CYTOKINE SIGNALING AND MITOGENIC RESPONSIVENESS OF B CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP

WOENSDAG 10 JUNI 1998 OM 15.45 UUR

door

Franciscus J.W. Smiers
geboren te Amsterdam

Promotiecommissie

Promotor:	Prof. dr B. Löwenberg
Co-promotor:	Dr I.P. Touw
Overige leden:	Prof. dr H.A. Büller Prof. dr J.M.J.J. Vossen Dr E. Vellenga

Teach thy tongue to say
I do not know
and thou shalt progress.

Maimonides, 1995
Ben Shahn (1899–1969)

Voor mijn ouders,
Lucilla en mijn gabbertjes

Contents

Abbreviations

AET	2-aminoethylisothiouronium bromide
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
B-ALL	B-cell acute lymphoblastic leukemia
BCP	B cell precursor
c-ALL	common acute lymphoblastic leukemia
CD	cluster of differentiation
CR	clinical remission
CRH	cytokine receptor homology (domain)
D	diversity
ECL	enhanced chemoluminescence
EPO-R	erythropoietin receptor
EFS	event free survival
FACS	fluoresence activated cell sorting
FITC	fluorescein isothiocyanate
FL	Flk2/Flt3-ligand
Flk2	fetal liver kinase-2
Flt3	fetal liver tyrosine kinase-3
GAM	goat anti mouse
γ_c	common gamma chain
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GDP/GTP	guanosine di/tri phosphate
Grb2	growth factor receptor binding protein 2
HGF	hematopoietic growth factor
^{3}H-TdR	tritium labeled thymidine
IGF-1	insulin growth factor 1
Ig_H	immunoglobulin heavy chain
Ig_L	immunoglobulin light chain
IL-n	interleukin-n
IRS1/2	insulin receptor substrate 1/2
JAK	Janus kinase/Just another kinase
J	joining
KL	kit ligand
MAPK	mitogen activated protein kinase
OS	overall survival
PBL	peripheral blood lymphocytes

PBS	phosphate buffered saline
(rt)PCR	(reversed transcriptase) polymerase chain reaction
PI3-K	phosphatidyl Inositol 3-kinase
PTB	protein tyrosine binding
PTK	protein tyrosine kinase
RAG	recombination activating gene
RF	reading frame
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SH2/3	src homology 2/3
STAT	signal transducer and activator of transcription
SOS	son of sevenless
T-ALL	T-cell acute lymphoblastic leukemia
TBS	Tris buffered saline
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TGF-β	transforming growth factor β
V	variable
WBC	white blood cell count

Chapter 1

General Introduction

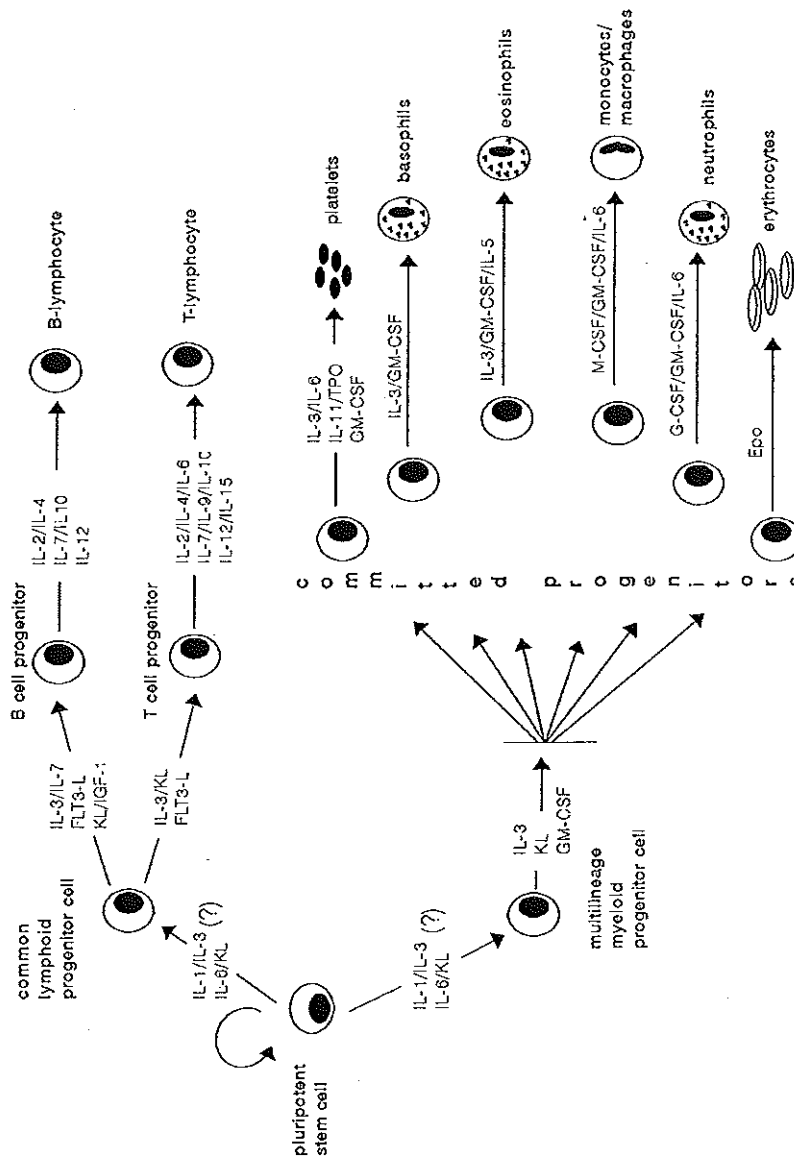

Figure 1 Schematic representation of hematopoiesis. The pluripotent stem cells give rise to committed lymphoid and myeloid progenitor cells. Commitment, proliferation and further development to fully differentiated cells occurs under the control of HGFs/cytokines. Major (but not all) HGFs involved in hematopoiesis are indicated.

1.1 Hematopoiesis

All blood cells originate from the hematopoietic stem cells, that are capable of both self renewal and differentiation towards the lineage committed progenitor cells (figure 1). The primary site of blood cell formation or hematopoiesis is the bone marrow. The bone marrow stromal cells provide a specific microenvironment required for hematopoietic cell development. In addition, proliferation and maturation of the committed progenitor cells are governed by a network of hematopoietic growth factors (HGF's) and cytokines (figure 1). A tight balance must be maintained to compensate for normal blood cell loss and to meet with the demands of extra cell production during hematopoietic stress and infectious disease.

