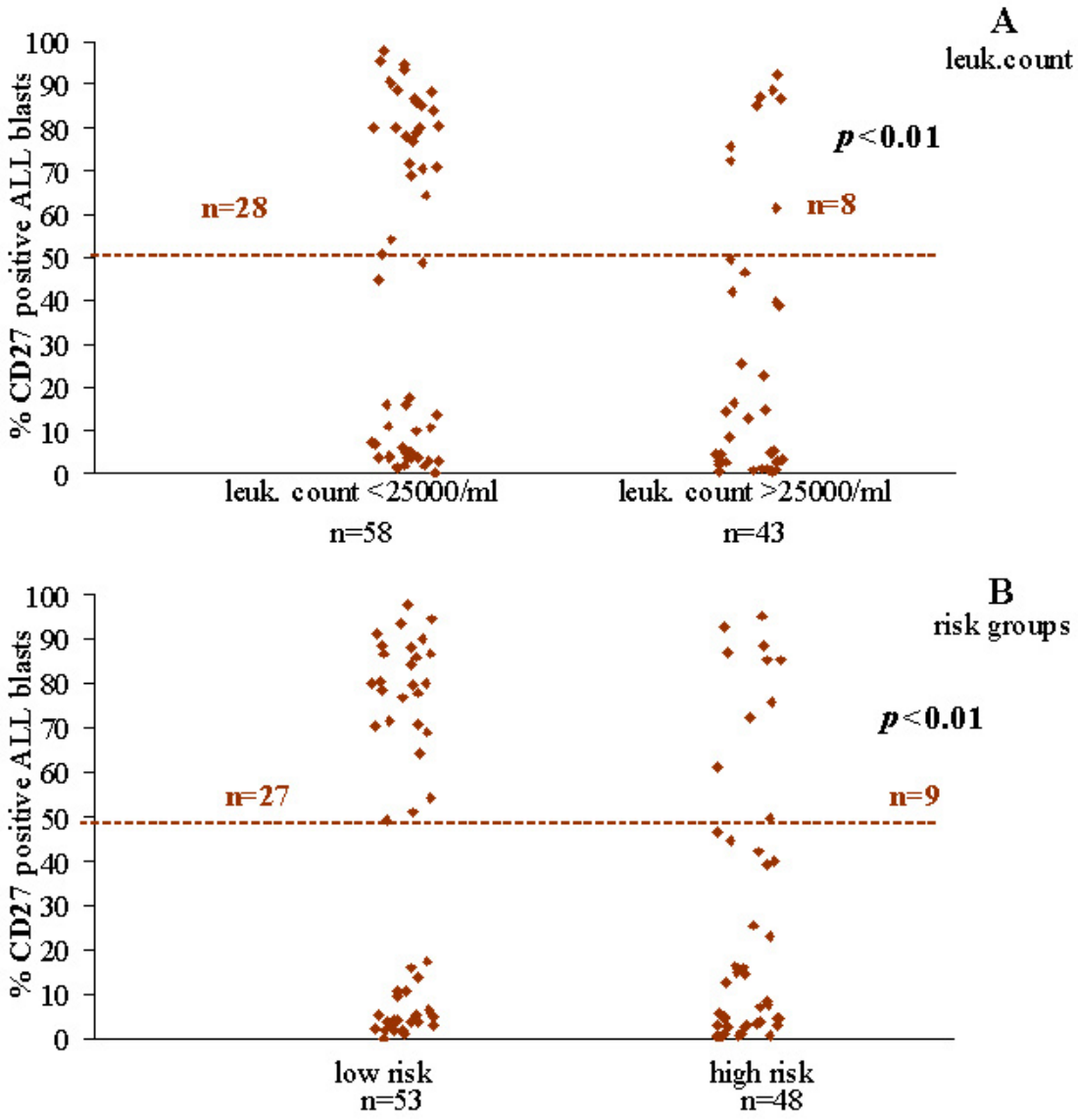


Figure 11:

CD27 expression and prognostic factors. Among patients with ALL whose blasts express more than 50% of CD27 probability to belong to group: (**A**) with leuk.count $\geq$25000/μl at diagnosis or (**B**) high risk group; was calculated and compared with general probability to belong to group: (**A**) with leuk.count $\geq$25000/μl at diagnosis or (**B**) high risk group. Probabilities were compared using a Chi-quadrat test with reliability index p. Difference with p <0.05 was considered significant.

Between baseline expression of CD70 on primary ALL blasts and prognostic factors leukocyte count and PVA score and relapse frequency we have found no correlation.

3.4.5 Prognostic factors and expression of costimulatory molecules on CD40-stimulated ALL blasts

Enhanced levels of costimulatory molecules on CD40-stimulated leukemia cells were shown to be important for antigen-presenting capacity of leukemia blasts. Therefore we have analysed whether the capacity of ALL blasts to up-regulate the costimulatory molecules (CD80, CD86 and CD70) after CD40-stimulation is correlated with any clinical parameters. We have analysed conditional probability to belong to different risk groups of patients depending on level of the costimulatory molecules on the CD40-stimulated ALL-blasts (see patients characteristic Table 8, page 51). Early pre-B-ALL samples were not used for analysis because of low number of patients.

As we have shown, CD86 is heterogeneously distributed on primary ALL blasts and its expression was not different between patients from low or high risk groups.

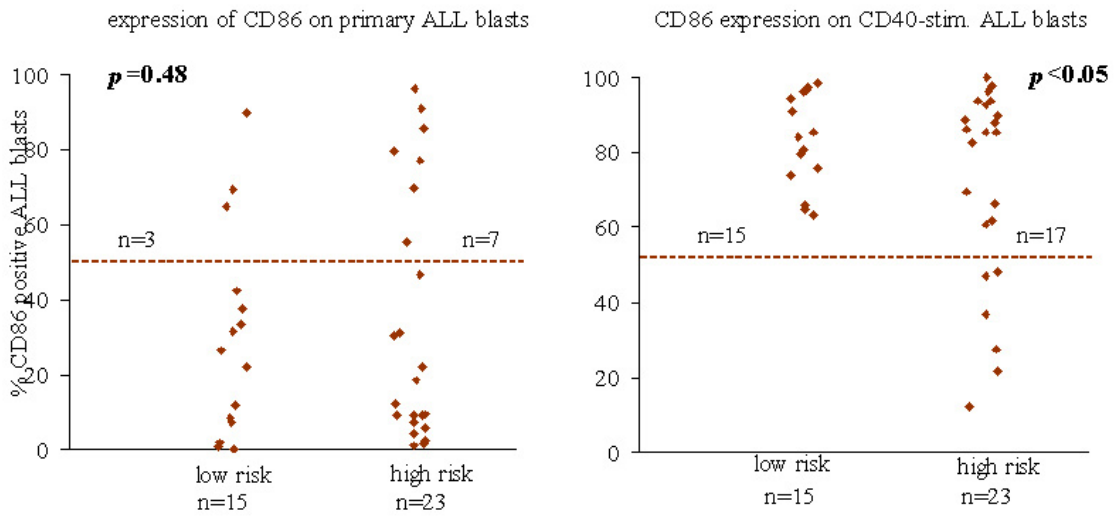


Figure 12:

CD86 expression on the primary and CD40-stimulated ALL blasts and risk groups. ALL blasts were cocultivated for 3-4 days with mitomycin C-treated $CD40L^+$-fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD86. Probabilities to belong to low risk group was calculated for patients whose blasts express CD86 at level more and less than 50%. The probabilities were compared using a Chi-quadrat test with reliability index p. Difference with $p < 0.05$ was considered significant.

In contrast, after the CD40-stimulation the ALL blasts from patients with low risk uniformly up-regulate CD86 at level more than 50% (Fig.12, page 55). This up-regulated level of CD86 is significantly higer ($p < 0.05$) when compared to CD86 expression on the

CD40-stimulated ALL blasts from high risk patients. Up-regulation of CD80 and CD70 have no correlation with any of prognostic factors or relapse probability.

3.4.6 Expression of costimulatory molecules and CD27 and CNS disease on ALL patients

Since it was described that some patients with leukemia have elevated level of soluble CD27 molecule (sCD27) in serum and cerebrospinal fluid, we have also analyse a possible association between the expression of CD27 and costimulatory molecules on ALL blasts and the probability to get CNS disease,

Among all patients studied in 8 patients CNS disease was detected at diagnosis and in one patient CNS disease was detected at relapse (Tab. 7, page 50). As seen in Fig.13 (page 57) the initial expression level of CD27, costimulatory molecules CD80, CD86 and CD70 was not different between the patient groups with or without CNS disease. However, because of the low number of patients with CNS disease (n=9) no significant correlation could be found.

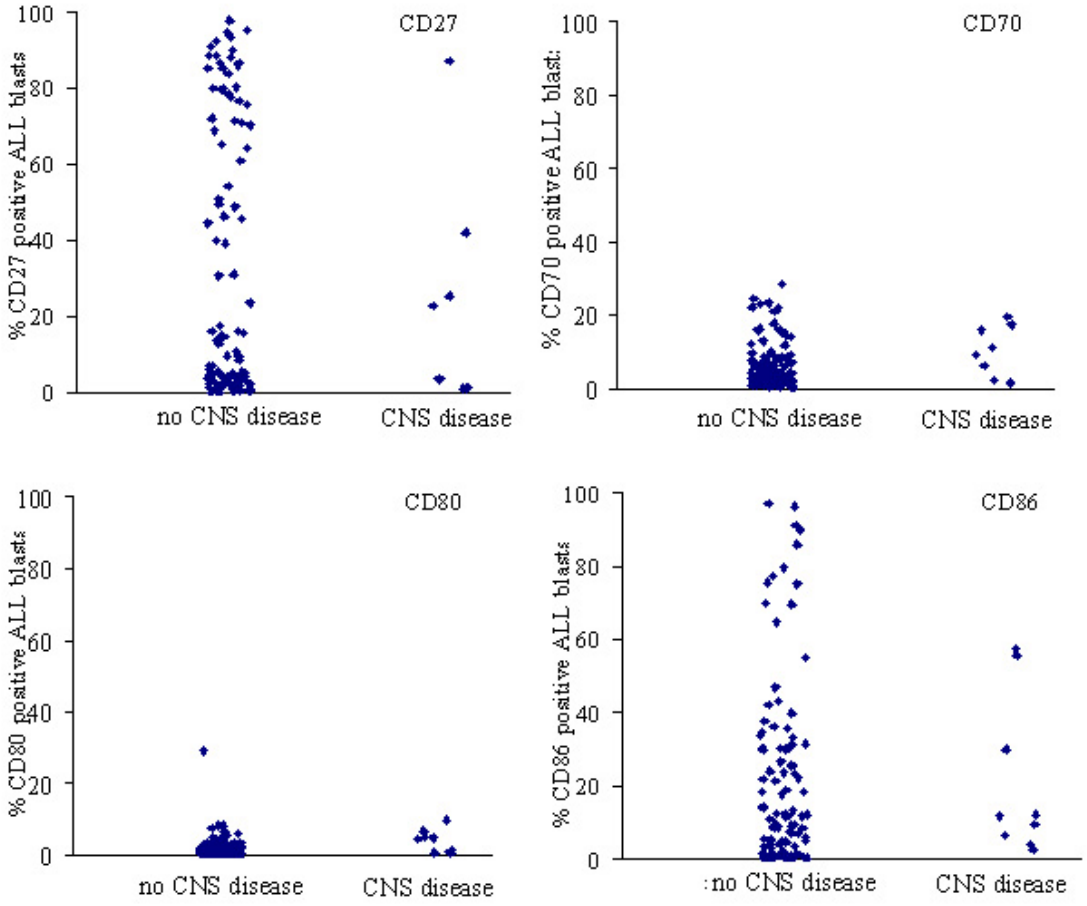


Figure 13:

Expression of costimulatory molecules and CD27 and CNS-involvement by ALL patients. ALL blasts were obtained from bone marrow or peripheral blood of ALL patients (n=102) and isolated by Ficoll-Hypaque density centrifugation. $CD19^+$ ALL blasts were double-stained with antibodies against CD80, CD86, CD70 and CD27 and subjected to flow cytometry analysis. CNS disease was detected in 8 patients.

3.4.7 Prognostic factors and expression of costimulatory molecules: summary

Our results show that low baseline expression of costimulatory molecule CD80 on the ALL blasts has a tendency to correlate with low probability of the patients to get a relapse (Tab. 9, page 58). We have also found that patients with high expression of CD27 on the ALL blasts have a significant higher probability to belong to the low risk group when compared to patients with low CD27 expression. As seen in Tab. 9, we have not found any association between prognostic factor PVA score and expression levels of all molecules studied. It is worth mentioning, that the analysed patient group consisted only of patients

with B-cell leukemias. Patients with T-cell leukemia were excluded from the analysis. Because of a low number of early pre-B- and B-ALL patients, their samples were also not used in this analysis. This means that the analysed group was artificially enriched with low risk patients. So the probability of belonging to a low risk group, calculated in our analysis can be slightly elevated when compared to a general probability for all patients with acute lymphoblastic leukemia of getting into a low risk group.

costimulatory	**prognostic factors**		**risk**	**relapse**
molecules and CD27	**PVA score**	**leukocyte count**	**group**	**probability**
CD80 n=100	n.a.	n.a.	n.a.	$p = 0.079$
CD86 n=100	n.a.	n.a.	n.a.	n.a.
CD70 n=101	n.a.	n.a.	n.a.	n.a.
CD27 n=101	n.a.	$^{*}\ p < 0.05$	$^{*}\ p < 0.05$	n.a.

Table 9:

Baseline expression of CD27 and costimulatory molecules and prognostic factors. n.a. - no significant association was found * - significant association was found (reliability index < 0.05.)

3.5 T-cell stimulatory capacity of CD40-stimulated ALL blasts

To investigate the stimulatory capacity of CD40-stimulated ALL blasts the primary mixed lymphocytes reactions (MLRs) with allogeneic T-cells were performed. The CD40-stimulated ALL blasts from 15 patients with c-ALL (pts numbers: 5-7, 10, 16, 19, 24, 26, 41-43, 61-62, 67 and 69), 3 patients with pre-B-ALL (pts numbers: 83, 97 and 101) and one patients with early pre-B-ALL (pt. number: 121) were used in experiments described in patents' characteristics in Tab. 2, pages 19-24.

Unstimulated and CD40-stimulated ALL blasts were cultured with allogeneic T-cells for 5 days. Maximal T-cell simulation was achieved with PHA (1μg/ml). H^3-Thymidin was added for last 18h of cultivation. Stimulation index (SI) was calculated for each individual experiment as described above (Materials and Methods, subsection 2.15, page 34).

The CD40-stimulated ALL blasts from one patient (pt.6) have stimulated T-cell proliferation with a SI 10-fold greater than ALL-blasts from other patients, therefore SI obtained in this sample was not used for analysis.

CD40-stimulated ALL blasts from all samples tested stimulated a 3-fold enhanced T-cell proliferation ($p<0.01$) compared to unstimulated ALL blasts as shown in Fig.14A (page 60). Maximal T-cell stimulation achieving with PHA-stimulation was SI=49$\pm$11.

In addition, increased production of IFN-γ by T-cells cultured with CD40-activated blasts (1764 IU/ml) compared to unstimulated ALL blasts (464 IU/ml) was detected as demonstrated in Fig.14B. IFN-γ production by T-cells cocultured with CD40-stimulated ALL blasts was determined only in 4 experiments (pts 16, 42, 67 and 121). Due to the low number of experiments the statistical analysis was not be performed.