1.2 Development of B cell precursors

When committed to the B cell lineage, primitive lymphoid cells enter a sequence of developmental steps, eventually resulting in the expression of functional immunoglobulin (Ig) heavy and light chain genes (1,2). Intermediate stages of B cell development can be recognized both by characteristic genomic and phenotypical changes. Different nomenclatures have been used to designate the subsequent stages of B cell development. In this thesis, the nomenclature according to Melchers et al is used (figure 2) (3). The most primitive stage is represented by the pro-B cell, in which the genes coding for Ig_{heavy} (Ig_H) and Ig_{light} (Ig_L) chains are still in the non rearranged or germ line configuration (figure 2). In the subsequent steps, first the Ig heavy chain variable (V) region genes are rearranged. At the pre-B-I cell stage one of the diversity (D) genes is juxtaposed to one of the joining (J) genes, with deletion of the interspacing DNA (4). This process of DJ_{heavy} (DJ_H) chain gene rearrangement is followed by fusion of one of the V genes to the rearranged DJ_H genes to form a continuous VDJ_H sequence coding for the variable region of the Ig heavy chain protein. D_H to J_H joining may give rise to three possible reading frames (RF)(5). In productively rearranged VDJ_H, the DJ_H gene is preferentially in RF-1 (6). The occurrence of DJ_H in RF-3 is counter selected by the generation of multiple stop codons in the rearranged gene, preventing translation of transcripts to proper proteins (5). DJ_H in RF-2 may form a DJC_μ protein which can be incorporated into the membrane, but is unable to give the progression signal for further maturation and prevents further rearrangement (7). By joining of DJ_H to one of the V genes, the cell enters the pre-B-II cell stage. The VDJ_H joint is coupled to the gene coding for the constant region of the IgM heavy chain.

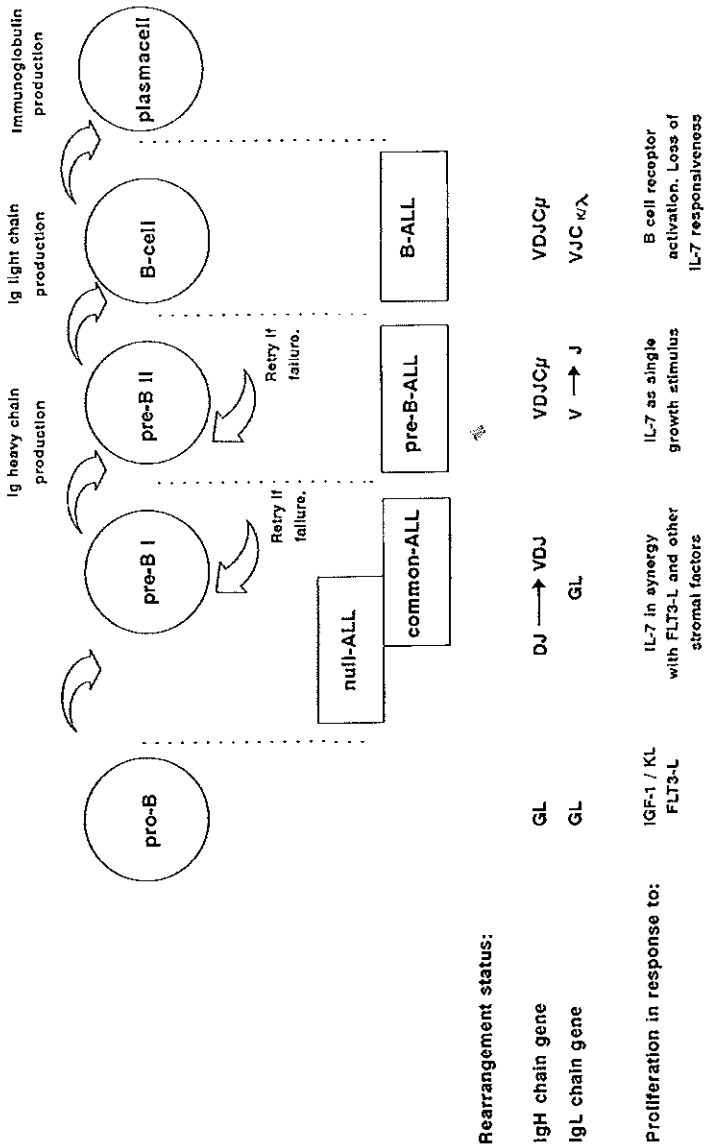

Figure 2 Simplified scheme of growth factor responses of normal B cell precursors in relation to rearrangement status of immunoglobulin genes. During B cell development the genes coding for Ig heavy and light chains are rearranged in an orderly fashion. Rearrangement of the Ig light chain will start only if the Ig heavy chain gene has been successfully rearranged. If rearrangement of either gene has been unsuccesful, the cell has the capacity to try again. If persistent failure of the genes occur, the cell will go into apoptosis. In normal B cell lymphopoiesis proliferative responses to HGFs are determined by the rearrangement status of the Ig genes. Also shown are the corresponding immunophenotypes of acute lymphoblastic leukemia cells. (GL = germ-line)

Thus, a complete μ heavy chain (Ig_H) is produced. If the rearrangement was non productive, i.e. not leading to expression of a $VDJC_\mu$ on the cell membrane, additional rearrangements of the VDJ genes may occur on the same or on the other allele (8-10) (figure 2). Membrane expression of a properly rearranged $VDJC_\mu$ terminates further VDJ_H gene rearrangement, thus preventing continued rearrangement of the VDJ genes on the other allele (allelic exclusion)(11,12). Because cells with VDJ_H in RF-2 or RF-3 are underrepresented in number in the pre-B-II cell pool, it is assumed that these cells die, or in case of RF-3 shift to RF-1 by continued rearrangement (3,6). In mice that are unable to express μ-protein due to targeted disruption of the μ gene, pre-B-I cells numbers are greatly reduced (13).

The Ig_H chain is first present in the cytosol, rapidly followed by expression on the membrane. Membrane expression of the Ig_H chain is possible only in conjunction with Ig_L chains. However, in Pre-B II-cells the Ig_L chain gene is not yet expressed. Instead, the cells express surrogate light chains, encoded by $V_{pre\ B}$ and λ_5 genes (14,15).

It is generally thought that rearrangement of the Ig_L gene κ or λ can only be initiated after successful rearrangement and membrane expression of the Ig_H chain (16-18).

Ig_L chain genes rearrange in a similar way as the Ig_H chain genes, but without the involvement of D genes, which are not present in the light chain loci. Rearrangement of the Ig_L chain genes occurs in the Pre B-II cell stage. Allelic exclusion prevents simultaneous expression of κ and λ light chains. Currently, this ordered fashion of rearrangements is under some debate since rearrangement of the κ light chain gene has been found in absence of rearrangement of the heavy chain gene (19,20). In any case, it seems unlikely that B cells lacking the Ig_H genes can persist, because improper rearrangement of either the Ig_H or Ig_L chain genes will arrest maturation and induce apoptosis, thereby preventing the accumulation of non functional B cell precursor cells (6). Indeed, mice lacking the Ig_H chains as a result of targeted disruption of the gene, showed a maturation arrest at the pre-B cell stage (13). Distinct stages of B cell precursor development are closely linked to different response patterns to exogenous growth stimuli. For instance during normal B cell development in mice, cells respond to IL-7 as single growth factor after productive rearrangement of the Ig_H chain gene, while in earlier stages responses are seen only in synergy with other stromal factors (21-23).

1.3 Acute lymphoblastic leukemia (ALL)

ALL is a neoplastic disease characterized by the clonal expansion of immature lymphoblastic cells. Accumulation of these cells in the bone marrow affects normal hematopoiesis. The clinical characteristics of ALL include anemia leading to fatigue and pallor, neutropenia with increasing susceptibility to infections, and thrombocytopenia resulting in enhanced bleeding tendency.

Among other prognostic determinants such as white blood cell count, age at diagnosis and cyto-genetics, immunophenotype has prognostic significance and may have implications for the therapeutic approach (24-26). Immunophenotypically, different types of ALL can be recognized: ALL of B cell precursor cells (BCP-ALL): null-, common- ,pre-B- and B-ALL and ALL of the T lineage (T-ALL): immature T-ALL, common thymocyte T-ALL, mature T-ALL.

Based on immunophenotypical characteristics, null- and common ALL cells may be considered leukemic representants of the normal pre-B-I cells (null-ALL: CD10-, CD19+, CD34+, Cytoplasmic(Cy)$_\mu$ -; c-ALL: CD10+, CD19+, CD34+/-, Cy$_\mu$-) (27)(Fig.2). According to this classification, pre-B ALL cells (CD10+, CD19+, CD34+/-, Cy$_\mu$+)(27) would be the leukemic representants of pre-B-II cells. B-ALL cells (SmIg+)(27) would be leukemic representants of immature B cells (Fig.2). Rearrangement of the Ig$_H$ chain gene in the absence of actual production of the Ig$_H$, indeed is found in most cases of null- and common-ALL (28,29). However, in a substantial number of null- and c-ALL cases (9-40%), rearrangement of either the kappa or lambda light chain gene is seen, which would be expected to be seen only in pre-B II cells that express μ protein (28,30,31). By definition pre-B ALL cells express functionally rearranged Ig$_H$ genes. This would imply that the correlation between immunophenotype and the corresponding rearrangement status seen in normal B cell precursors is frequently lost in acute lymphoblastic leukemia.

If BCP-ALL cells would behave like representants of normal developmental stages of B cells and if in human and mice a similar development dependent growth factor response pattern exists, one would expect BCP-ALL cells to respond to IL-7 as single growth stimulus in pre B ALL, or in synergy with either IGF-1 or KL in null-ALL or c-ALL. Whether these phenotypically defined maturation stages of ALL have different requirements for growth factor stimulation, has not been establioshed. This question has been addressed in Chapter 2.

1.4 Hematopoietic growth factor (HGF) and cytokine responses of normal and leukemic BCP

Most HGFs are active in more than one hematopoietic differentiation lineage and/or at multiple stages of maturation. HGFs that govern the development of lymphoid cells show this pleiotropy as well. For instance, interleukin (IL-) 7 is a mitogenic factor for B cell precursor (BCP) cells, thymocytes and cytotoxic T cells (32-36) and in vitro experiments suggest a role for IL-7 in the rearrangement process of the T cell receptor gene (37-39) and possibly the Ig_H chain gene in BCP cells (40).

In the mouse model, the response of BCPs to IL-7 was shown to depend strictly on the Ig rearrangement status (21,41). BCP cells become responsive to IL-7 in synergy with Kit Ligand (KL) during Ig_H chain gene rearrangement, to IL-7 as single growth stimulus during Ig_L chain genes rearrangement and finally lose the capacity to respond to IL-7 after succesful rearrangement of Ig_H and Ig_L chain genes (figure 2) (21,41-46).

Insulin-like growth factor (IGF-1) is a potent growth stimulus at the pro-B cell stage, but not in the more mature stages of B cell development (47,48). Other HGFs of which mitogenic effects in BCP cells have been reported are IL-2 and IL-3 (49-51).

Opposite effects of HGFs on normal and leukemic BCP cells have been reported as well. For instance, proliferation of both normal and leukemic BCP cells is inhibited by IL-4. In contrast, IL-4 induces proliferation in mature B cells in synergy with CD40 activation (36,52-55). Modulation of effects of other cytokines is a characteristic feature of most HGFs.

Failure to respond to specific growth and differentiation factors during specific stages of maturation or excessive synthesis of growth factors may contribute to leukemogenesis. In experimental models it was shown that uncontrolled synthesis of HGFs may lead to the development of leukemia. For instance, the overexpression of IL-7 may result in leukemia and lymphomas (60,61). Recently, the expression of multiple growth factors in leukemic cells was reported, but its relevance for development of disease still has to be established - (62-64). On the other hand leukemic transformation may result in growth factor responses different from normal. The experiments described in chapters 2 and 3 were performed to compare the proliferative responses of BCP-ALL cells to different growth stimuli with those of normal BCP cells.

1.5 Effects of Interleukin 7 in combination with other growth stimuli on normal and leukemic BCP cells

1.5.1 Interleukin 7

IL-7 induces proliferation in normal and malignant BCP cells during distinct stages of development. Originally, IL-7 was considered BCP growth factor and have a role in maintenance of BCPs to compensate for the high loss of cells during the inefficient re-arrangement procedure. IL-7 induces N-myc expression (65,66), downregulates expression of genes involved in Ig gene recombination events RAG1 and RAG2 genes as well as the gene coding for terminal deoxynucleotidyl transferase (TdT)(67). Based on these results, it has been suggested that IL-7 also promotes the differentiation of BCP cells.

Recently a role of IL-7 in promotion of rearrangement of the Ig heavy chain gene has been suggested as well (40). The function of IL-7 in B cell development was further elucidated in mice lacking the IL-7R α chain gene. In these mice both B and T cell development was severely impaired. B cell development was arrested at the pre-B-I cell stage of maturation, whereas T cell development was affected before CD4 and CD8 expression and before rearrangement of the T cell receptor β chain (68). Similar results were found in mice deficient of IL-7 either by disruption of the IL-7 gene or by continuous application of IL-7R neutralizing antibodies (69-71). Strikingly, some mature peripheral B and T cells were still detectable, suggesting that B and T cell development without the involvement of IL-7 is possible. However, it remains to be determined whether these lymphocytes are fully functional. The important role of IL-7 in T proliferation and differentiation of thymocytes and cytotoxic T cells (37, 72-76) underlines the pleiotropic features of IL-7.

1.5.2 CD20 and CD40 activation in BCP cells

CD20 is an integral membrane protein exclusively expressed on BCP cells and mature B cells, but not plasma cells. No natural ligand is known and its precise function is uncertain, although the structure of CD20 suggests a function as ion channel (88). In support of the latter motion, a role for CD20 in transmembrane Ca^{++} fluxes in B and T cells was demonstrated (89). Activation of CD20 by monoclonal antibodies induces cell cycle progression from G0 to G1, but in synergy with e.g. CD40, CD20 activation elicits a full mitogenic signal in B lymphocytes (90,91).

Differentiation and proliferation of BCP cells is dependent on cell-cell contact, allowing membrane bound ligands to bind to their receptors. CD40 is a receptor already expressed at an early stage of B cell development. Its ligand is expressed on activated T cells (92). Activation of CD40 in normal BCP cells induces proliferation in synergy with other growth stimuli like IL-3, IL-7 or IL-10 (93,94), suggesting a role of activated T cells in B lymphopoiesis (95).

1.5.3 Bone marrow stroma and BCP cells

Proliferation and differentiation of normal murine and human BCP cells is dependent on the bone marrow micro environment. Although stromal cells produce many of the growth factors involved in lymphopoiesis like IL-7, KL and IGF-1, these cytokines cannot completely replace the role of stromal cells (32,46,48,77,78). In vitro culture systems using bone marrow stromal cells showed that the differentiation of murine BCP cells is dependent on direct contact with the stromal cells (79-82). Similar in vitro culture systems for human BCP cells permitted some proliferation, but not differentiation (82-86). Moreover, these cultures of BCP depended on the presence of considerable concentrations of fetal calf serum or horse serum, contaminating the culture system with undefined growth modulatory substances. Only recently a serum free in vitro culture system using human fetal bone marrow has been proposed that allows for differentiation of hematopoietic stem cells to lymphoid cells and differentiation of BCP cells to mature B cells (87).