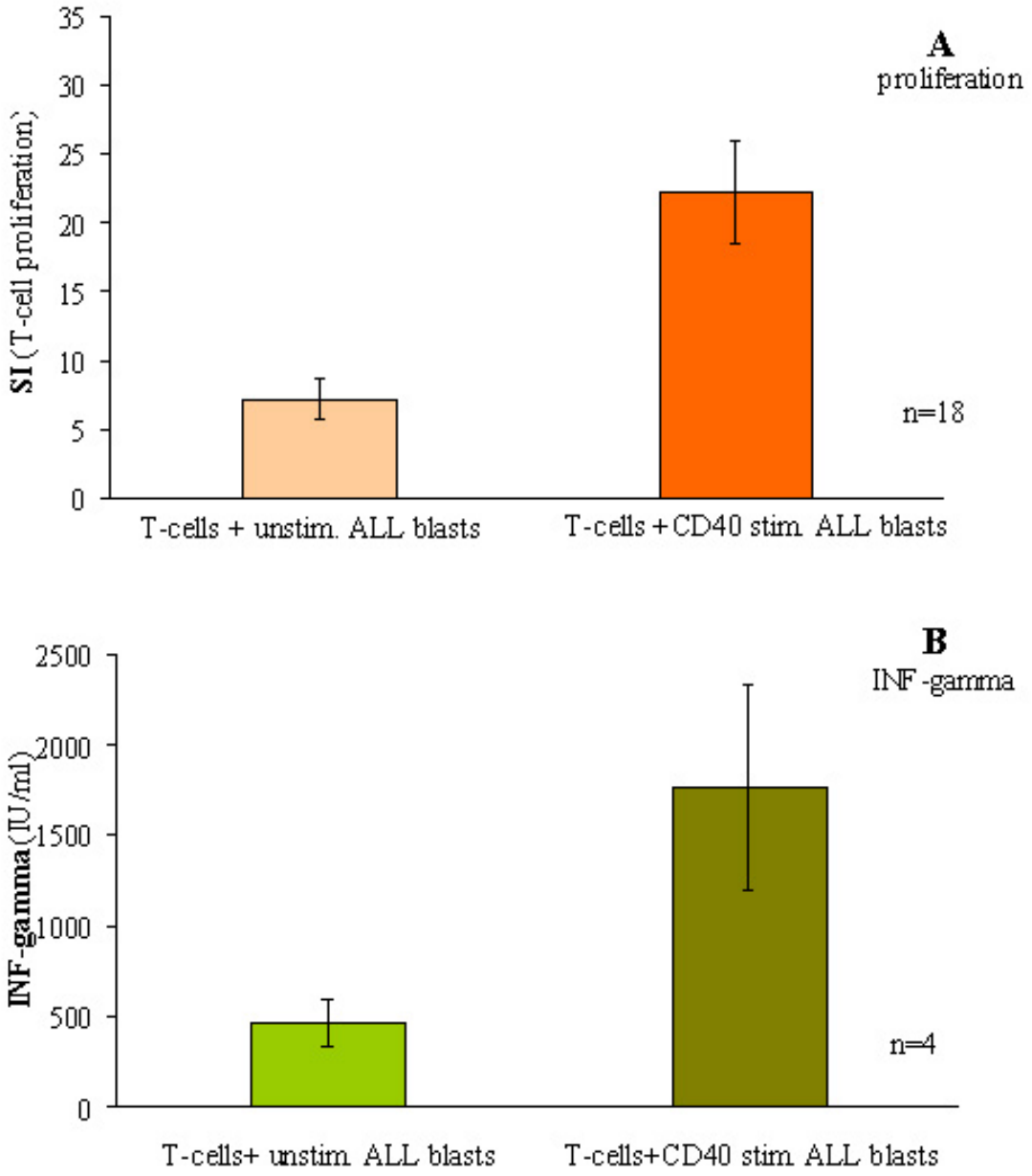


Figure 14:

IFN-γ secretion and T-cell proliferation in primary MLR with unstimulated or CD40-stimulated ALL blasts. Allogeneic T-cells and unstimulated or CD40-stimulated ALL blasts (ratio 2:1) were cocultured for 5 days. **A** - proliferation of T-cells was measured as H^3-Thymidin incorporation during the last 18 h of culture. Results represent the mean±SE of stimulation index (SI). **B** - IFN-γ secretion was assessed in supernatants after 5 days of culture. Results represent the mean±SE of IFN-γ concentration.

The functional significance for an effective T-cell stimulation of each of these costimulatory molecules was demonstrated by inhibition experiments using the blocking antibodies anti-

CD80, anti-CD86 and anti-CD70 against the respective costimulatory molecules (Fig.15A, page 61). In all cases tested the blockade of CD80/CD86 costimulation lead to the reduced T-cell proliferation ($p<0.05$).

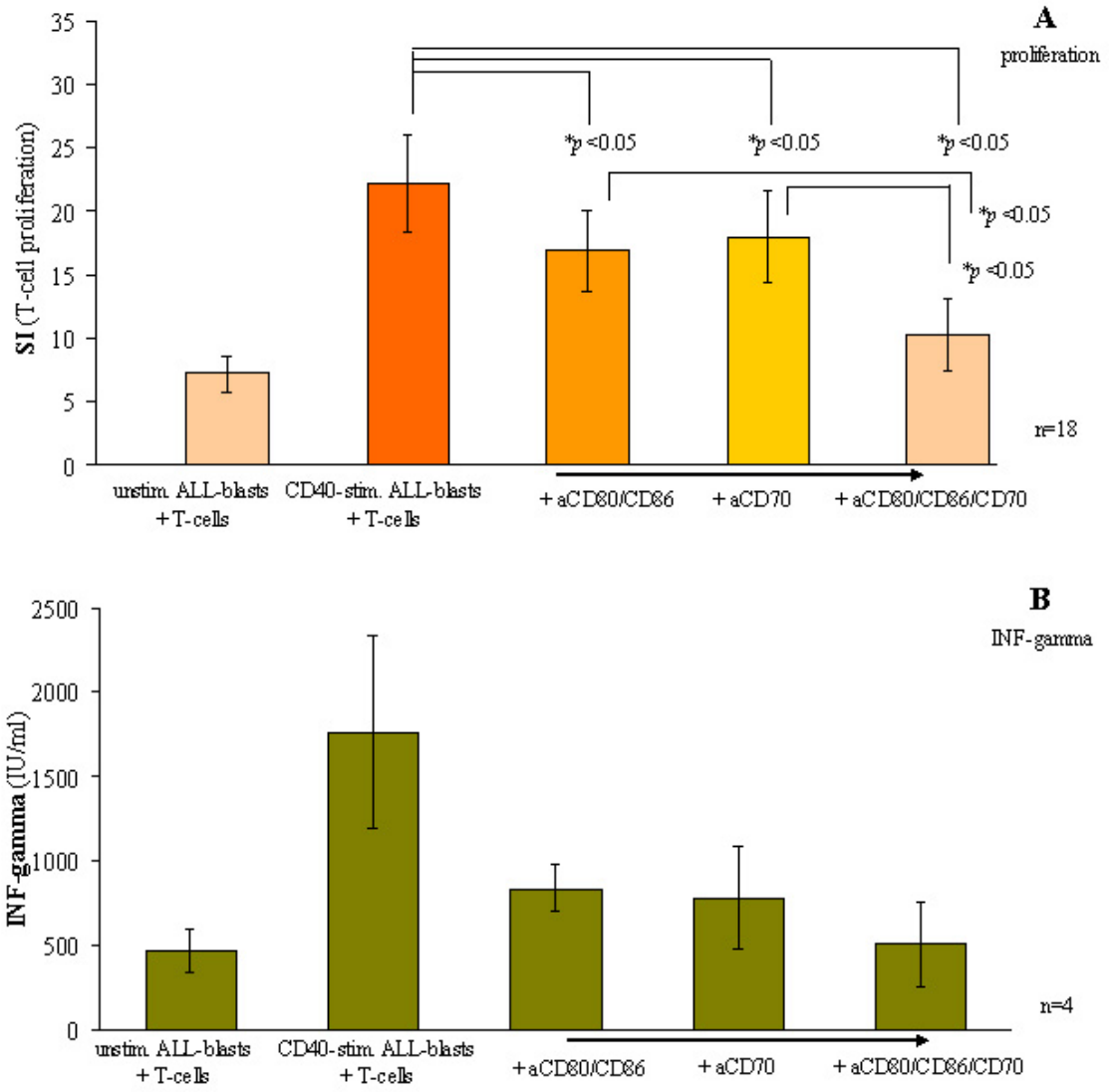


Figure 15:

Role of costimulatory molecules on the IFN-γ secretion and proliferation of T-cells induced by CD40-stimulated ALL blasts. Allogeneic T-cells and CD40-stimulated ALL blasts (ratio 2:1) were cocultured for 5 days. Blocking antibodies anti-CD80/anti-CD86 (2μg/ml), anti-CD70 (4μg/ml) or anti-CD80/anti-CD86/anti-CD70 (2μg/ml) were added at initiation of culture. **A** - proliferation of T-cells was measured as H^3-Thymidin incorporation during the last 18 h of culture. Results represent the mean±SE of stimulation index (SI). **B** - IFN-γ secretion was assessed in supernatants after 5 days of culture. Results represent the mean±SE of IFN-γ concentration.

Proliferation of T-cells stimulated with CD40-activated ALL blasts in presence of anti-

CD70 antibodies also results in significant inhibition of T-cell proliferation ($p<0.05$). More important, blockade of all costimulatory molecules simultaneously have an additive effect and results in 3-fold reduced T-cell proliferation ($p<0.05$).

Blockade of costimulatory molecules CD80/CD86, CD70 and its combination resulted also in a decrease of IFN-γ secretion in effectors T-cells cultured with CD40-stimulated ALL blasts (836, 781, 502 IU/ml respectively) as shown in Fig. 15B, page 61.

Interestingly, T-cells that have been stimulated with unstimulated ALL blasts also showed increased proliferation and IFN-γ production when compared with unstimulated T-cells.

As shown in Fig.16A and B (page 63) also a 2-fold reduced proliferation of the effector T-cells stimulated with unstimulated ALL-blasts in the presence of the blocking antibodies anti-CD80, anti-CD86 and anti-CD70 and its combination was detected as well as a decrease of IFN-γ secretion (292, 89, 70 IU/ml respectively).

So unstimulated ALL blasts, which expressed low levels of costimulatory molecules can also induce T-cell proliferation and IFN-γ production that can be inhibited by addition of blocking antibodies against costimulatory molecules.

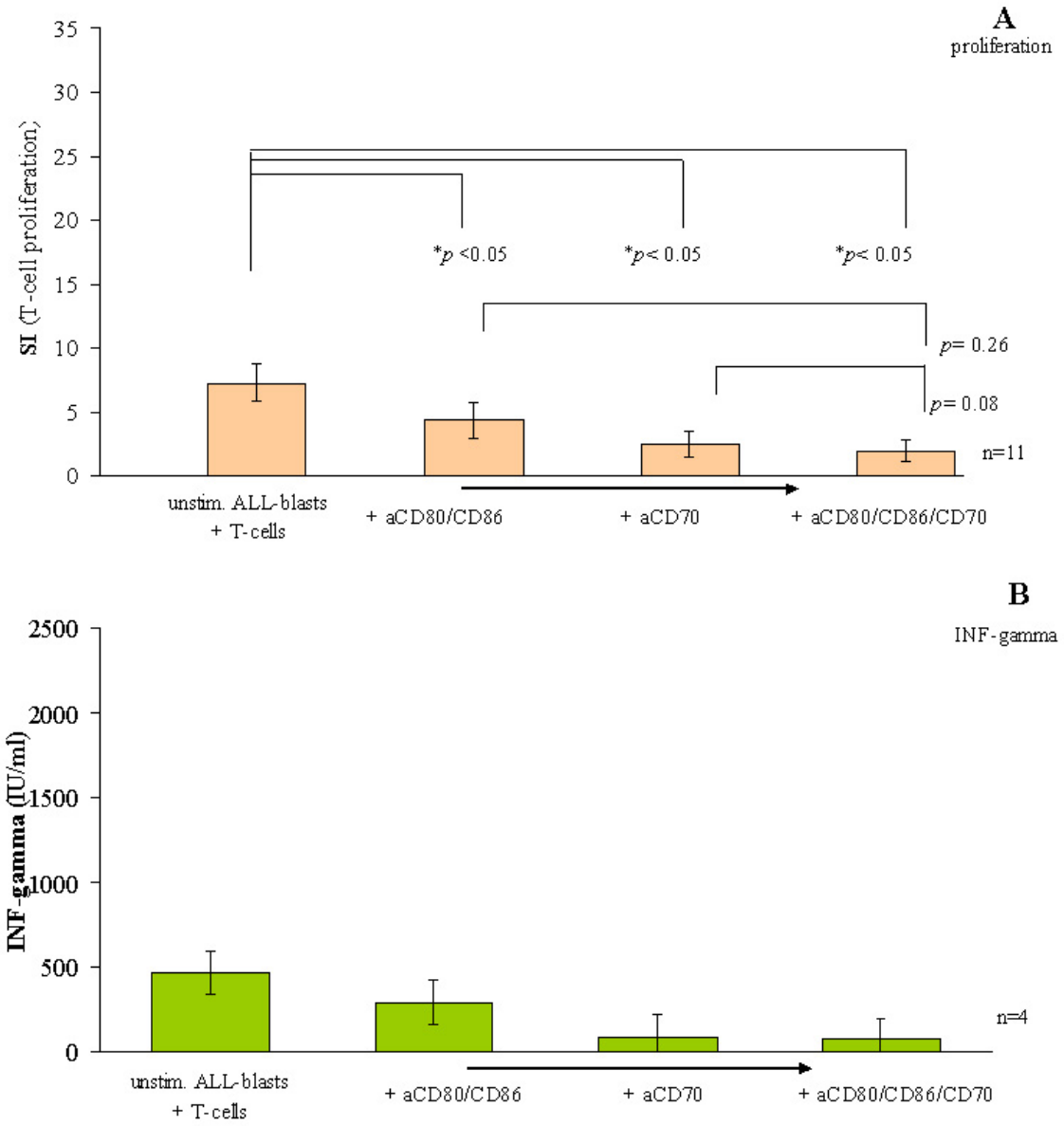


Figure 16:

Role of costimulatory molecules in T-cell proliferation and IFN-γ production induced by unstimulated ALL blasts. Allogeneic T-cells and unstimulated ALL blasts (ratio 2:1) were cocultured for 5 days. Blocking antibodies anti-CD80/anti-CD86 (2μg/ml), anti-CD70 (4μg/ml) or anti-CD80/anti-CD86/anti-CD70 (2μg/ml) were added at initiation of culture. **A** - proliferation of T-cells was measured as H^3-Thymidin incorporation during the last 18 h of culture. Results represent the mean±SE of stimulation index (SI). **B** - IFN-γ secretion was assessed in supernatants after 5 days of culture. Results represent the mean±SE of IFN-γ concentration.

IL-10 production by T-cells cocultured with unstimulated/CD40-stimulated ALL blasts was also determined in cases of 4 patients (pts 16, 69, 42, 121). In contrast, IL-10 production was comparable in T-cells cultured with either unstimulated (731 IU/ml) or CD40-

stimulated ALL blasts (927 IU/ml) and was not reduced in the settings with blocking antibodies against costimulatory molecules.

As shown in Fig.16A and B (page 63) also a 2-fold reduced proliferation of the effector T-cells stimulated with unstimulated ALL-blasts in the presence of the blocking antibodies anti-CD80, anti-CD86 and anti-CD70 and its combination was detected as well as a decrease of IFN-γ secretion (292, 89, 70 IU/ml respectively).

So unstimulated ALL blasts, which expressed low levels of costimulatory molecules can also induce T-cell proliferation and IFN-γ production that can be inhibited by addition of blocking antibodies against costimulatory molecules.

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Taken together our results evidenced, that participation of CD70 costimulation in the process of T-cell activation is on the same level of importance as that provided by CD80 and CD86 molecules. Moreover, the costimulation mediated through both CD28-CD80/CD86 and CD27-CD70 pathways are necessary for the sufficient T-cell stimulation.