One of the molecules expressed on bone marrow stroma cells and involved in cell to cell contact is FLK2/FLT3 ligand (FL)(96, 97). Binding of FL to its tyrosine kinase receptor (FLK2/FLT3) induces proliferation of hematopoietic stem cells and committed progenitors (98-102). FL itself, but much more strongly in synergy with IL-7 and KL, supports the proliferation and limited differentiation of B cell progenitors (98, 100,103,104). However, addition of human bone marrow stroma cells greatly enhances both proliferation and differentiation (103), indicating that still other bone marrow stroma components are active in BCP development. The effects of FL on the proliferation of BCP-ALL cells have also been studied to some extend. These experiments revealed a heterogeneous stimulation pattern with induction of DNA synthesis in response to FLT3 activation in a minority of the samples tested. Interestingly in some cases synergy was observed with IL-7, but not with KL, IL-3 or IL-6 (105,106).

Experiments dealing with the role of CD20 and CD40 activation on proliferation of ALL cells in combination with other stimuli are presented in chapter 3.

1.6 The IL-7 receptor complex

HGFs act on cells through binding to specific membrane receptors. Receptors for most HGFs form a superfamily of integral membrane glycoproteins based on structural homologies of the extracellular but not the cytoplasmic domains (107-109). The extracellular domains of members of this family contain in general four highly conserved cystein residues and a five residue motif of Trp-Ser-X-Trp-Ser (WSXWS) within a region of approximately 200 amino acids (110,111) . This region is called the Cytokine Receptor Homology domain (CRH) and is crucial for ligand binding.

The IL-7R is a heteromeric complex, comprising the IL-7R α chain and the common γ (γ_c) chain. IL-7R α chains display a low affinity for IL-7 (112,113). Only in combination with the γ chain, the high affinity binding site is formed (114). The presence of the γ_c chain in the complex is also essential for proliferative signaling (115-117). The γ_c chain is a common receptor chain for multiple receptors, including those for IL-2, IL-4, IL-9 and IL-15.

The human IL-7R α chain is a protein of 459 amino acids with a predicted molecular weight of 75 kD and is expressed on BCP cells, mature and immature T cells and on monocytes and macrophages (112,113,118,119). Alternative splicing can generate three IL-7Rα transcripts, encoding a soluble receptor and two membrane-bound forms: one fully length, the other with an altered, truncated cyotoplasmic domain. Both membrane bound forms bind IL-7 ,but only the full length form is capable of signal transduction (112).

In the intracellular part of certain HGF receptors, conserved stretches of amino acids can be recognized designated respectively boxes 1, 2 and 3 (120,121). Boxes 1 and 2 are involved in mitogenic signaling (120,122-126), while the function of box 3 remains obscure. Moreover, these regions are most likely involved in binding of signaling molecules. The intracellular part of the IL-7R contains the box 1 consensus sequence formed by amino acids 269 to 282. In the IL-7R, box 2 and box 3 consensus sequences are lacking. Furthermore, the cytoplasmic domain of the IL-7R α chain contains three tyrosine residues at positions 401,449 and 456. Phophorylated tyrosine residues form potential recruitment sites for intracellular molecules containing Src homology-2 (SH2) or protein tyrosine binding (PTB) domains (127, 128).

The possibility that ALL cells may overexpress functionally inactive splice variants of the IL-7Rα chain, has been investigated. These experiments are presented in chapter 2.

1.7 Signaling through the IL-7 receptor complex

Like other HGF-Rs, the IL-7R complex does not contain intrinsic tyrosine kinase domains, but recruitment of cytoplasmic protein tyrosine kinases (PTKs) enables rapid tyrosine phosphorylation of a broad set of protein substrates, including the IL-7Rα chain itself (109,129-134). Three major signaling routes generally involved in HGF and cytokine signaling that depend on protein tyrosine phosphorylation have been identified: **1)** the JAK/STAT pathway **2)** the Ras/ MAPK pathway and **3)** the Phosphatidylinositol- 3-kinase (PI3-kinase) pathway. These three pathways have also been found to play a major role in IL-7 signaling (figure 3).

Constitutive association of JAK family members to HGF receptors prior to ligand binding has been demonstrated (135-140). JAK kinases bind to the membrane proximal cytoplasmic region of receptor proteins (137,139,141-143). This membrane proximal region, containing box 1 and box 2 motifs, is essential for mitogenic signaling following receptor activation (124,125,144-147), suggesting a critical role of JAK tyrosine kinase activation in mitogenesis.

In heteromeric receptor complexes the individual receptor chains have different specificity for JAK binding and activation. The IL-7R complex has been shown to activate JAK1 and JAK3 in T cells (148). JAK3 binds to the common γ chain and JAK1 to the IL-7R α chain (148). Activation is thought to occur via cross-phosphorylation of the JAK proteins that are brought in each other's proximity as the result of receptor complex formation (149-151).

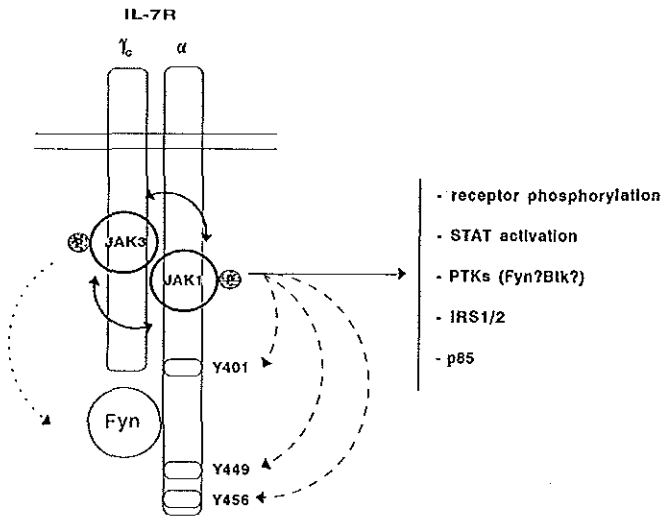

Figure 3a JAK1 and JAK3 phosphotyrosine kinases are activated resulting in the tyrosine phosphorylation of a number of substrates among which the IL-7R complex itself and STAT molecules. Activated STAT molecules form complexes that directly translocate to the nucleus, bind to distinct DNA sequences and activate transcription of specific genes. The activated receptor tyrosines from the IL-7R complex form docking sites for several signaling molecule complexes like PI3-kinase and IRS1/2. The binding domain of IRS1/2 is not exactly known.

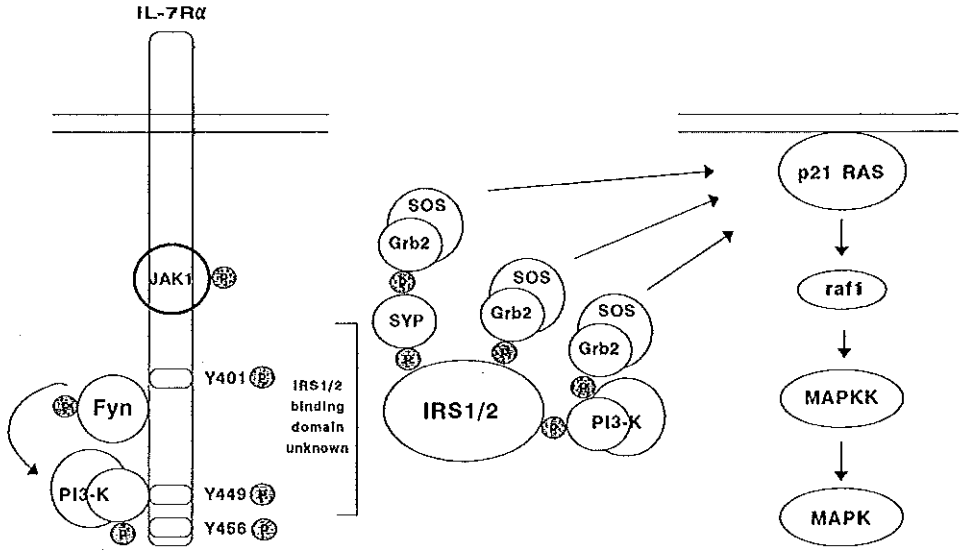

Figure 3b: following receptor binding IRS1/2 becomes phosphorylated on tyrosines and in turn form new docking sites. Grb2/SOS will be activated through binding to IRS1/2 and potentially activate the Ras signaling pathway resulting in activation of gene transcription. PI3-kinase can either be activated through binding to the phosphorylated receptor tyrosine 449 or through binding to the activated IRS1/2 molecule. Activation of IRS1/2 is mos likely terminated by binding of the protein tyrosine phosphatase Syp to IRS1/2, which on itself is ivolved in activation of p21 Ras by recruitment of Grb2/SOS complex.

Following JAK activation, STAT (Signal Transducer and Activator of Transcription) proteins will be tyrosine phosphorylated and form complexes through P-Tyr/SH2 interactions (152,153). STAT complexes will translocate to the nucleus and bind to specific consensus DNA sequences in the promotor regions of cytokine inducible genes. Tyrosine phosphorylation of STAT molecules suffices for STAT dimerization and DNA binding, but to activate transcription the STAT complex needs to be phosphorylated on serine residues as well (154,155). Until now six different STAT family members have been identified and implicated in HGF signaling, but the formation of homo- and heterodimeric STAT complexes may further expand the functional diversity of STATS. Different combinations of STAT molecules are involved in signaling through different cytokine receptors although extensive overlap in STAT activation exists (156). Binding affinity of STAT complexes for specific DNA sequences depends on the STAT complexes formed. Together with the cell-type specific expression and activation of STAT molecules this determines

the response specificity to various cytokines in cells of different origin or in different stages of differentation. Receptor specific STAT activation and complex formation was originally thought to be regulated by the specific activation of the JAK/Tyk kinase family (153,157,158). Recently however it was suggested that specificity of activation of STAT molecules is largely determined by recruitment to modular tyrosine based motifs in the signal-transducing receptor components (142,159-164).

Other substrates for at least two members of the JAK kinase family (JAK1 and JAK3) are insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) and PI3-kinase . The JAK kinases may physically bind to IRS1/2 and possibly play a role in tyrosine phosphorylation of these molecules (165,166).

IRS-1 is a 130 KDa protein that is activated upon stimulation of the insulin receptor or IGF-1-R. Activation of IRS-1/2 also plays a crucial role in signaling via the IL-2R, IL4-R, IL-7R, IL-9R and the IL-15R (165,167). IRS-1/2 contains 18 potential sites for tyrosine phosphorylation from which at least 6 sites serve as sites for SH2 domain containing proteins. IRS-1/2 has an ATP binding site that is homologous to the ATP binding site in the kinase domain of JAK1 but does not have kinase activity itself. Upon receptor activation, IRS-1/2 is directly phosphorylated and serves as a docking molecule for SH2 domain containing proteins, e.g. kinases, phosphatases like Syp and other docking molecules like Nck. In addition, IRS-1/2 binds the Ras pathway activating complex Grb2/SOS and the p85 subunit of PI3-kinase. The binding of the p85 subunit of PI3 kinase and the Grb2/SOS complex results in activation of both complexes.

A second major pathway involved in cytokine receptor signaling is the p21 Ras dependent MAPK (Mitogen Activated Protein Kinase) pathway (168-175). Ras is a GTPase and has a critical role in proliferation control. With the activation of Ras a phosphorylation cascade of molecules participating in the Ras signaling chain is started, resulting in the phosphorylation of MAPK. Activated MAPK translocates to the nucleus and activates transcription either direct or indirect by activation of nuclear transcription factors.

Following receptor activation phosphorylated tyrosine residues can interact with the Src homology 2 (SH2) adaptor protein Shc, which in turn will become tyrosine phosphorylated (176-179). The Grb2-SOS complex connects with phosphorylated Shc, upon which the catalytic acitvity of SOS becomes activated and in turn activates Ras by ex-changing GDP for GTP (176,179-181). However, IL-7 induces MAPK activation independent of Shc (182), suggesting the use of alternative MAPK activation pathways e.g. via PI3-kinase or IRS-1/2 (183-186). Alternatively, JAK1 and JAK2 have been found directly associated with Grb2/SOS, offering another potential Shc independent mechanism of Ras activation (187-189).

PI3-kinase is a protein complex that comprises a p85 regulatory and p110 katalytic subunit. PI3-kinase is as a serine/threonine kinase involved in phospholipid metabolism and most likely in mitogenic signaling. Importantly, PI3-kinase is activated in response to IL-7 and coupled to the IL-7R, either directly via association with the IL-7R alpha chain (190-192), indirectly via interaction with the Src kinase $p59^{fyn}$, via both mechanisms (193-195). For association of PI3-kinase to the IL-7Rα, phosphorylation of tyrosine 449 (Y449) of the IL-7Rα chain was shown to be indispensible (40). Both prevention of tyrosine phosphorylation and substitution of Y449 for phenylalanine completely inhibited PI3-kinase activity and proliferation (40). The role of PI3-kinase in proliferative signaling in B cells is further demonstrated by the complete block of IL-7 induced proliferation by the specific PI3-kinase inhibitor Wortmannin (40,196). Moreover, IL-7 induced proliferation is completely inhibited in cells bearing the IL-7Rα/Y449F mutant, but the capacity to rearrange the Ig_H chain gene leading to B cell differentiation was preserved, suggesting that differentiation occurs independently of PI3-kinase activation (40).

The role of $p59^{fyn}$ in B cell development is still unclear. The cytoplasmic tail of the IL-7R recruits $p59^{fyn}$ constitutively in a SH2 independent way (194). PI3 kinase binds to the IL-7R associated $p59^{fyn}$ through its SH3 domain (193,197,198). Upon activation of the IL-7R, both PI3-kinase and $P59^{fyn}$ become activated as well (193). However, mice lacking $p59^{fyn}$ by targeted disruption of the coding gene, show normal B cell development and B cell mediated immune responses are not affected in these animals (199). In addition, normal numbers of functional peripheral T cells are generated, implicating that T cell development is not strictly Fyn dependent (199,200).

In different cell types the association of Grb2 and SOS to PI3 kinase was demonstrated, suggesting possible activation of the Ras pathway (183,201-204). Illustrative for the complex nature of these signaling routes is the observation that the reverse activation route was demonstrated as well: PI3-kinase activation by Ras (184,185,205).