3.6 Generation of anti-leukemia specific cytotoxic T-cells

3.6.1 Characteristics of cells used for generation of anti-leukemia cytotoxic T-cells

Since for the stimulation of specific anti-leukemia cytotoxic T-cell response, T-cells need in addition to the antigen-specific signal, the second costimulatory signal, we have investigated whether leukemia specific cytotoxic T lymphocytes (CTL) could be generated using CD40-stimulated ALL blasts that expressed the costimulatory molecules CD80, CD86 and CD70. In this setting we used HLA-matched donor T-cells as effector cells to be stimulated by CD40-stimulated or unstimulated ALL blasts. Patients characteristic whose blasts were used for further experiments are summarized in Table 10.

patients number	**leukemia subtype**	**sex**	**age, yrs**	**PVA score**	**leuk. count x$10^6/\mu$l**	**risk groups**
pt.121	early pre-B-ALL	f	4	9	104	high
pt.19	c-ALL	m	6	5	103	high
pt.43	c-ALL	m	14	7	6.3	high
pt.7	c-ALL	m	1	6	28.3	high
pt.87	pre-B-ALL	m	6	5	30.5	high
pt.95	pre-B-ALL	m	4	0	71	high

Table 10:

Characteristics of patients whose ALL blasts were used as stimulator cells for the generation of cytotoxic anti-leukemia T-cell lines. m - male; f - female; yrs - years; PVA - *in vitro* resistance-profile of ALL blasts against chemotherapeutic drugs; leuk. count - leukocyte count.

The generation and expansion of anti-leukemia T-cell lines was performed as depicted in Fig.2, (materials and methods, subsection 2.16, page 35). ALL blasts were for 3-4 days cultured on mitomycin C-treated $CD40L^+$- or $CD40L^-$- fibroblasts and used as stimulator cells for the generation of cytotoxic anti-leukemia T-cells.

The expression of costimulatory molecules on ALL blasts used for stimulation and restimulation are shown in Tab. 11 (page 66).

patient number	**initial expression***			**ALL blasts****					
				unstimulated			**CD40-stimulated**		
	$CD80^+$	$CD86^+$	$CD70^+$	$CD80^+$	$CD86^+$	$CD70^+$	$CD80^+$	$CD86^+$	$CD70^+$
pt.121	3.6	36	7.8	10±0.5	28±0.5	9±0.5	18±1	82±1	31±2
pt.19	0.7	9	1.8	5±1	55±5	8±0.9	23±8	88±3	40±6
pt.43	1.5	1	0.6	11±3	22±2	8±1.3	24±2	38±3	28±2
pt.7^A	6	12	4	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
pt.87	0.8	56	2.2	8±1	64±3	16±4	67±17	94±1	58±5
pt.95^B	2.7	8.9	8.3	n.t.	n.t.	5.6±0	n.t.	n.t.	12±2

Table 11:

Expression of costimulatory molecules before and after CD40-stimulation on ALL blasts using as stimulator cells for generation of cytotoxic anti-leukemia T-cells. ALL blasts were cocultivated 3-4 days with mitomycin C-treated $CD40L^+$- fibroblasts in presence of IL-4 (200U/ml). $CD19^+$ blasts were double-stained with antibodies directed against CD80, CD86, CD70, CD27 and subjected for flow cytometry analysis. Expression of the surface molecules was not tested: ^{A} - due to reduced cell viability; ^{B} - due to the low cell number. Results represent: * - the percentage of double-positive blasts; ** - the mean percentage of double-positive blasts±SE.

Allogeneic HLA-matched donor or autologous PB or PB stem cells were used as effector cells. Characteristics of cells which were used as effector cells for each patients are shown in Table 12, page 67.

stimulator cells (pts. number)	**source of effector cells**		**phenotype**	
			CD3$^+$	**CD3$^+$CD8$^+$**
pt.121	MUD	PBST	71%	21%
pt.19	MRD	PB	n.t.	n.t.
pt.43	MRD*	PB	70%	24%
pt.7	MRD	PB	63%	n.t.
pt.87	auto	PBST	9%	8%
pt.95	auto	PB	55%	22%

Table 12:

Characteristic of effector cells used for the generation of anti-leukemia T-cells. MRD - matched related donor, MRD* - syngeneic donor, MUD - matched unrelated donor, PBST - peripheral blood stem cells, PB - peripheral blood cells. n.t. - not tested. Results represent % of double-positive cells.

3.6.2 Role of CD70 for the expansion of T-cell lines using CD40-stimulated or unstimulated ALL blasts.

ALL blasts cells from 6 patients were used to generate cytotoxic anti-leukemia T-cell lines. As summarized in Table 13 (page 68), T-cell lines were established in 4 from 6 experiments. Phenotypic analysis showed that the all CD3$^+$T-cell lines contained mixed population of CD4$^+$ and CD8$^+$ T-cells (Table 13). The expanded T-cells uniformly expressed activation markers CD25 and CD69 (data not shown). Also the percentage of CD56$^+$-NK cells in the generated T-cell lines was determined and was in all cases <20%.

In four experiments (pts 121, 19, 43, 7) allogeneic PB or PBST cells were used as effector cells. In three but one (pt 7) cases marked T-cell proliferation was observed and T-cell lines were established. In the case of patient 7 poor T-cell proliferation can be explained by the low percentage of viable ALL blasts from cryoconserved probes which were used as stimulator cells. In two experiments autologous PB or PBST cells were used as effector cells to be stimulated with CD40-stimulated or unstimulated ALL blasts. In one experiment (pt 87) T-cell proliferation was observed, but at lower level when compared to the T-cell proliferation in other experiments when allogeneic effector cells were used. In the case of patient 95, no proliferation was detected.

To determine the role of the CD70 molecule in the expansion of cytotoxic T-cells, T-cell lines

patients number	**T-cell lines***	**T-cell expansion** (x-fold)	**Phenotype**		
			$CD8^+$	$CD4^+$	$CD56^+$
pt.121	T-cells+ALL^-	30	33%	90%	7%
	T-cells+ALL^+	10	59%	34%	15%
pt.19	T-cells+ALL^-	80	17%	62%	15%
	T-cells+ALL^+	80	12%	76%	12%
pt.43	T-cells+ALL^-	250	37%	19%	18%
	T-cells+ALL^+	250	34%	26%	6.6%
pt.7	T-cells+ALL^-	no prolif.	n.t.	n.t.	n.t.
	T-cells+ALL^+	no prolif.	n.t.	n.t.	n.t.
pt.87	T-cells+ALL^-	3	11%	88%	0.9%
	T-cells+ALL^+	9	28%	71%	6.5%
pt.95	T-cells+ALL^-	no prolif.	n.t.	n.t.	n.t.
	T-cells+ALL^+	no prolif.	n.t.	n.t.	n.t.

Table 13:

Characteristic of generated T-cell lines. * - T-cells+ALL^- - effector cells stimulated with unstimulated ALL blasts, T-cells+ALL^+ -effector cells stimulated with CD40-stimulated ALL blasts. no prolif. - no proliferation was detected.

were generated in the presence of blocking anti-CD70 antibodies (4μg/ml). One example for proliferation of allogeneic effector cells (patient 43) after stimulation with unstimulated and CD40-stimulated ALL blasts is shown in Fig. 17 (page 69).

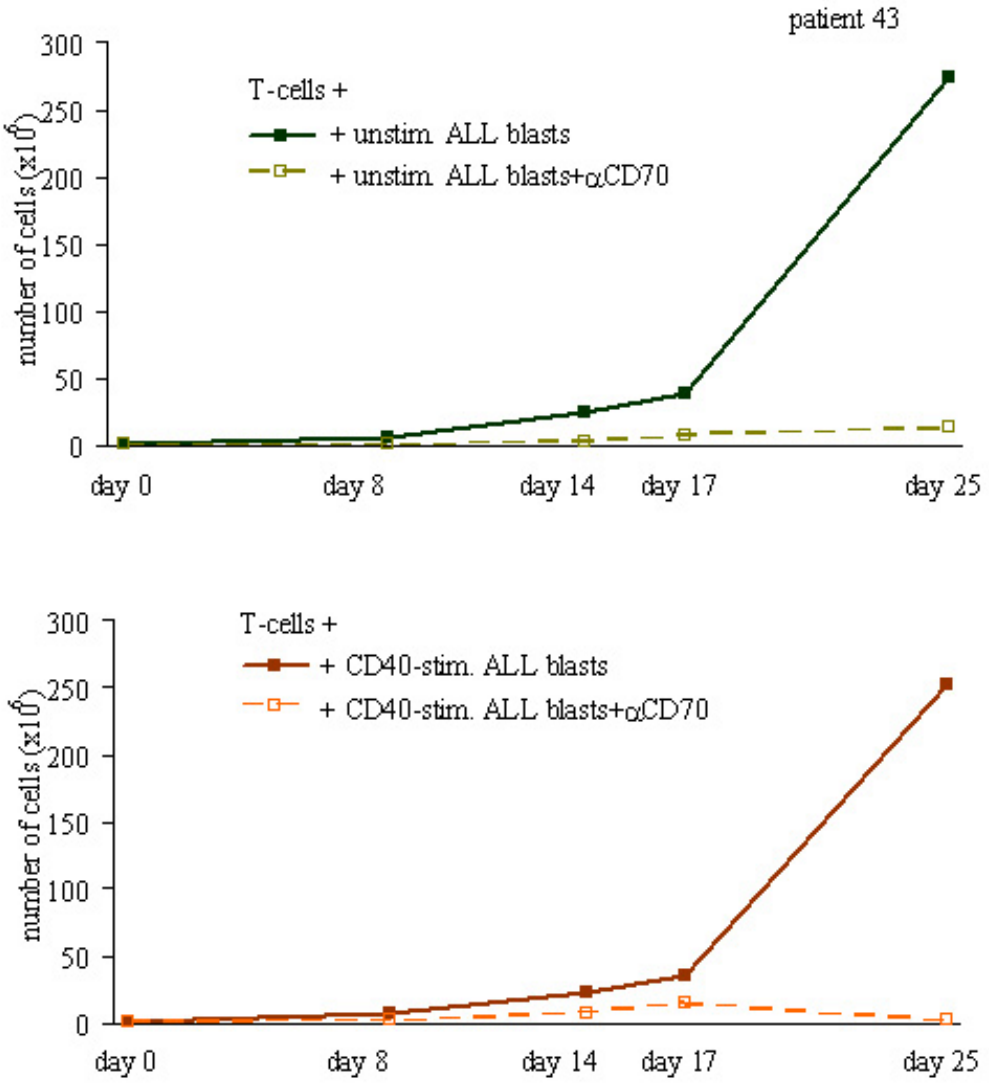

Figure 17:

Role of CD70 for allogeneic T-cell expansion after stimulation with unstimulated or CD40-stimulated ALL blasts. T-cell lines were stimulated and restimulated with unstimulated or CD40-stimulated leukemia cells in the presence of IL-7 (10ng/ml) and IL-2 (50U/ml). Blocking anti-CD70 antibodies (4μg/ml) were added at the initiation of culture and at each restimulation.

CD40-stimulated ALL blasts as well as unstimulated ALL blasts induced 200-fold proliferation of effector cells in this case and T-cell expansion was significantly reduced in comparison to the unmanipulated culture (Fig. 17). Interestingly, in the presence of blocking anti-CD70 antibodies the T-cell proliferation was decreased in T-cell lines generated with

CD40-stimulated as well as with unstimulated ALL blasts. The same results were obtained in other two experiments (pts 121 and 19) in which allogeneic cells were used as effector cells. However, in the case of pt 121 T-cell proliferation induced CD40-stimulated ALL blasts was lower when compared to T-cell proliferation induced with unstimulated ALL blasts. No definitive blocking effect of anti-CD70 antibodies was observed in this case.
In one case when autologous cells (pt 87) were used as effector cells, only a 3-9-fold proliferation was observed which was reduced to 10% in the presence of blocking anti-CD70 antibodies (Fig.18). When a control IgG_3 antibodies were used (4μg/ml), no reduced cell proliferation was detected.

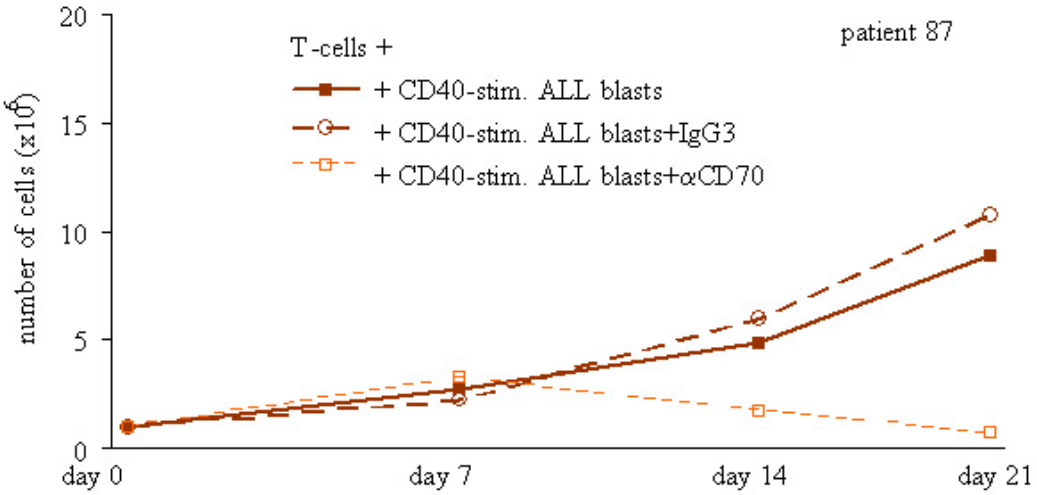

Figure 18:

Role of CD70 for autologous T-cell expansion after stimulation with CD40-stimulated ALL blasts. T-cell lines were stimulated and restimulated with CD40-stimulated leukemia cells in the presence of IL-7 (10ng/ml) and IL-2 (50U/ml). Blocking anti-CD70 antibodies (4μg/ml) or isotype antibodies IgG3 (4μg/ml) were added at the initiation of culture and at each restimulation.

In the primary mixed lymphocyte reaction we have previously demonstrated that the blockade of the CD70 molecule can only partially reduce T-cell proliferation that were stimulated with ALL blasts. In contrast, the long term cultured T-cell lines stimulated with ALL blasts in the absence of CD70 costimulation showed a complete reduction of proliferation.

3.6.3 Anti-leukemia cytotoxicity of T-cell lines generated using unstimulated or CD40-stimulated ALL blasts.

The lytic activity of generated T-cell lines was determined using a standard ^{51}Cr-release assay. The assay was performed in the effector to target ratios of 100:1, 50:1, 25:1, 12.5:1 and 6.25:1 by limiting dilution procedure. To asses the capacity of the generated T-cell lines to lyse leukemia cells, T-cells were tested against the following targets: (1) unstimulated ALL cells; (2) CD40-stimulated ALL cells; (3) autologous nonmalignant cells (PHA-blasts); (4) NK-sensitive K562 cells.

patients number	**CTL-lines***	**% killing of ****			**NK-mediated killing**
		unst. ALL	CD40-stim. ALL	PHA-blasts	
pt.121	T-cells+ALL$^-$	14%	19%	4%	10%
	T-cells+ALL$^+$	38%	33%	3%	9%
pt.19	T-cells+ALL$^-$	32%	26%	6%	8%
	T-cells+ALL$^+$	39%	40%	7%	17%
pt.43	T-cells+ALL$^-$	0%	25%	19%	74%
	T-cells+ALL$^+$	3%	39%	24%	30%
pt.87	T-cells+ALL$^-$	0%	0%	0%	0%
	T-cells+ALL$^+$	12%	7%	8%	8%

Table 14:

Characteristics of generated T-cell lines. * - T-cells+ALL$^-$ - effector cells stimulated with unstimulated ALL blasts; T-cells+ALL$^+$ - effector cells stimulated with CD40-stimulated ALL blasts. ** - % specific lysis was evaluated by a ^{51}Cr-release assay. The results represent lysis at an effector:target ratio of 25:1 in % of specific lysis.