Defects in downstream signaling events might contribute to loss of mitogenic control and inability to differentiate. To investigate normal signaling properties of the single human IL-7R α chain we constructed a chimeric receptor containing the cytoplasmic and transmembrane part of the IL-7R and the extracellular part of the human G-CSFR.

Lack of mitogenic response in a subset of BCP-ALL patients was shown not to be due to defective expression of members of the IL-7R complex. We therefore investigated signaling routes after activation of the both the natural IL-7R and the chimeric receptors. Results and implications are discussed in chapters 4 and 5.

1.8 Relevance of in vitro proliferation in clinical practice of acute leukemias

The biological significance for HGFs in normal hematopoiesis is well established. In leukemia control over proliferation and differentiation is lost. This might result in or be the result of aberrant responses to HGFs. In vitro proliferation assays have been used to study the responses of leukemic cells to various HGFs. In acute myeloid leukemia (AML), in vitro proliferation in absence of HGFs is an unfavorable prognostic factor for relapse after remission (206).

Proliferation and differentiation of B cell precursor cells in vivo require presence of HGFs. In vitro proliferation responses of acute lymphoblastic leukemia cells in absence or presence of cytokines are highly heterogeneous. In chapter 6 we have attempted to correlate in vitro HGF responses of ALL to clinical outcome.

References

1. Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575.
2. Alt F, Blackwell T, DePinho R, Reth M, Yancopoulos G. Regulation of genome rearrangement events during lymphocyte development. Immunol.Rev. 1986;89:5.
3. Melchers F, Karasuyama H, Haasner D, et al. The surrogate light chain in B-cell development. Immunology Today 1993;14(2):60-68.
4. Usuda S, Takemori T, Matsuoka M, et al. Immunoglobulin V gene replacement is caused by the intramolecular DNA deletion mechanism. EMBO J 1992;11(2):611-618.
5. Ichihara Y, Hayashida H, Miyazawa S, Kurosawa Y. Only D_{FL16}/D_{SP2}, and D_{Q52} gene families exist in mouse immunoglobulin heavy chain diversity gene loci, of which D_{FL16} and D_{SP2} originate from the same primordial D_H gene. Eur J Immunol 1989;19:1849-1854.
6. Gu H, Kitamura D, Rajewsky K. B cell development regulated by gene rearrangement: arrest of maturation by membrane-bound D mu protein and selection of D_H element reading frames. Cell 1991;65(1):47-54.
7. Reth M, Alt F. Novel immunoglobulin heavy chains are produced from DJ_H gene segment rearrangements in lymphoid cells. Nature 1984;312:418-423.
8. Alt F, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implication from a chromosome with evidence of three D-JH fusions. Proc.Natl.Acad.Sci USA 1982;79:4118.
9. Alt F, Yancopoulos G, Blackwell T, et al. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J 1984;3:1209.
10. Yancopoulos G, Alt F. Regulation of the assembly and expression of variable-region genes. Annu.Rev.Immunol 1986;4:339.
11. Reth M, Ammirati P, Jackson S, Alt F. Regulated progression of a cultured pre-B-cell line to the B-cell lineage. Nature 1985;317:353-355.
12. Nussenzweig M, Shaw A, Sinn E, et al. Allelic exclusion in transgenic mice that express the membrane form of Immunoglobulin μ. Science 1987;236:816-819.
13. Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature 1991;350:423-426.
14. Karasuyama H, Kudo A, Melchers F. The proteins encoded by th V preB and lambda5 pre-B specific genes can associate with each other and with μ heavy chain. J Exp Med 1990;172:969-972.
15. Nishimoto N, Kubagawa H, Ohno T, Gartland G, Stankovic A, Cooper M. Normal pre-B cells express a receptor complex of μ heavy chains and surrogate light-chain proteins. Proc.Natl.Acad. Sci.USA 1991;88:6284-6288.
16. Iglesias A, Kopf M, Williams G, Buhler B, Köhler G. Molecular requirements for the μ-induced light chain gene rearrangement in pre-B cells. EMBO J 1991;10(8):2147-2156.
17. Tsubata T, Tsubata R, Reth M. Crosslinking of the cell surface immunoglobulin (μ-surrogate light chain complex) on pre-B cells induces activation of V gene rearrangements at the immunoglobulin k locus. Int Immunol 1992;4:637-641.
18. Shapiro A, Schlissel M, Baltimore D, DeFranco A. Stimulation of k light chain gene rearrangement by the immunoglobulin μ heavy chain in a pre-B cell line. Mol Cell Biol 1993;13(9):5679-5690.
19. Kitamura D, Rajewsky K. Targeted disruption ofμ chain membrane exon causes loss of heavy-chain allelic exclusion. Nature 1992;356:154-156.

20. Ehlich A, Schaal S, Gu H, Kitamura D, Muller W, Rajewsky K. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. Cell 1993;72(5):695-704.
21. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med 1991;173(5):1213-25.
22. Faust EA, Saffran DC, Toksoz D, Williams DA, Witte ON. Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from pre-B cells. J Exp Med 1993;177(4): 915-23.
23. Era T, Ogawa M, Nishikawa S, et al. Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J 1991;10(2):337-42.
24. Pui C-H, Behm F, Crist W. Clinical and biological relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. Blood 1993;82(2):343-362.
25. Crist W, Boyett J, Jackson J, et al. Prognostic significance of the pre-B cell immunophenotype and other presenting features in B lineage childhood acute lymphoblastic leukemia. Blood 1989;74:1252-1259.
26. Basso G, Rondelli R, Covezzoli A, Putti M. The role of immunophenotype in acute lymphoblastic leukemia of infant age. Leukemia & Lymphoma 1994;15(1-2):51-60.
27. Dongen JJ van, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33(5-6):298-314.
28. Beishuizen A, Hählen K, Hagemeijer A, et al. Multiple rearranged immunoglobulin genes in childhood acute lymphoblastic leukemia of precursor B-cell origin. Leukemia 1991;5:657-667.
29. Korsmeyer S, Arnold A, Bakhshi A. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T and B cell precursor origins. J Clin Invest 1983;71:301-313.
30. Dongen JJ van, Wolvers-Tettero I. Analysis of immunoglobulin and T cell receptor genes. Part II: possibilities and limitations in the diagnosis and managemement of lymphoproliferative diseases and related disorders. Clin Chim Acta 1991;198:93-174.
31. Felix C, Poplack D, Reaman G. Characterization of immunoglobulin and T-cell receptor gene patterns in B-cell precursor acute lymphoblastic leukemia of childhood. J Clin Oncol 1990;8:431-442.
32. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 1988;333(6173):571-3.
33. Saeland S, Duvert V, Pandrau D, et al. Interleukin-7 induces the proliferation of normal human B-cell precursors. Blood 1991;78(9):2229-38.
34. Skjonsberg C, Erikstein BK, Smeland EB, et al. Interleukin-7 differentiates a subgroup of acute lymphoblastic leukemias. Blood 1991;77(11):2445-50.
35. Touw I, Pouwels K, van Agthoven T, et al. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 1990;75(11):2097-101.
36. Smiers F, Paassen Mv, Pouwels K, et al. Heterogeneity of proliferative responses of human B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells to interleukin 7 (IL-7): no correlation with immunoglobulin gene status and expression of IL-7 receptor or IL-2/IL-4/IL-7 receptor common gamma chain genes. Leukemia 1995;9:1039-1045.