As seen in Tab. 14, only in the case of patient 87 low cytotoxic killing activity of generated T-cell lines against ALL blasts was detected. Also NK-mediated killing was absent. The other T-cell lines that have been generated using CD40-stimulated or unstimulated ALL blasts (patients 121, 192 and 43) showed specific lysis of unstimulated as well as of CD40-stimulated ALL blasts (Tab. 14). As shown in Fig.19A (page 73) T-cell lines generated using CD40-stimulated ALL blasts demonstrated specific cytotoxicity against

CD40-stimulated (33-40% specific lysis) as well as unstimulated ALL blasts (3-39% specific lysis). Interestingly, T-cell lines that were repeatedly restimulated with unstimulated ALL blasts also recognize and lyse CD40-stimulated ALL blasts (19-26% specific lysis) as well as unstimulated ALL blasts (0-32% specific lysis) as shown in Fig.19B. It is worth to note that all generated T-cell lines demonstrate the higher cytolytic activivty against CD40-stimulated ALL blasts when compared to unstimulated ALL blasts. Moreover, target lysis achieved by T-cells generated using unstimulated ALL cells was lower when compared to target lysis demonstrated by T-cells generated using CD40-stimulated ALL blasts. This correspond to clinical data and showed that unstimulated ALL blasts can stimulate anti-leukemia response only at low intensity.

To attempt to demonstrate anti-leukemia specifity of generated T-cell lines, phytohemagglutinin (PHA)-activated T-cell blasts as autologous nonmalignant cells were tested. As shown in Fig.19 A and B, cytolysis was significantly lower or absent toward this nonmalignant target when compared to unstimulated or CD40-stimulated ALL blasts. This indicates that the cytotoxicity is directed against leukemia-associated antigens.

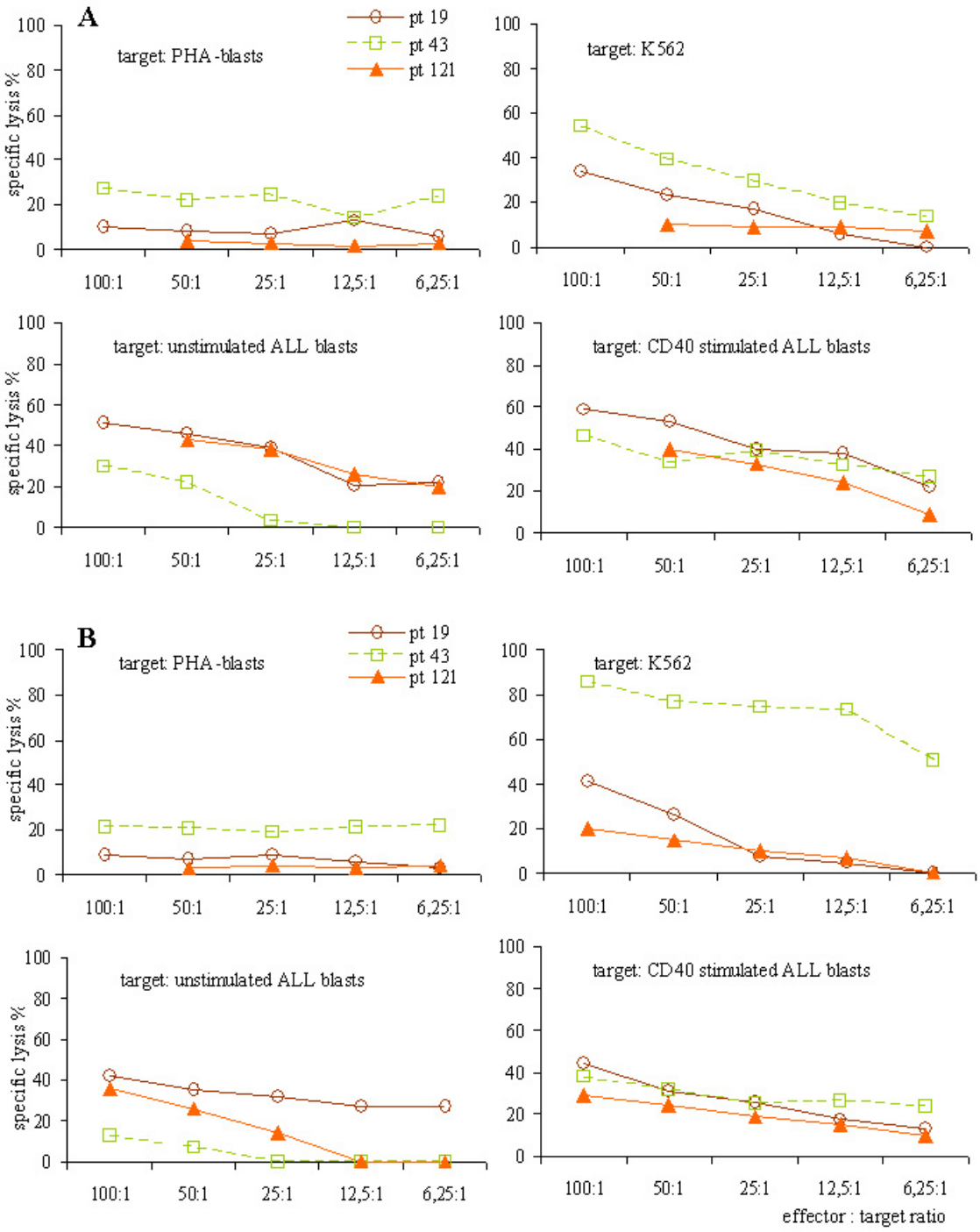

Figure 19:

Leukemia specific CTL generated with CD40-stimulated ALL blasts. T-cell lines were stimulated and 2 times restimulated with **A** - CD40-stimulated ALL blasts or **B** - unstimulated ALL blasts in the presence of IL-7 (10ng/ml) and IL-2 (50U/ml).

To test the NK-mediated killing in generated T-cell lines the NK-sensitive K562 cells were used (Tab. 14 and Fig.19). The T-cell line generated with CD40-stimulated as well as with unstimulated ALL blasts demonstrated the NK-mediated cytotoxicity, however at low level. Patient 43 was an exception in which high NK-mediated killing was detected. Interestingly, after thawing and one restimulation these T-cell lines showed decreased NK-mediated cytotoxity (Fig.20, page 74), wheres the anti-leukemia cytotoxity against CD40-stimulated ALL blasts remained at the same level (see to compare Fig.19 on page 73).

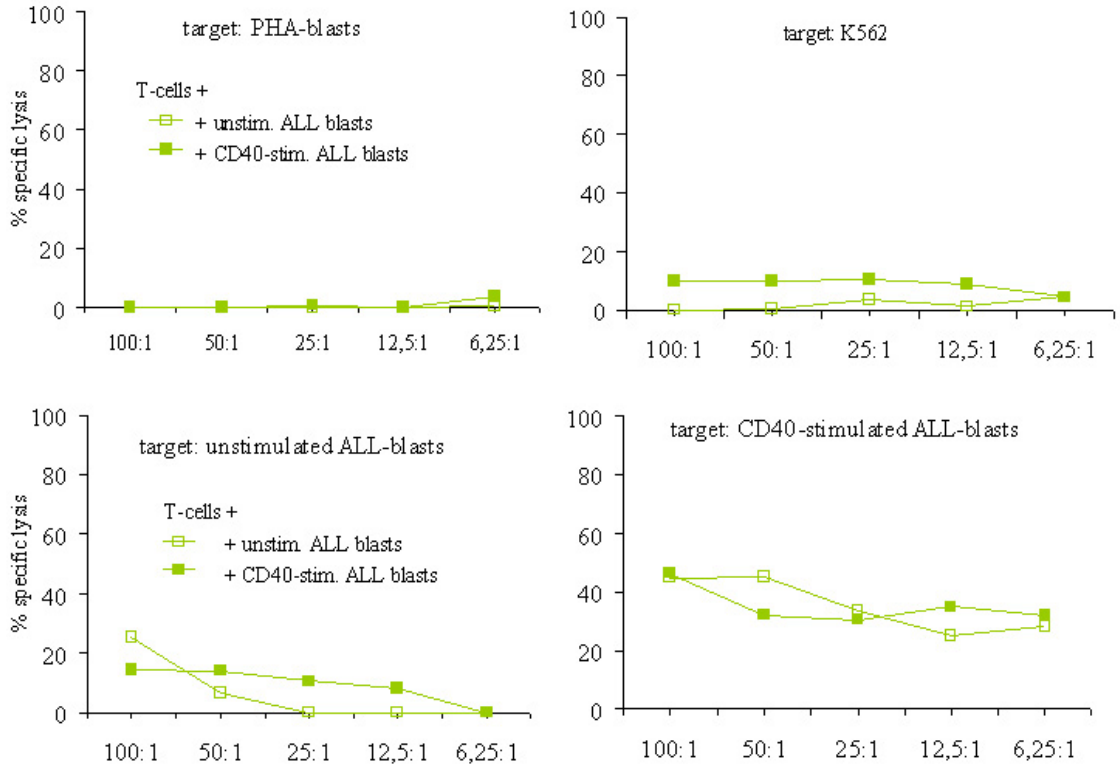

Figure 20:

Cytotoxicity of thawed and restimulated T-cell lines from pt.43. T-cell lines were stimulated and restimulated two times with CD40-stimulated or unstimulated leukemia cells in the presence of IL-7 (10ng/ml) and IL-2 (50U/ml). After freezing and thawing T-cell lines were restimulated once with CD40-stimulated or unstimulated leukemia cells in the presence of IL-7 (10ng/ml) and IL-2 (50U/ml).

3.6.4 Role of CD70 for the anti-leukemia cytotoxic activity of T-cell lines generated with unstimulated or CD40-stimulated ALL blasts.

We have previously shown (see 3.6.2, page 67), that blockade of CD70 costimulation led to the reduced proliferation of T-cell lines stimulated either with unstimulated or CD40-stimulated ALL blasts. Nevertheless, in two experiments (pts 43 and 19) the T-cell lines generated in the presence of blocking anti-CD70 antibodies were established, however only very low cell numbers were obtained. To determine the role of CD70 for cytotoxicity of T-cell lines generated using unstimulated or CD40-stimulated ALL blasts, cytotoxic activity of T-cell lines generated in the absence of CD70 costimulation was tested.

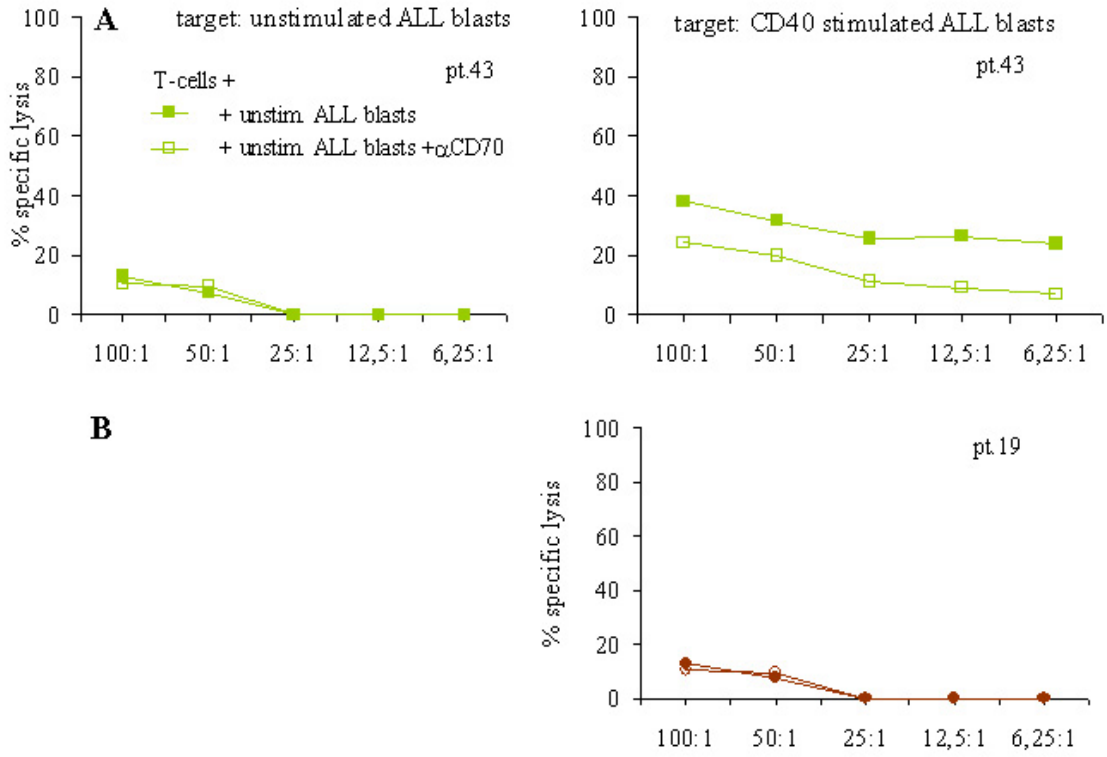

Figure 21:

Role of CD70 molecule for the anti-leukemia cytotoxity of T-cell lines generated with unstimulated ALL blasts. T-cells were stimulated and restimulated with unstimulated ALL blasts. Blocking anti-CD70 antibodies (4μg/ml) were added at the initiation of culture and at each restimulation. **A** - T-cell line generated using cells from patient 43. **B** - T-cell line generated using cells from patient 19. Due to the low cell number the cytotoxicity was tested only against target CD40-stimulated ALL cells.

As seen in Fig.21 (page 75), T-cells generated with unstimulated ALL blasts in the presence of blocking anti-CD70 antibodies demonstrated a reduced specific lysis of CD40-stimulated ALL blasts, whereas unstimulated ALL blasts were recognized and lysed by these T-cell lines at the same level as the T-cell lines generated in the presence of blocking anti-CD70 antibodies. Also the T-cell lines generated using the CD40-stimulated ALL blasts in presence of blocking anti-CD70 antibodies demonstrated decreased cytotoxic activity against CD40-stimulated ALL blasts (Fig.22, page 76). Due to the low cell number of generated T-cell lines, a cytotoxic assay was possible only for the effector:target ration of 25:1, 12.5:1 and 6.25:1. Specific lysis of unstimulated ALL blasts was already very low at the effector:target ratio 25:1, therefore no definitive blocking effect was observed in this case.

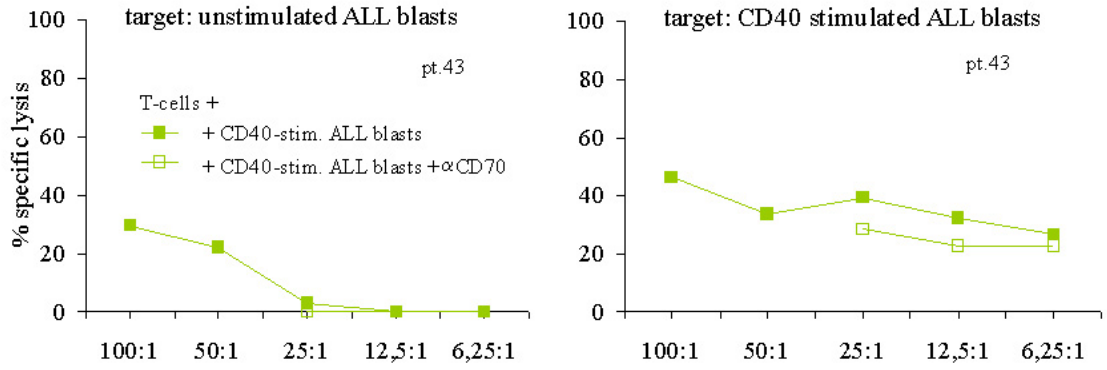

Figure 22:

Role of CD70 for anti-leukemia cytotoxity of T-cell lines generated with CD40-stimulated ALL blasts. T-cell lines were stimulated and restimulated with CD40-stimulated ALL blasts. Blocking anti-CD70 antibodies (4μg/ml) were added at the initiation of culture and at each restimulation.

Thus, CD70 costimulation is important not only for expansion, but also for cytotoxic activity of the specific anti-leukemia T-cell lines. Proliferation and cytotoxicity of T-cells were decreased when CD70 was blocked during generation of T-cell lines by stimulation with CD40-stimulated as well as with unstimulated ALL blasts.

3.7 CD95 system in ALL blasts

3.7.1 CD95 expression on primary ALL blasts

It was hypothesed that low expression of CD95 on leukemia cells could contribute to the pathogenesis of leukemia. We have therefore investigated the constitutive expression level of CD95 on primary ALL blasts. The CD95 expression was assessed in a total of 48 patients with pre-B-ALL (n=13), c-ALL(n=30) and early pre-B-ALL (n=5) (see patients characteristic in table 20, page 87). As shown in Fig.23 (page 77), ALL blasts express detectable but low (<15%) level of CD95 molecule in all cases tested. This expression level was low independent of pre-B or common- subtype of leukemia, but was extremely low in cases of early pre-B-ALL (<5%).

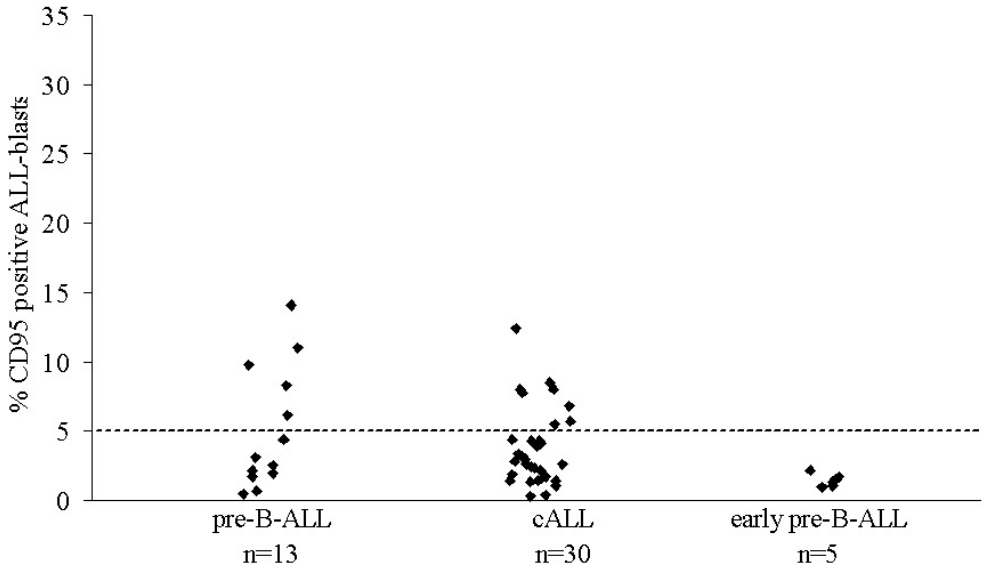

Figure 23:

CD95 expression on primary ALL-blasts. ALL blasts were obtained from bone marrow or peripheral blood of patients with more than 80% blast cell count and isolated by Ficoll-Hypaque density centrifugation. $CD19^+$ cells were stained with antibodies directed against CD95 and subjected to flow cytometry analysis.

3.7.2 CD95-mediated apoptosis in primary ALL blasts

To examine the sensitivity of primary ALL blasts to CD95-mediated apoptosis ALL blasts from 23 patients (see patients characteristics in table 21, page 88) have been incubated

for 16 hours with or without anti-CD95 antibodies (1μg/ml). ALL blasts from all samples tested showed 44±5% of apoptotic cells in presence of anti-CD95 antibodies and this was comparable with the percentage of apoptotic cells (45±5%) in ALL blasts cultured for 16h without anti-CD95 antibodies (Tab. 15, page 78). The fact that the CD95-expression level was low in all these samples and that incubation with anti-CD95 antibodies did not augment specific apoptosis in primary ALL blasts, demonstrates that spontaneous death of leukemia cells after 16h incubation *in vitro* is not CD95-mediated.

leukemia subtype	**initial CD95 expression on ALL blasts** *	**percentage of apoptotic cells** ** **after 16h culture with**	
		medium alone	anti-CD95 antibodies
c-ALL n=10	3.5±0.8	46±7	46±7
pre-B-ALL n=10	5.3±1.1	42±6	42±6
early pre-B-ALL n=3	1.4±0.3	47±18	48±16

Table 15:

Spontaneous and CD95-mediated apoptosis on primary ALL blasts. * - primary $CD19^+$ blasts were double-stained with antibodies against CD95. Values represent the mean of percentage±SE of double-positive primary ALL blasts. ** - primary ALL blasts were cultured for 16 hours with or without of anti-CD95 antibodies (1μg/ml). Values represent the mean of percentage±SE of apoptotic cells.

Interestingly, we have observed, that ALL blasts from samples that have been delivered from other clinics and therefore pre-incubated for minimum 24 hours at room temperature (during the travel time), exhibited significantly higher spontaneous apoptosis after 16h culture *in vitro* when compared to ALL blasts immediately isolated from freshly obtained PB or BM.

samples origin	**initial apoptosis, %**	**percentage of apoptotic cells after 16h culture**
Dusseldorf n=9	5.03±1.3	30.4±5.6
other clinics n=14	10.1±2.3	53.3±5.5

Table 16:

Survival of primary ALL blasts after 16 hours culture. Primary ALL blasts were cultured for 16 hours with medium alone. Values represent the mean of percentage±SE of apoptotic cells.

3.8 Survival and CD95 expression on ALL blasts after CD40 engagement

Ligation of CD40 on normal B-cells induces the proliferation and CD95 expression. Since the majority of ALL blasts express high level of CD40 molecule (Fig. 4, page 38), we have investigated the effect of CD40 ligation on survival and CD95 expression in ALL blasts.

3.8.1 Survival of ALL blasts during cultivation on fibroblasts

As we have shown ALL blasts demonstrated reduced viability after 16h in culture on plastic (Tab. 15, page 78). Therefore we first examined the survival of ALL blasts during cultivation on $CD40L^-$- or $CD40L^+$-fibroblasts.

Cell viability was detected by annexin V-FITC and PI staining. Viable cells exhibited a annexin V^- and PI^- phenotype. ALL blasts from 5 patients (pts numbers: 6, 43, 61, 106 and 116) were cocultured on $CD40L^-$- and $CD40L^+$-fibroblasts. Number of viable cells was

determined at different time points as indicated in Fig.24 (page 80). In the first 24 hours of cultivation cell viability in both unstimulated and CD40-stimulated ALL blasts was decreased to 58% and 59% respectively. Further cultivation up to 72 hours lead to minor changes in cell viability. After 72 hours of cultur unstimulated ALL blasts showed a lower survival rate when compared to CD40-stimulated ALL blasts, however this difference was not significant. Number of viable ALL cells after 72 hours of cultivation on the $CD40L^-$- and $CD40L^+$-fibroblats was 41±15% and 55±12% respectively (Fig.24, page 80).

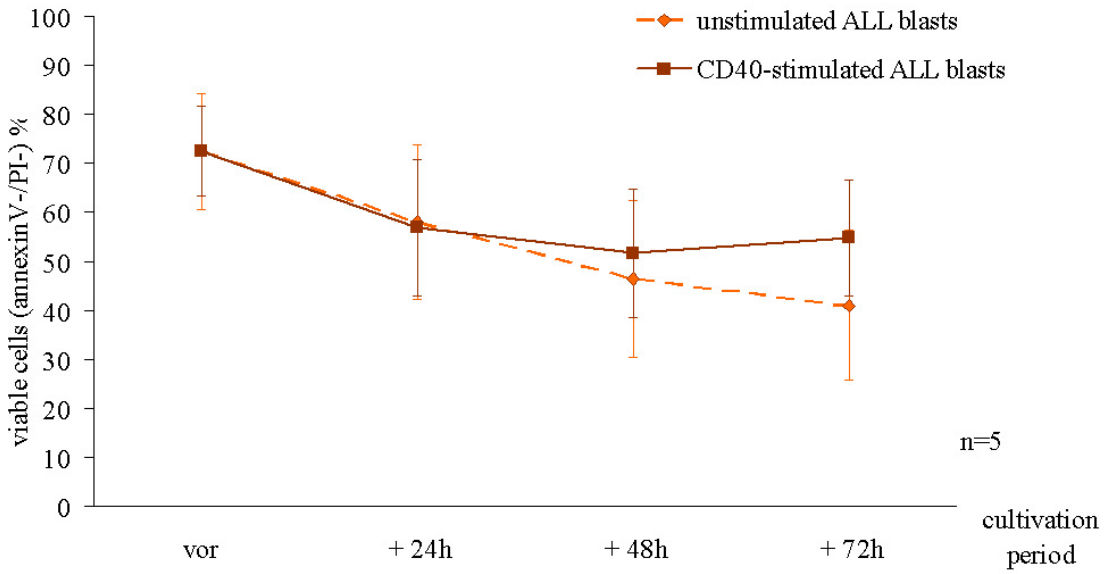

Figure 24:

Survival of ALL blasts on the $CD40L^-$- or $CD40L^+$-fibroblasts. ALL blasts were cocultured on $CD40L^-$- or $CD40L^+$-fibroblasts. Cell viability was detected by annexin V-FITC and PI-staining at time points as indicated. Values represent the mean percentage±SE of viable cells.

3.8.2 Dynamic of CD95 expression on ALL blasts during the CD40-stimulation

Next we have studied the effect of CD40-stimulation on the CD95 expression on ALL blasts. ALL blasts from 5 patients (pts numbers: 2, 43, 67, 106 and 107) were cocultured on $CD40L^-$- or $CD40L^+$-fibroblasts and CD95 expression was determined at different time points as indicated in Fig.25 (page 81). ALL cells upregulated CD95 expression already after 16 hours of CD40 activation from 2.6±1.1% up to 16.4±1.3% ($p<0.001$). The expression was further increased during ongoing cultivation over a period of 72 hours up to 61.3±5.2% ($p<0.001$).

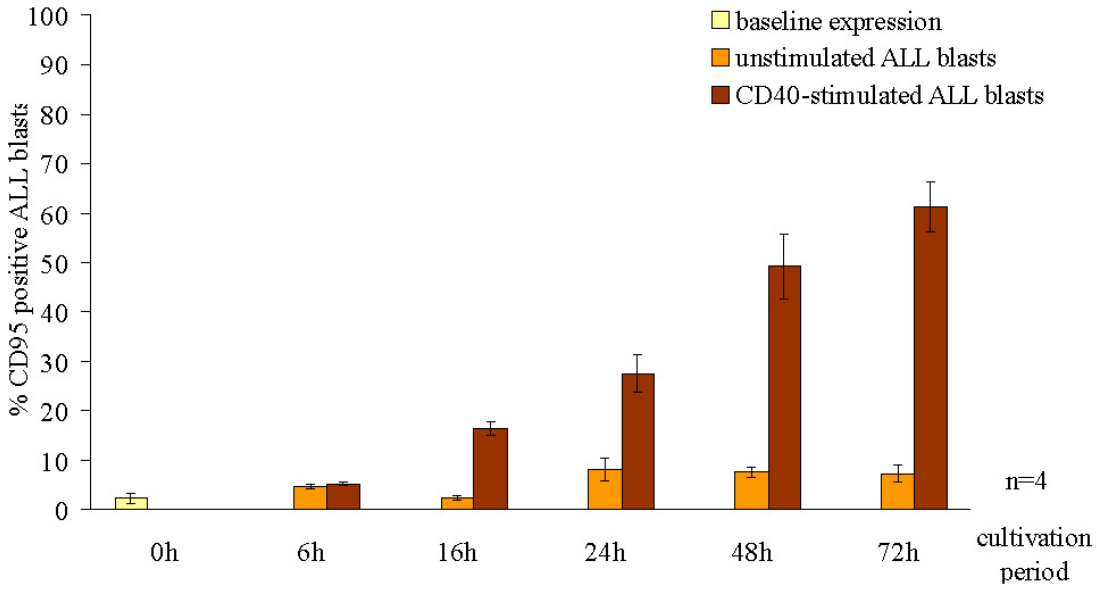

Figure 25:

Dynamic of the CD95 expression on the CD40-stimulated ALL blasts. ALL blasts were cocultured on $CD40L^-$- or $CD40L^+$-fibroblasts. $CD19^+$ blasts were double-stained with antibodies against CD95. CD95 expression was determined at different time points as indicated via flow cytometry analysis. Values represent the mean percentage±SE of double-positive cells.

In contrast, culture of ALL blasts with $CD40L^-$-fibroblasts for 24 hours results in a minor up-regulation of CD95 expression at 8.1±2.3%, however this expression was not increased after further cultivation on $CD40L^-$-fibroblasts.

3.8.3 CD95 expression on CD40-stimulated ALL blasts

Due to the fact that optimal up-regulation of CD95 expression on ALL blasts was achieved after 72h of CD40-stimulation, we have examined the capacity of ALL blasts from a large patient group to express CD95 molecule in response to CD40-stimulation during 72 hours. ALL blasts from a total of 39 patients with pre-B-ALL (n=12), c-ALL (n=22) and early pre-B-ALL (n=5) (see patients characteristic in Table 22, page 89) were used for CD40-stimulation. Cells were cultured on $CD40L^-$- or $CD40L^+$-fibroblasts for 72-96 hours. Blasts from patients with pre-B- and c-ALL up-regulated CD95 expression up to 65.4±4.3% (p<0.001) after 72-96 hours of CD40-stimulation (Fig.26, page 82). The CD95 expression on CD40-stimulated ALL blasts from patients with pre-B-ALL was comparable to CD95-expression on ALL-cells from patients with c-ALL.

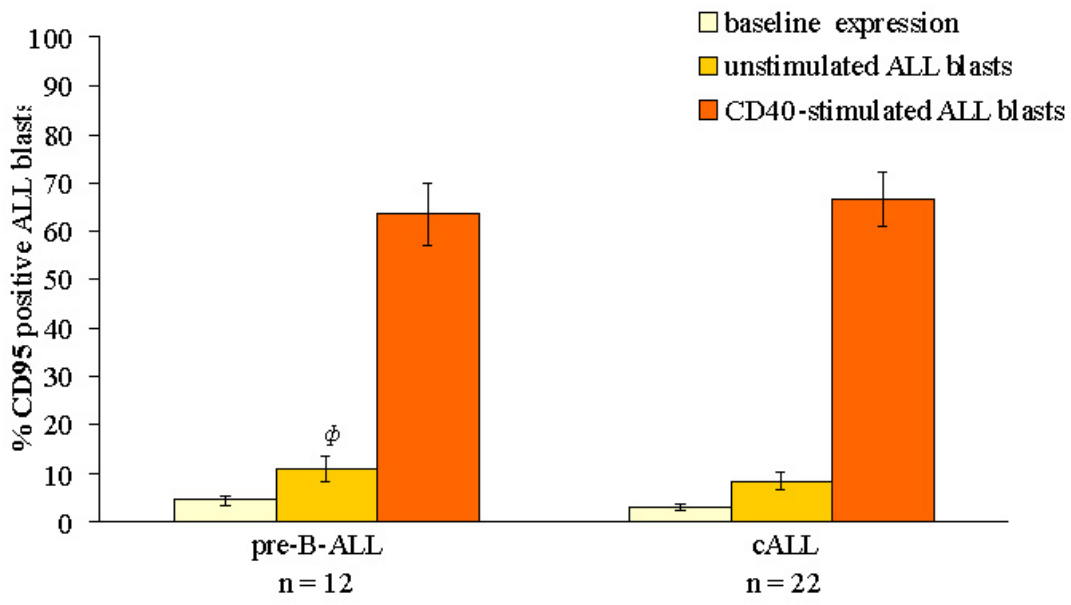

Figure 26:

CD95 expression on the CD40-stimulated ALL-blasts. ALL blasts were cocultured on $CD40L^-$- or $CD40L^+$-fibroblasts for 72h. $CD19^+$ blasts were double-stained with antibodies against CD95. Values represent the mean percentage±SE of double-positive cells. ^{A}- CD95 expression was not tested due to low cell viability in 2 cases.