37. Muegge K, Vila MP, Durum SK. Interleukin-7: a cofactor for V(D)J rearrangement of the T cell receptor beta gene. Science 1993;261(5117):93-5.
38. Maki K, Sunaga S, Komagata Y, et al. Interleukin 7 receptor-deficient mice lack gammadelta T cells. Proc Natl Acad Sci U S A 1996;93(14):7172-7.
39. He YW, Malek TR. Interleukin-7 receptor alpha is essential for the development of gamma delta + T cells, but not natural killer cells. J Exp Med 1996;184(1):289-93.
40. Corcoran AE, Smart FM, Cowling RJ, Crompton T, Owen MJ, Venkitaraman AR. The interleukin-7 receptor alpha chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. EMBO J 1996;15(8):1924-32.
41. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J Immunol 1989;142(11):3875-83.
42. Palacios R, Nishikawa S. Developmentally regulated cell surface expression and function of c-kit receptor during lymphocyte ontogeny in the embryo and adult mice. Development 1992;115(4):1133-47.
43. Rolink A, Kudo A, Karasuyama H, Kikuchi Y, Melchers F. Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J 1991;10(2):327-36.
44. Hayashi S, Kunisada T, Ogawa M, et al. Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J Exp Med 1990;171(5):1683-95.
45. Funk PE, Varas A, Witte PL. Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. J Immunol 1993;150(3):748-52.
46. McNiece IK, Langley KE, Zsebo KM. The role of recombinant stem cell factor in early B cell development. Synergistic interaction with IL-7. J Immunol 1991;146(11):3785-90.
47. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood 1992;80(5):1207-12.
48. Gibson LF, Piktel D, Landreth KS. Insulin-like growth factor-1 potentiates expansion of interleukin-7-dependent pro-B cells. Blood 1993;82(10):3005-11.
49. Wormann B, Gesner TG, Mufson RA, LeBien TW. Proliferative effect of interleukin-3 on normal and leukemic human B cell precursors. Leukemia 1989;3(6):399-404.
50. Touw I, Delwel R, van Zanen G, Löwenberg B. Acute lymphoblastic leukemia and non-Hodgkin's lymphoma of T lineage: colony-forming cells retain growth factor (interleukin 2) dependence. Blood 1986;68(5):1088-94.
51. Touw I, Delwel R, Bolhuis R, van Zanen G, Löwenberg B. Common and pre-B acute lymphoblastic leukemia cells express interleukin 2 receptors, and interleukin 2 stimulates in vitro colony formation. Blood 1985;66(3):556-61.
52. Pandrau D, Saeland S, Duvert V, et al. Interleukin 4 inhibits in vitro proliferation of leukemic and normal human B cell precursors. J Clin Invest 1992;90(5):1697-706.
53. Okabe M, Kuni-eda Y, Sugiwura T, et al. Inhibitory effect of interleukin-4 on the in vitro growth of Ph1-positive acute lymphoblastic leukemia cells. Blood 1991;78(6):1574-80.
54. Banchereau J, Rousset F. Growing human B lymphocytes in the CD40 system. Nature 1991;353(6345):678-9.

55. Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science 1991;251(4989):70-2.
56. Kehrl J, Roberts A, Wakefield L, Jakowlew S, Sporn M, Fauci A. Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. J Immunol 1986;137:3855-3862.
57. Smeland E, Blomhoff H, Holte H, et al. Transforming growth factor β (TGFβ) inhibits G1 to S transition, but not activation of human B lymphocytes. Exp Cell Res 1987;171:213.
58. Rehman J, LeBien T. Transforming growth factor-β regulates normal human pre-B cell differentiation. Int Immunol 1994;6(2):315-322.
59. Kehrl J, Taylor A, Delsing G, Roberts A, Sporn M, Fauci A. Further studies of the role of transforming growth factor β in human B cell function. J Immunol 1989;143:1868.
60. Rich BE, Campos-Torres J, Tepper RI, Moreadith RW, Leder P. Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J Exp Med 1993;177(2):305-16.
61. Overell RW, Clark L, Lynch D, et al. Interleukin-7 retroviruses transform pre-B cells by an autocrine mechanism not evident in Abelson murine. Mol Cell Biol 1991;11(3):1590-7.
62. Kurzrock R, Kantarjian H, Wetzler M, et al. Ubiquitous expression of cytokines in diverse leukemias of lymphoid and myeloid lineage. Exp Hematol 1993;21(1):80-5.
63. Benjamin D, Sharma V, Knobloch TJ, Armitage RJ, Dayton MA, Goodwin RG. B cell IL-7. Human B cell lines constitutively secrete IL-7 and express IL-7 receptors. J Immunol 1994;152(10):4749-57.
64. Benjamin D, Park CD, Sharma V. Human B cell interleukin 10. [Review]. Leuk Lymphoma 1994;12(3-4):205-10.
65. Morrow MA, Lee G, Gillis S, Yancopoulos GD, Alt FW. Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes. Genes Dev 1992;6(1):61-70.
66. Smith RK, Zimmerman K, Yancopoulos GD, Ma A, Alt FW. Transcriptional down-regulation of N-myc expression during B-cell development. Mol Cell Biol 1992;12(4):1578-84.
67. Billips LG, Nunez CA, Bertrand F3, et al. Immunoglobulin recombinase gene activity is modulated reciprocally by interleukin 7 and CD19 in B cell progenitors. J Exp Med 1995;182(4):973-82.
68. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 1994;180(5):1955-60.
69. Freeden-Jeffry Uv, Vieira P, Lucian L, McNeil T, Burdach S, Murray R. Lymphopenia in interleukin(IL-)7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 1995;181:1519-1526.
70. Grabstein K, Waldschmidt T, Finkelman F, et al. Inhibition of murine B and T lymphopoiesis in vivo by anti-interleukin 7 monoclonal antibody. J Exp Med 1993;178:257-264.
71. Sudo T, Nishikawa S, Ohno N, et al. Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc Natl Acad Sci U S A 1993;90(19):9125-9.
72. Herbelin A, Machavoine F, Schneider E, Papiernik M, Dy M. IL-7 is requisite for IL-1-induced thymocyte proliferation. Involvement of IL-7 in the synergistic effects of granulocyte-macrophage colony-stimulating factor or tumor necrosis factor with IL-1. J Immunol 1992;148(1):99-105.
73. Okazaki H, Ito M, Sudo T, et al. IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing alpha beta or gamma delta T-cell receptors in vitro: synergism with IL-2. J Immunol 1989;143(9):2917-22.