As seen in table 17 (page 83) blasts from patients with early pre-B-ALL (n=5) upregulate CD95 expression (p=0.05) in all cases tested, however at low intensity when compared to upregulation of CD95 expression on CD40-stimulated ALL blasts from patients with pre-B- and c-ALL. The markedly up-regulation of CD95 on CD40-stimulated ALL blasts from patients with early pre-B-ALL was observed only in one case (pt.121).

The blasts from cryoconserved samples upregulate CD95 after CD40 stimulation at the same level when compared to CD40-stimulated freshly isolated ALL blasts. It is worth to underline that CD40-stimulated ALL blasts from samples delivered from other clinics and therefore pre-incubated for 24h at room temperature express the CD95 at the same level as CD40-stimulated ALL blasts from fresh samples obtained in our clinic.

patient number	**CD95 expression on early pre-B-ALL blasts**		
	baseline expression	**unstimulated cells**	**CD40-stimulated cells**
pt.118	0.9	7.2	30.8
pt.119	1.3	1.7	9.2
pt.120	1.0	0.4	25.7
pt.121	2.1	7.5	90
pt.123	1.7	6.3	15.7

Table 17:

Effect of CD40-stimulation on CD95 expression on early pre-B-ALL blasts. ALL blasts were cocultured on $CD40L^-$- or $CD40L^+$-fibroblasts for 72h. $CD19^+$ blasts were double-stained with antibodies against CD95 and subjected to flow cytometry analysis. Values represent percentage of double-positive cells.

3.9 Functional activity of CD95 expression induced by CD40-stimulation on ALL blasts

It has been previously described that ligation of CD95 on CD40-stimulated normal B-cells augmented apoptosis. Indeed, in our experiments cultivation of CD40-stimulated normal B-cells (n=4) in presence of anti-CD95 antibodies for 16 hours lead to 36.7±5.5% of specific apoptosis (see Table 23, page 90). We have further investigated whether CD95 molecule expressed on CD40-stimulated ALL blasts may also induce apoptosis. ALL blasts from 16 patients (see patients characteristic Table 23, page 90) were first stimulated by CD40 ligation for 72 hours and were then incubated with 1μg/ml anti-CD95 antibodies for 16h. Apoptotic cells were detected using annexin V-FITC and PI-staining. Percentage of specific apoptosis was calculated as described above (see Materials and Methods, subsection 2.18.2, page 37).

Ligation of CD95 on CD40-stimulated ALL blasts augmented specific apoptosis at level more than 10% in 12 of 16 cases tested. CD40-stimulated ALL blasts which were cultured with anti-CD95 antibodies for 16 hours demonstrated 19.1±3% of specific apoptosis (Fig.27, page 84). It was significanlty greater (p<0.05) when compared to specific apoptosis detected in cultur of unstimulated cells with anti-CD95 antibodies.

	CD95 expression *	**specific apoptosis** **
unstimulated normal B-cells, n=4	9.9±5.9	0.5±2.1
CD40-stimulated normal B-cells, n=4	59.3±13.1	36.7±5.5

Table 18:

CD95 expression and CD95-mediated apoptosis on CD40-stimulated normal B-cells. Normal peripheral B-cells were cultured on CD40L$^+$-fibroblasts for 72h. CD19$^+$ B-cells were double-stained with antibodies against CD95 and subjected for flow cytometry analysis. CD40-stimulated and unstimulated B-cells were further incubated in the presence of anti-CD95 antibodies (1μg/ml) for 16h. Percentage of apoptotic cells was determined using annexin V-FITC and PI-staining. * - values represent the mean of percentage±SE of double-positive cells. ** - values represent the mean of percentage of specific apoptosis±SE.

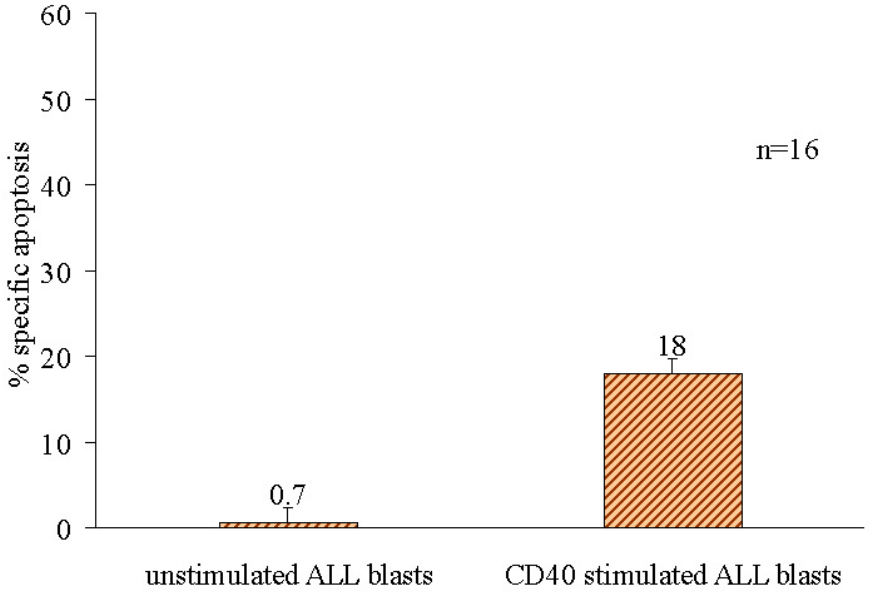

Figure 27:

CD95-mediated apoptosis in CD40-stimulated ALL blasts. ALL cells (n=16) were cultured on CD40L$^+$-fibroblasts for 72h. CD19$^+$ blasts were double-stained with antibodies against CD95 and subjected for flow cytometry analysis. CD40-stimulated and unstimulated ALL cells were further incubated in the presence of anti-CD95 antibodies (1μg/ml)for 16h. Percentage of apoptotic cells was determined using annexin V-FITC and PI-staining. Values represent the mean of percentage±SE.

Increased sensitivity to CD95-mediated apoptosis was correlated (correlations coefficient = 0.65) with the high density of CD95 molecule expressed on the surface of CD40-stimulated ALL blasts. The level of CD95 (29±7%) on CD40-stimulated blasts (n=4) that were resistant

to CD95 triggering (< 10% of specific apoptosis) was significantly lower (p<0.05) when compared to CD95 levels (70±6%) on sensitive CD40-stimulated blasts (n=12), as indicated in Table 19, page 85.

CD40-stimulated ALL blasts	
CD95 expression *	**percentage of specific apoptosis** **
29±7 (n=4)	1.8±3
70±6 (n=12)	23±3

Table 19:

Correlation between expression level of CD95 molecule on CD40-stimulated ALL blasts and sensitivity to CD95-mediated apoptosis. ALL cells were cultured on CD40L$^+$-fibroblasts for 72h. CD19$^+$ blasts were double-stained with antibodies against CD95 and subjected for flow cytometry analysis. CD40-stimulated and unstimulated ALL blasts were further incubated in the presence of anti-CD95 antibodies (1μg/ml) for 16h. Percentage of apoptotic cells was determined using annexin V-FITC and PI-staining. * - values represent the mean of percentage±SE of double-positive cells. ** - values represent the mean of percentage of specific apoptosis±SE.

Despite the fact that CD40-stimulated ALL blasts expressed CD95 at similar high level when compared to CD40-stimulated normal B-cells, addition of anti-CD95 antibodies (1μg/ml) to normal CD40-stimulated B-cells resulted in 36.7±5.5% specific apoptosis whereas leukemia cells showed significantly lower (18.0±3.4%) level of specific apoptosis in this setting.

3.10 Effect of protein synthesis inhibitor to CD95-mediated apoptosis of CD40-stimulated ALL blasts

Thus, CD40-stimulated ALL blasts express CD95, but show decreased sensitivity to CD95-mediated apoptosis, when compared to sensitivity to CD95-mediated apoptosis of CD40-stimulated normal B-cells. Therefore we next investigated the possible role of anti-apoptotic proteins in complete or partial resistance of CD40-stimulated ALL blasts toward CD95-mediated apoptosis. To investigate the role of anti-apoptotic proteins for CD95-mediated apoptosis in CD40-stimulated ALL basts, the inhibitor of protein synthesis cycloheximide

(CHX) was used. It has been shown that CHX can block the production of *de novo* synthesized proteins in cells.

ALL blasts from 4 patents with c-ALL (pts numbers: 8, 34, 43 and 51) and from 3 patents with pre-B-ALL (pts numbers: 90-91 and 100) were cultured with on $CD40L^-$- and $CD40L^+$-fibroblasts for 72h. CD40-stimulated blasts were further cultured with anti-CD95 (0.1μg/ml) antibodies in the presence or absence of CHX (0.1μg/ml). Incubation of CD40-stimulated ALL blasts with anti-CD95 antibodies in the presence of CHX lead to increased percent of apoptotic cells already after 24 hours when compared to ALL blasts cultured with anti-CD95 antibodies alone. This difference was greater after 48 hours cultivation of CD40-stimulated ALL blasts in presence of both anti-CD95 antibodies and CHX. As a control unstimulated ALL-blasts were used (Fig.28, page 86). They expressed low level of CD95 and no specific apoptosis was detected in the presence neither of anti-CD95 antibodies nor CHX.

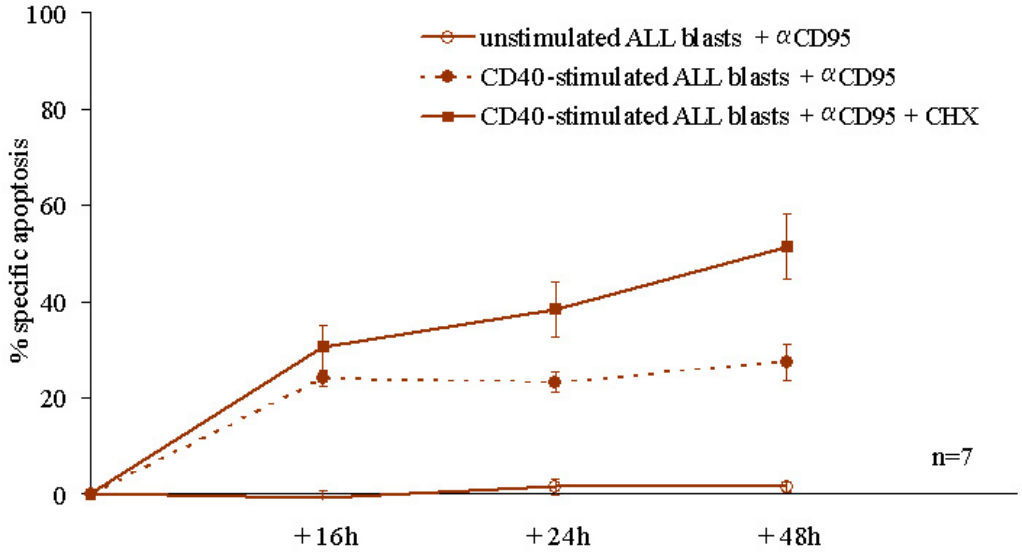

Figure 28:

Protein synthesis inhibitor sensitize CD40-stimulated ALL blasts to apoptosis. CD40-stimulated and unstimulated ALL blasts were cultured with anti-CD95 antibodies (1μg/ml) for 16h in the presence or absence of CHX (0.1μg/ml). Percentage of apoptotic cells was determined using annexin V-FITC and PI-staining. Values represent the mean of percentage of specific apoptosis±SE.

3.11 Clinical relevance of the expression and function of CD95 on ALL blasts

3.11.1 Patients' characteristics

Since resistance of ALL cells *in vivo* to chemotherapy-induced apoptosis could be associated with resistance of ALL cells to CD95-mediated apoptosis we have analysed correlation of CD95 expression and its functional activity on primary and CD40-stimulated ALL cells with generally accepted prognostic factors.

CD95 expression was determined on ALL blasts from a n=30 patient with c-ALL (pts numbers: 2, 5, 6-8, 10, 13, 16, 18-19, 27, 29, 34, 35, 40, 43, 45, 51, 58, 61, 67, 69-77), from n=13 patients with pre-B-ALL (pts numbers: 88, 90-91, 96, 100-103, 106, 108, 112, 116-117) and from n=5 patients with early pre-B-ALL (pts numbers: 118-121 and 123). Characteristics of patients whose blasts were used to determine the initial CD95 expression include some important prognostic factors that are summarized in Tab. 20, page 87.

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age (yrs); (range)	PVA score ≥ 7	leuk. count $\geq 25000/\mu$l	low	high
c-ALL n=30*	20	10	3; (0-16)	7	18	10	20
			n=4($\geq$10yrs); n=1(<1yrs)				
pre-B-ALL n=13**	10	3	4; (1-16)	2	6	5	8
			n=2($\geq$10yrs); n=0(<1yrs)				
early pre-B-ALL n=5***	2	3	2; (0-8)	1	4	0	5
			n=0($\geq$10yrs); n=2(<1yrs)				

Table 20:

Characteristics of ALL patients used for determination of initial CD95 expression. m - male; f - female; yrs - years; PVA - *in vitro* resistance-profile of ALL blasts against chemotherapeutic drugs; leuk.count - leukocyte count. PVA score was not tested: * - in 10 cases (8pts with diagnosis date<1997; 1pt - infant, in 1pt was not tested). ** - in 1 case (1pt with diagnosis date<1997). *** - in 2 cases (infants).

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age(yrs); range	PVA score ≥ 7	leuk. count $\geq 25000/\mu$l	low	high
c-ALL n=10	6	4	3.5; (1-14) n=1($\geq$10yrs); n=0($<$1yrs)	3	6	3	7
pre-B-ALL n=10	8	2	3; (1-10) n=1($\geq$10yrs); n=0($<$1yrs)	2	4	4	6
early pre-B-ALL n=4*	1	2	2; (0-4) n=0($\geq$10yrs); n=1($<$1yrs)	1	3	0	3

Table 21:

Characteristics of patients those primary ALL blasts were used to determine spontaneous and CD95-mediated apoptosis. * - PVA score not tested in 1 case (infant).

The spontaneous and CD95-mediated apoptosis was determined on primary ALL blasts from 10 patients with ALL (pts numbers: 6, 8, 16, 18, 34, 40, 43, 51, 67 and 69), 10 patients with pre-B-ALL (pts numbers: 88, 90-91, 96, 100, 102-103, 106, 108 and 112) and 3 patients with early pre-B-ALL with pts numbers 118-119 and 121. Patients' characteristics is shown in Tab. 21, page 88.

ALL blasts from n=22 patients with c-ALL (pts numbers: 2, 5, 6-8, 10, 13, 16, 18-19, 27, 29, 34, 40, 43, 45, 51, 58, 61, 67, 69-70), n=12 patients with pre-B-ALL (pts numbers: 88, 90-91, 96, 100- 103, 106, 108, 112, 116) and n=5 patients with early pre-B-ALL (pts numbers: 118-121 and 123) were used to determine CD95 expression after CD40-stimulation (see patients' characteristics in table 22, page 89).

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age (yrs); (range)	PVA score ≥ 7	leuk. count $\geq 25000/\mu$l	low	high
c-ALL n=22*	15	7	3.5; (1-14)	6	13	7	15
			n=2(≥10yrs); n=0(<1yrs)				
pre-B-ALL n=12**	10	2	3.5; (1-16)	2	5	5	7
			n=2(≥10yrs); n=0(<1yrs)				
early pre-B-ALL n=5***	2	3	2; (0-8)	1	4	0	5
			n=0(≥10yrs); n=2(<1yrs)				

Table 22:

Characteristics of ALL patients whose blasts were used for CD40-stimulation. PVA score was not tested: * - in 3 cases (2pts with diagnosis date<1997; in 1pt was not tested) ** - in 1 case (1pt with diagnosis date<1997). *** - in 1 case (infant).

CD40-stimulated and unstimulated ALL blasts from n=7 patients with c-ALL (pts numbers: 8, 16, 34, 43, 51 and 67), from n=7 patients with pre-B-ALL (pts numbers: 90-91, 96, 102, 106, 108 and 112) and from 2 patents with early pre-B-ALL (pts numbers: 118-119) were used to determine the CD95-mediated apoptosis. Characteristics of patients is summarized in Tab. 23, page 90.

leukemia subtype	**sex**		**risk factors**			**risk groups**	
	m	f	median age(yrs); range	PVA score ≥ 7	leuk. count $\geq 25000/\mu$l	low	high
c-ALL n=7	4	3	3; (2-14) n=1($\geq$10yrs); n=0($<$1yrs)	3	3	3	4
pre-B-ALL n=7	6	1	4; (2-10) n=1($\geq$10yrs); n=0($<$1yrs)	2	1	4	3
early pre-B-ALL n=2*	1	1	1; (0-2) n=0($\geq$10yrs); n=1($<$1yrs)	1	2	0	2

Table 23:

Characteristics of patients those CD40-stimulated ALL blasts were used to determine CD95-mediated apoptosis.* - PVA score not tested in 1 case (infant).

It is important to note that a high cell number was necessary to perform our functional tests. Therefore mainly high-risk patients were included in our experiments. In other hand the sufficient cell number was also possible to obtain from patients with low risk prognosis, for example from older patients. Thus, high-risk patients as well as low risk patients were included in our specific cohort of patients. Consequently it was possible to investigate the association between prognostic risk factors and CD95 expression and its functional activity on primary and CD40-stimulated ALL blasts.

3.11.2 Association between CD95 expression and function on primary and CD40-stimulated ALL blasts and prognosis factors

There was found no correlation between baseline CD95 expression on primary ALL blasts and any prognostic factor and relapse frequency. Primary ALL blasts showed very different percentage of spontaneous apoptosis after 16h of culture *in vitro*, but no correlation between clinical parameters and spontaneous apoptosis was found.

High CD95 expression on CD40-stimulated ALL blasts was significant correlated ($p < 0.05$) with low risk prognosis. ALL blasts from patients with low-risk (n=13) demonstrated high level of CD95 after CD40-stimulation (77±4%) and CD40-stimulated leukemia blasts from high-risk patients (n=26) expressed only 53±6% of CD95 (Fig.29, page 91).

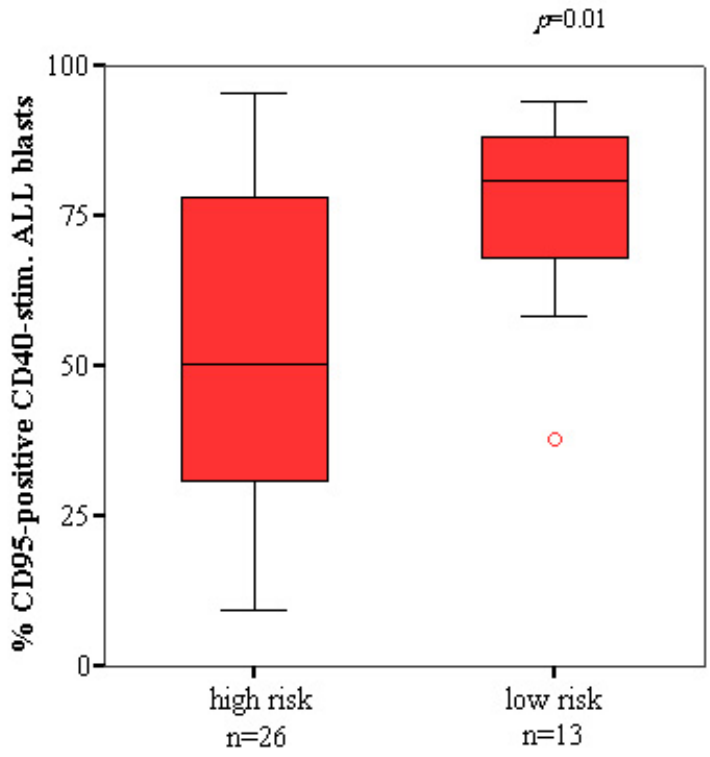

Figure 29:

CD95 expression on the CD40-stimulated ALL blasts and risk groups. ALL blasts were cultured for 3 days on $CD40L^-$- or $CD40L^+$-fibroblasts. $CD19^+$ blasts were double-stained with antibodies directed against CD95 and subjected for flow cytometric analysis. The Mann-Whitney test was used to determine significance of difference. Results represent the median percentage of double-positive cells±SD.

High expression levels of CD95 on CD40-stimulated ALL blasts was associated with sensitivity to CD95-mediated apoptosis (correlation coefficient = 0.65). However no correlation was found between risk factors and sensitivity to CD95-mediated apoptosis of CD40-stimulated ALL blasts was found. Interestingly, CD40-stimulated ALL cells from four patients that showed low sensitivity to CD95-mediated apoptosis (<10% of specific apoptosis) and expressed CD95 at low level (29±7%) belong to high risk group.

As summarized in Fig.30, ALL blasts from patients with low risk prognosis (empty dots on figure) expressed higher level of CD95 (>50%) after CD40-stimulation and showed higher sensitivity to CD95-induced apoptosis compared to CD40-stimulated ALL-blasts from patients with high-risk prognosis (full dots on figure).

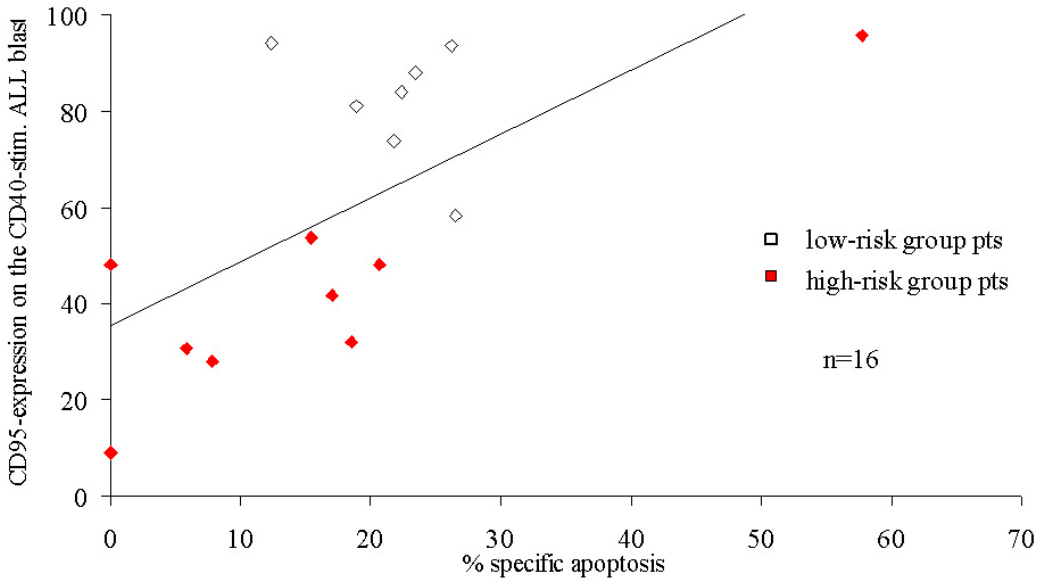

Figure 30:

CD95 expression and CD95-mediated apoptosis in CD40-stimulated ALL blasts. ALL blasts were cultured for 3 days on $CD40L^-$- or $CD40L^+$-fibroblasts. Apoptosis was triggered by addition of anti-CD95 antibodies (1μg/ml) for 16h. CD95-mediated apoptosis was evaluated using annexin V-FITC and PI-staining.

The fact that CD40-stimulated CD95 expression on the ALL blasts correlate with prognostic factors indirectly demonstrate that apoptosis through the CD95 pathway can be involved in leukemogenesis and elimination of leukemia cells.

4 Discussion

In most leukemia patients the intensified radio/chemotherapy can reach a remission. However, for patients with refractory and relapsed disease the development of novel immunotherapy is necessary. Despite the facts that immunological effector mechanisms have been shown to be involved in eradicating leukemia diseases and that leukemia cells usually express specific antigens that can serve as target structures for anti-leukemia immune responses [7, 8, 9, 10, 11], acute leukemias tend to escape immune surveillance in the clinical setting. The lack of costimulatory molecules expression is one of the postulated mechanisms by which leukemia cells escape the immune surveillance. In addition to CD28-CD80/CD86 interaction that provide necessary second costimulatory signal for the efficient T-cell activation [26, 27], the CD27-CD70 costimulatory pathway now is also of particular interest.

4.1 Expression of CD70 and CD27 on ALL cells can be modified via *in vitro* cocultivation of primary ALL cells with CD40L-expressing fibroblasts

In consonance with the other findings [50, 51] we also detected the low expression levels of CD80 and heterogeneous expression levels of CD86 on primary ALL cells (Fig. 5, page 40). Moreover, we have for the first time examine the CD70 expression on the blasts from great number (n=101) of ALL patients. The CD70 molecule was barely detectable on the surface of freshly isolated ALL cells (Fig. 5, page 40). Expression of CD27 was in contrast very heterogeneously with 40% of ALL patients expressing CD27 at a level of >20% (Fig. 5, page 40). Low expression of CD70 has also been detected on other malignant B-cells such as follicular lymphoma cells and chronic lymphocytic leukemia cells [79, 89], but till now was not reported for ALL cells.

It is well documented that the lack of CD80 and CD86 expression on ALL cells explains their poor antigen-presenting capacity [50, 51]. Since CD70 is an important costimulatory molecule involved in antigen-dependent T-cell activation, our observation of low CD70 expression may also contribute to the reduced immunogenicity of ALL cells.

The findings that B-cell-lineage acute lymphoblastic leukemias express CD40 [105] offers

the possibility to modify the leukemia cells by cocultivation with CD40L-expressing feeder-cells or with the CD40L-trimer. Since CD40-stimulation of leukemia cells was reported to upregulate expression of costimulatory molecules [48, 108, 109, 110, 111], we have analysed the CD40 expression in our specific cohort of patients with ALL and found that more than 80% cases from ALL patients express CD40 (Fig. 4, page 38). In accordance with other studies we found that ALL blasts increased CD80 and CD86 expression following CD40-stimulation (Fig. 6, page 43). Moreover, we demonstrated that CD40-stimulation upregulated the expression of CD70 in ALL blasts from 43 patients with a concomitant down-regulation of the CD27 expression (Fig. 8, page 45). Upregulation of CD70 has been also reported for CLL and FL cells [82, 89], however only in small patients groups.

Interestingly, we observed that also unstimulated ALL blasts cultured on non-transfected $CD40L^-$-fibroblasts, showed minor upregulation of costimulatory molecules CD80, CD86 and CD70 (Fig. 6 and 8). This could be partly due to the presence of some adhesive molecules on the fibroblasts or secretion of soluble factors by fibroblasts. These factors seem to increase the survival of ALL blasts and could stimulate expression of costimulatory molecules, however, with a much low intensity.

4.2 The CD70-CD27 pathway synergistically enhances the CD80 and CD86-CD28 pathway of antigen presentation by ALL cells

In many studies it was demonstrated that enhanced through CD40-activation immunogenicity of leukemia B-cells can be explained by up-regulation of the expression of CD80 and CD86 costimulatory molecules on their surface [113, 114, 115]. We have investigated whether increased CD70 expression on the CD40-stimulated ALL blasts is also participated in the improved antigen-presenting capacity of ALL cells.

In order to dissect the role of the costimulatory molecules CD80/CD86 and CD70 for the antigen-presenting capacity of CD40-stimulated ALL cells, we examined the effect of the respective specific blocking antibodies on allogeneic T-cell proliferation and cytokine production. Our results confirm the previous finding [48, 109, 107] that CD40-stimulated ALL blasts induce the allogeneic T-cell proliferation that can be reduced by the blocking of

CD80/CD86 costimulation (Fig. 15, page 61). Moreover, we have shown that blocking of the CD27-CD70 interaction alone also inhibited T-cell proliferation and IFN-γ secretion, however only partially (Fig. 15). The blocking of both CD28-CD80/CD86 and CD27-CD70 costimulatory pathways consistently had additive effect on the antigen presenting capacity of CD40-stimulated ALL cells, confirming that both CD80/CD86 and CD70 molecules were involved (Fig. 15). The similar results has been obtained on CD40-stimulated FL cells cultured with allogeneic T-cells [89]. It was demonstrated that blockade of CD70-CD27 interaction led only to a 25% reduced T-cell proliferation. An additive effect was observed when CD70-CD27 and CD80/CD86-CD28 were blocked toghether, resulting in more than 70% inhibition of T-cell proliferation [89].

Also T-cells that have been stimulated with unstimulated ALL blasts showed reduced proliferation in presence of combination of blocking CD80/CD86/CD70 antibodies (Fig. 16, page 63). This can be explained by the fact that unstimulated ALL blasts also express CD80, CD70 and CD86 molecules however at low level. Thus, presence of costimulatory molecules also at low levels can partially improve the T-cell stimulatory capacity of ALL blasts.

Thus, our observation demonstrate that expression of both CD70 and CD80/CD86 on ALL cells seems to be essential for an efficient T-cell stimulation. Our results, together with other studies [83, 89] confirm that in the induction of T-cell proliferation CD80/CD86 and CD70 act synergistic, that suggest that cooperation of two signals - through CD28 and CD27 pathways may be essential for optimal T-cell stimulation.

4.3 The CD70-CD27 pathway is necessary for the generation of anti-leukemia cytotoxic T-cells

In the next part of our study we have examined the potential of CD40-stimulated ALL blasts to activate cytotoxic anti-leukemia specific T-cells (CTLs). We have investigated the requirement of the T-cell costimulation through CD27 by CD70 on ALL cells for the expansion and cytolytic activity of anti-leukemia CTLs.

By priming with CD40-stimulated ALL blasts and repetitive restimulation and cytokine-driven expansion, T-cell lines have been established in 4 from 6 cases studied (Tab. 13,

page 68). In 4 cases the allogeneic donor peripheral blood (Tab. 12, page 67) was used as effector cells for the T-cell lines generation. In this allogeneic setting we have established 3 T-cell lines. The low percentage of viable ALL blasts used for stimulation and restimulation is one possible reason why we have failed to expand the T-cell line in one case (pt.7). Using the identical technique we have tried to generate anti-leukemia T-cell lines using as effector cells the autologous peripheral blood cells from 2 patients. T-cells were expanded only in one case (Tab. 13). In another case (pt.95), autologous effector cells used for stimulation were obtained from peripheral blood of chemotherapy-treated patient and were probably functionally incomplete. However, the poor proliferation of autologous effector cells in this case could be explained by low expression of CD70 on the ALL blasts that were used as stimulator cells (Tab. 11, page 66).

In fact, we have demonstrated that proliferation of effector cells required CD70-mediated costimulation. The proliferation of effector cells stimulated with CD40-stimulated ALL cells was completely abrogated by addition of anti-CD70 antibodies at the initiation of culture (Fig. 17 and 18, page 69 and 70). Interestingly, that expansion of T-cells stimulated with unstimulated ALL blasts was also significantly reduced when anti-CD70 antibodies were added (Fig. 17 and 18). Thus, blockade of even low expression of CD70 on ALL blasts that have been used for stimulation led to inhibition of T-cell proliferation. Therefore, our results demonstrate that CD70 costimulation is an absolute requirement for the expansion of T-cells stimulated with ALL blasts.

To demonstrate anti-leukemia specificity of the generated T-cell lines, the ability of T-cells to lyse CD40-stimulated, unstimulated ALL blasts and autologous non-malignant cells (PHA blasts) was tested. 3 from 4 expanded T-cells were capable of lysing both unstimulated and CD40-stimulated ALL blasts (Tab. 14, page 71), although the efficiency of lysis was variable between patients. The fact that generated CTLs did not lyse PHA blasts, suggested that CTLs responses were directed against leukemia-specific target. It is important to note that T-cell line generated from the autologous effector cells were unable to lyse ALL blasts. It is unknown why we have failed to generate anti-leukemia cytotoxic T-cells in this case, because CD40-stimulated ALL blasts used for stimulation have uniformly expressed costimulatory molecules CD80/CD86 and CD70. One possible reason to explain this is the low percentage of $CD3^+$ cells in the initial effector cell population.

The inability of CD40-stimulated ALL blasts to expand cytotoxic anti-leukemia T-cells in the autologous setting in our patients cohort may be also explained by the fact that additional allo-stimulation can enhance the development of cytotoxic T-cell response. Although, it is possible that effector cells should be restimulated more than two times for generation an effective anti-leukemia cytotoxic T-cell response *in vitro* in autologous settings. We have shown that effector cells from syngeneic donor stimulated and three times restimulated with ALL blasts demonstrated cytotoxic anti-leukemia activity. Moreover, the high NK-mediated cytotoxicity of generated T-cell lines which was observed after the second restimulation has been reduced after the thirty restimulation. The anti-leukemia cytotoxicity of these T-cell lines has been slightly enhanced after the thirty restimulation.

To investigate the role of CD70 costimulation in the cytolytic activity of CTLs generated using CD40-stimulated ALL blasts we have tested the ability of T-cell lines generated in the presence of blocking anti-CD70 antibodies to lyse the leukemia target. Due to the fact that proliferation of T-cells was strong inhibited in the presence of anti-CD70 antibodies only very low number of T-cells was obtained for the cytotoxic activity test. Nevertheless, we have demonstrated that cytotoxicity of T-cells generated using CD40-stimulated or unstimulated ALL blasts was weak, when CD27-CD70 costimulation was inhibited. Moreover, the T-cell lines generated in presence of blocking anti-CD70 antibodies, showed lower cytotoxic activity against both unstimulated and CD40-stimulated ALL cells.

It has been shown that CD27-CD70 interaction plays a crucial role in immunoglobulin production attributed to T-B-cell interaction [149] and enhancement of NK or T-cell activity [86]. Taken together our results suggest that CD27-CD70 costimulatory pathway is also necessary for the proliferation and augmentation of cytotoxic activity of CTLs.

The molecular mechanisms by which CD70 costimulation provide the costimulatory signal for the T-cell is remain to be study. It has been reported that the enhancement of CTL activity by CD27-CD70 interaction is largely perforin/granzyme dependent [150]. Since, the expression of perforin by the EBV transformed B-cells-induced CTLs in the presence of anti-CD70 blocking antibodies was diminished [150], it would be interesting to investigate whether the perforin/granzyme pathway is responsible in the reduced cytolytic activity of the T-cells expanded using the CD40-stimulated ALL cells in the presence of anti-CD70

antibodies.

Also the mechanisms by which CTLs lyse leukemia cells is not well studied. We have observed that anti-leukemia CTLs lysed more efficiently CD40-stimulated than unstimulated ALL cells. It could not be explained by high expression levels of costimulatory molecules on the CD40-stimulated ALL cells, because presence of blocking CD80/CD86 and CD70 antibodies were unable to prevent lysis of target cells (data not shown). One possible explanation of this fact is that CD40-stimulated ALL cells express higher level of CD95 molecule when compared to unstimulated ALL cells. In fact, it is known that in addition to perforin/granzyme pathway, CD95-CD95L interaction is also involved in T-cell-mediated cytotoxicity [151, 152, 153]. In CLL cells, it has been demonstrated that allogeneic CTLs mediate apoptosis of both unstimulated and CD40-stimulated CLL cells in CD95-independent manner via the granule exocytosis pathway [116]. However, recently the same group has demonstrated that autologous cytolytic $CD4^+$ T-cells affected cytolysis of CLL cells via CD95-dependent pathway and were not restricted via class I molecules of the MHC [154]. Since generated in our study cytotoxic T-cell lines represent a $CD4^+$ and $CD8^+$ phenotype, both perforin/granzyme and CD95-CD95L pathways could be involved in cytotoxic activity against ALL blasts. It would be interesting to investigate what kind of pathway is involved in the recognition and T-cell mediated lyse of CD40-stimulated or unstimulated ALL cells.

4.4 A high CD27 expression rate correlates with patients belonging to the low risk group

Since the costimulatory signals are necessary for the sufficient T-cell stimulation, the density of the costimulatory molecules on the cell surface and the number of those cells within a population are of particular interest.

It has been reported for AML that the 5% CD86 expression on the leukemia cells was sufficient to costimulate an allogeneic T-cell response *in vitro* [155]. Most recently it was shown that duration of first remission is significantly prolonged when 10% CD80 and/or CD86 expression is present on AML blatsts [155]. Also in ALLs the CD86 expression required to delivery a costimulatory signal and prevent the induction of anergy was found to

be low [115]. We investigated therefore whether the initial expression of the costimulatory molecules CD80, CD86, CD70 and CD27 on primary ALL blasts correlate with prognostic factors or favorable clinical outcome.

Despite the fact that CD80 molecule was low expressed on primary ALL blasts, we found that patients which blasts expressed CD80 at level more than 3% have a tendence to lower probability to get relapse, when compared to other patients (Fig. 10, page 52). Our observation suggests that presence of CD80 at even low level can be sufficient for T-cell stimulation. We have not found any correlation between CD80 expression on primary and CD40-stimulated ALL blasts and other prognostic factors such as PVA score and leukocyte count at diagnosis. However, the PVA score display the sensitivity of ALL blasts only to selected chemotherapeutic drugs - prednisolon, vincristine and asparaginase. Recently in one study in CLLs has been described that low or negative level of CD80 associated with resistance of CLL cells to purine analogs (2'-chlorodeoxyadenosine and fludarabine) [156]. It could be also interesting to investigate whether the low level of CD80 on the ALL blasts correlate with sensitivity to chemotherapeutic drugs others than are used for determination of PVA score.

As CD86 has been heterogeneously distributed on primary ALL blasts we have analysed whether high expression of CD86 correlate with clinical outcome. We have found no significant correlation between CD86 expression on primary ALL blasts and prognosis factors. However, CD40-stimulated ALL blasts from patients with low risk prognosis increase CD86 expression in all cases tested, but ALL blasts from patients with high risk prognosis (Fig. 12, page 55). Thus, capacity of CD40-stimulated ALL blasts up-regulate CD86 is the significant criteria for the patients to belong to a low risk prognosis group. Surprisingly, but we have also found that patients whose blasts initially expressed more than 50% of CD86 have a shorter disease-free survival period compared to the patients whose blasts express low level of CD86 (<50%) (p=0.062) (data not shown). The similar observation has been already described in AMLs [157], however it was not replicated in another publication [107]. Moreover, it was demonstrated that plasma collected from patients with AML and CLL analysed only at the time of presentation of relapse contained elevated level of soluble CD86 [158]. Taken together, it is suggests that the high initial expression of CD86 seems to be correlate with poor prognosis in leukemias. In other hand, the high

expression of CD86 induced by activation correlate with low risk prognosis. The further investigations are needed to clear the clinical relevance of CD86 expression on ALL cells and its the biological role in the progression of leukemia.

With respect to CD70 expression, we have not found significant correlation between CD70 expression levels on primary and CD40-stimulated ALL blasts and any prognostic factors and probability to get a relapse. However, we have demonstrated that high levels of CD27 which is heterogeneously expressed on primary ALL blasts significant correlate with probability of patients to belong to low prognosis group (Fig. 11), page 54.

The correlation of high expression levels of CD27 with low risk prognosis has been described for multiple myeloma (MM) patients [159]. It was shown that malignant plasma cells from MM patients in the situation of complete clinical remission display a significantly higher CD27 expression compared to those obtained from newly diagnosed, relapsed and refractory MM patients [159]. In CLLs a strong correlation was found between soluble CD27 level in serum and tumor load [80, 160]. Taken together these data support the hypothesis that loss of CD27 is associated with more aggressive disease. Further study of the regulation of surface and soluble CD27 and its association with the clinical outcome could provide new insights into the role of CD27 in the biological and clinical aspects of ALL. Its potential prognostic value should be tested in prospectives studies.

4.5 Induction of CD95 expression does not completely sensitize ALL cells for CD95-mediated apoptosis

The understanding of mechanisms are involved in the resistance of leukemia cells to apoptosis can facilitate the development of novel therapeutic strategies for ALL treatment. We have examined in our study the expression of CD95 and its functional activity on ALL blasts.

Our results show that freshly isolated ALL blasts expressed detectable, but low levels of CD95 (Fig. 23, page 77), that consider with other reports [143, 138, 130, 124, 133]. Similar to observations on lymphoma and chronic leukemia cells, initially showed low level of CD95 and resistance to CD95-mediated apoptosis [130, 143], we have not detected significantly increased apoptosis of primary ALL blasts after CD95 ligation (Tab. 15, page 78). In contrast,

primary leukemia cells from patients with T-ALL and AML exhibit heterogeneous CD95 expression pattern and are quite sensitive to CD95-mediated apoptosis [134, 132, 135]. Taken together, the low expression on primary leukemia cells seems to be associate with low expression levels of CD95. However, we have not found quantitative correlation between initial CD95 expression on primary ALL blasts and sensitivity to CD95. In addition, no significant correlation was found between initial CD95 expression on primary ALL blasts and selected risk parameters (PVA score and leukocyte count at diagnosis).

As it has been shown for CLL and NHL cells [143], CD40-stimulation of ALL blasts lead to CD95 up-regulation (Fig. 25, page 81). We have found that, CD40-stimulation of ALL blasts for 16 and 24 hours results in CD95 expression, which although is not sufficient for CD95-mediated apoptosis (data not shown). It was already described many reports that CD40-stimulation for 48 hours of CLL cells induce CD95 expression, but did not sensitize the cells to CD95-mediated apoptosis [116, 143]. In contrast, it was recently shown that only prolonged (72 hours) CD40 ligation is sufficient to render CLL cells to be sensitive against CD95-mediated apoptosis [154]. Thus, although CD95-expression occurs early after CD40 activation of leukemia cells, susceptibility to CD95-mediated apoptosis develops slowly.

We have found, that the maximal CD95 expression on ALL blasts could be reached after 72-96 hours of CD40 activation (Fig 26, page 82). At that time CD40-stimulated ALL blasts showed enhanced sensitivity toward CD95-mediated apoptosis (Fig 27, page 84). It is worth to note that the enhanced susceptibility in CD95-mediated apoptosis was found to be associated with increased density of CD95 expression on CD40-stimulated ALL blasts (Tab. 19, page 85). Most importantly, capacity of ALL blasts to express CD95 in response to CD40-stimulation and sensitivity to CD95-mediated apoptosis both correlate with a favorable prognosis (Fig 29 and 30, page 91 and 92). Blasts from patients with high risk prognosis express CD95 only at low levels after CD40-stimulation and demonstrate low sensitivity to CD95-mediated apoptosis. It is worth to underline that among four patients whose CD40-stimulated ALL cells exhibited very low level of CD95 and shown resistance toward CD95-mediated apoptosis, two patients have diagnosis early pre-B-ALL. It suggests together with our early observations, that ALL blasts from patients with early pre-B-ALL are not quite sensitive for the CD40-stimulation.

4.6 Anti-apoptotic proteins are involved in low sensitivity of ALL cells to CD95-mediated apoptosis

Despite CD40 activated ALL blasts express high level of CD95 they demonstrate decreased sensitivity to CD95-mediated apoptosis also after long time incubation with anti-CD95 antibodies, when compared to CD40-stimulated normal mature B-cells (Tab. 18 and Fig 27, page 84).

It appears that three elements are essential for CD95-mediated apoptosis: 1) expression of CD95 molecule at sufficient densities, 2) cross-linking of CD95 molecule and 3) a permissive cellular background which allows CD95 ligation to generate signals resulting in apoptosis. As CD40-stimulated ALL blasts express high level of CD95 and no mutation in CD95 receptor gene hes been detected [161], their partially resistance is largely attributed to anti-apoptotic intracellular factors affecting the CD95 signaling pathway. In T-ALLs it was demonstrated that additional treatment with protein synthesis inhibitor resulted in enhanced sensitivity to CD95-mediated apoptosis [134]. In our experiments, incubation of partially resistant ALL cells with the protein synthesis inhibitor cycloheximide reversed resistance and induced sensitivity to anti-CD95 antibodies (Fig 28, page 86). Our data suggested that resistance to anti-CD95 mediated apoptosis in ALL is maintained by an active cellular program and could be overcome by blockade of anti-apoptotic proteins. In fact, there is accumulating evidence that, after signaling by extracellular factors, the death-survival decision depends on the balance between pro- and anti-apoptotic intracellular proteins, especially the members of Bcl-2 gene family. The anti-apoptotic bcl-2 protein was increased in AML, T- and B-ALL, and its expression was found to be increased in AML and ALL cells, when compared with that of normal progenitor counterparts [136, 131, 132, 143]. The absence of a quantitative correlation between bcl-2 expression in ALL cells and sensitivity to CD95 triggering apoptosis [132, 143] suggests that the expression and the complex interaction of other anti-apoptotic proteins may being involved in the intracellular apoptotic signaling process. The expression of anti-apoptotic proteins, such as the recently described survivin molecule [162, 19], in ALL is presently under investigation.

Since the expression of CD95 ligand on T-cells is another pathway in addition to the perforin/granzyme pathway, by which cytotoxic T-cells kill target cells, CD40-stimulated ALL

blasts may also be more sensitive to cytotoxic killing than resting leukemia cells. Encouraging results were reported in a phase I clinical trial of gene therapy for patients with CLL [118]. It was purposed that the sensitivity of CD40-stimulated CLL cells to CD95-mediated apoptosis may account in part for the noted reduction in leukemia cell counts and lymph node size in CLL patients treated with CD40L-transduced CLL cells. We have demonstrated that CD40-stimulated ALL blasts exhibit sensitivity toward CD95-mediated apoptosis and this can be enhanced by blocking of protein synthesis cycloheximide. Therefore, further investigation of agents that can modify the expression of factors associated with the partial resistance of CD40-stimulated ALL blasts to CD95-mediated apoptosis are necessary.

5 Summary

The characterization of important molecules expressed on leukemias cells has allowed a further understanding of basic biologic mechanisms including apoptosis pathways or immune responses directed against individual malignant cell. For generation of leukemia cell vaccines the manipulation of the expression-level of costimulatory molecules for achieving the most efficient T-cell stimulation, or of CD95 expression for enhanced apoptosis of leukemia cells seem to be promising targets.

In this work, the importance of the CD70-CD27 costimulatory pathway involved in antigen-presentation and CD95-mediated apoptosis was investigated in primary ALL cells that have been stimulated *in vitro* by CD40L expressing feeder cells. By flow-cytometry analysis it was found that expression of CD70 could be significantly enhanced after CD40-stimulation.

The importance of the CD70-CD27 pathway for ALL cells was characterized here for the first time to synergistically enhance the CD80/CD86-CD28 mediated antigen presentation. In addition, using a protocol for in vitro generation of T-cell lines it was demonstrated that the CD70-CD27 pathway is a prerequisite for generation of cytotoxic T cells (CTL). For the first time the expression of costimulatory molecules and their clinical relevance were investigated on the large number of primary blasts from newly diagnosed patients with ALL. It was found that high CD27 expression rate correlates with patients belonging to the low risk group.

It has been shown, that ALL cells up-regulated CD95 at high level after CD40-stimulation and demonstrated sensitivity toward CD95-mediated apoptosis. Moreover, the CD95-mediated apoptosis in CD40-stimulated ALL blasts can be enhanced by cycloheximide-treatment most likely due to blockade of anti-apoptotic proteins. More important, it was found that high level of CD95 on CD40-stimulated ALL blasts and their sensitivity to CD95-mediated apoptosis are associated with low risk prognosis.
The data observed here for ALL are encouraging for up-coming clinical trials of leukemia vaccines.

6 References

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Sie wurde weder für die andere Prüfungen verwendet noch an anderen Fakultäten eingereicht, auch nicht auszugsweise.

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