74. Watson JD, Morrissey PJ, Namen AE, Conlon PJ, Widmer MB. Effect of IL-7 on the growth of fetal thymocytes in culture. J Immunol 1989;143(4):1215-22.
75. Lynch DH, Miller RE. Interleukin 7 promotes long-term in vitro growth of antitumor cytotoxic T lymphocytes with immunotherapeutic efficacy in vivo.J Exp Med 1994;179(1):31-42.
76. Tsuda S, Rieke S, Hashimoto Y, Nakauchi H, Takahama Y. IL-7 supports D-J but not V-DJ rearrangement of TCR-β gene in fetal liver progenitor cells. J Immunol 1996;156:3233-3242.
77. Namen AE, Schmierer AE, March CJ, et al. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. J Exp Med 1988;167(3):988-1002.
78. Billips LG, Petitte D, Dorshkind K, Narayanan R, Chiu CP, Landreth KS. Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis. Blood 1992;79(5):1185-92.
79. Ryan DH, Nuccie BL, Abboud CN, Liesveld JL. Maturation-dependent adhesion of human B cell precursors to the bone marrow microenvironment. J Immunol 1990;145(2):477-84.
80. Ryan DH, Nuccie BL, Abboud CN. Inhibition of human bone marrow lymphoid progenitor colonies by antibodies to VLA integrins. J Immunol 1992;149(11):3759-64.
81. Miyake K, Weissman IL, Greenberger JS, Kincade PW. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J Exp Med 1991;173(3):599-607.
82. Manabe A, Murti KG, Coustan-Smith E, et al. Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells. Blood 1994;83(3):758-66.
83. Manabe A, Yi T, Kumagai M, Campana D. Use of stroma-supported cultures of leukemic cells to assess antileukemic drugs. I. Cytotoxicity of interferon alpha in acute lymphoblastic leukemia. Leukemia 1993;7(12):1990-5.
84. Moreau I, Duvert V, Caux C, et al. Myofibroblastic stromal cells isolated from human bone marrow induce the proliferation of both early myeloid and B-lymphoid cells. Blood 1993;82(8):2396-405.
85. Rawlings DJ, Quan SG, Kato RM, Witte ON. Long-term culture system for selective growth of human B-cell progenitors. Proc Natl Acad Sci U S A 1995;92(5):1570-4.
86. McGinnes K, Quesniaux V, Hitzler J, Paige C. Human B-lymphopoiesis is supported by bone marrow-derived stromal cells. Exp Hematol 1991;19(4):294-303.
87. Pribyl J, LeBien T. Interleukin 7 independent development of human B cells. Proc.Natl.Acad.Sci.USA 1996;93:10348-10353.
88. Tedder TF, Schlossman SF. Phosphorylation of the B1 (CD20) molecule by normal and malignant human B lymphocytes. J Biol Chem 1988;263(20):10009-15.
89. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. J Cell Biol 1993;121(5):1121-32. 90.Gordon J, Millsum MJ, Guy GR, Ledbetter JA. Synergistic interaction between interleukin 4 and anti-Bp50 (CDw40) revealed in a novel B cell restimulation assay. Eur J Immunol 1987;17(10):1535-8.
91. Ledbetter JA, Shu G, Gallagher M, Clark EA. Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen BP50 (CDW40). J Immunol 1987;138(3):788-94.

92. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. Nature 1992;357(6373):80-2.
93. Larson AW, LeBien TW. Cross-linking CD40 on human B cell precursors inhibits or enhances growth depending on the stage of development and the IL costimulus. J Immunol 1994;153(2): 584-94.
94. Saeland S, Duvert V, Moreau I, Banchereau J. Human B cell precursors proliferate and express CD23 after CD40 ligation. J Exp Med 1993;178(1):113-20.
95. Renard N, Duvert V, Blanchard D, Banchereau J, Saeland S. Activated CD4+ T cells induce CD40-dependent proliferation of human B cell precursors. J Immunology 1994;152:1693-1701.
96. Hannum C, Culpepper J, Campbell D, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature 1994;368(6472):643-8.
97. Lyman SD, James L, Escobar S, et al. Identification of soluble and membrane- bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs. Oncogene 1995;10(1):149-57.
98. Lyman SD, James L, Vanden Bos T, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell 1993;75(6):1157-67.
99. Muench MO, Roncarolo MG, Menon S, et al. FLK-2/FLT-3 ligand regulates the growth of early myeloid progenitors isolated from human fetal liver. Blood 1995;85(4):963-72.
100. Hirayama F, Lyman SD, Clark SC, Ogawa M. The flt3 ligand supports proliferation of lymphohematopoietic progenitors and early B-lymphoid progenitors. Blood 1995;85(7):1762-8.
101. Jacobsen SE, Okkenhaug C, Myklebust J, Veiby OP, Lyman SD. The FLT3 ligand potently and directly stimulates the growth and expansion of primitive murine bone marrow progenitor cells in vitro: synergistic interactions with interleukin (IL) 11, IL-12, and other hematopoietic growth factors. J Exp Med 1995;181(4):1357-63.
102. McKenna HJ, de Vries P, Brasel K, Lyman SD, Williams DE. Effect of flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. Blood 1995;86(9):3413-20.
103. Namikawa, R MM de Vries JE,Roncarolo M. The FLK2/FLT3 ligand synergizes with interleukin-7 in promoting stromal cell independent expansion and differentation of human fetal pro-b cells in vitro. Blood 1996;87(5):181-1890.
104. Ray RJ, Paige CJ, Furlonger C, Lyman SD, Rottapel R. Flt3 ligand supports the differentiation of early B cell progenitors in the presence of interleukin-11 and interleukin-7. Eur J Immunol 1996;26(7):1504-10.
105. Eder M, Hemmati P, Kalina U, et al. Effects of Flt3 ligand and interleukin-7 on in vitro growth of acute lymphoblastic leukemia cells. Experimental Hematology 1996;24:371-377.
106. McKenna H, Smith F, Brasel K, et al. Effects of flt3 ligand on acute myeloid and lymphocytic leukemic blast cells from children. Experimental Hematology 1996;24:378-385.
107. Budel L, Dong F, Löwenberg B, Touw I. Hematopoietic growth factor receptors: structure variations and alternatives of receptor complex formation in normal hematopoiesis and in hematopoietic disorders. [Review]. Leukemia 1995;9(4):553-61.
108. Heldin CH. Dimerization of cell surface receptors in signal transduction. [Review]. Cell 1995;80(2):213-23.

109. Miyajima A, Kitamura T, Harada N, Yokota T, Arai K. Cytokine receptors and signal transduction. [Review]. Annu Rev Immunol 1992;10:295-331.
110. Bazan JF. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. Biochem Biophys Res Commun 1989;164(2):788-95.
111. Cosman D. The hematopoietin receptor superfamily. [Review]. Cytokine 1993; 5(2):95-106.
112. Goodwin RG, Friend D, Ziegler SF, et al. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell 1990;60(6):941-51.
113. Park LS, Friend DJ, Schmierer AE, Dower SK, Namen AE. Murine interleukin 7 (IL-7) receptor. Characterization on an IL-7-dependent cell line. J Exp Med 1990;171(4):1073-89.
114. Kondo M, Takeshita T, Ishii N, et al. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4 [see comments]. Science 1993;262(5141):1874-7.
115. Kawahara A, Minami Y, Taniguchi T. Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2, IL-4, and IL-7 signalling. Mol Cell Biol 1994;14(8):5433-40.
116. Nakamura Y, Russell SM, Mess SA, et al. Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. Nature 1994;369(6478):330-3.
117. Nelson BH, Lord JD, Greenberg PD. Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. Nature 1994;369(6478):333-6.
118. Jacobsen FW, Rusten LS, Jacobsen SE. Direct synergistic effects of interleukin-7 on in vitro myelopoiesis of human CD34+ bone marrow progenitors. Blood 1994;84(3):775-9.
119. Jacobsen FW, Veiby OP, Jacobsen SE. IL-7 stimulates CSF-induced proliferation of murine bone marrow macrophages and Mac-1+ myeloid progenitors in vitro. J Immunol 1994;153(1):270-6.
120. Murakami M, Narazaki M, Hibi M, et al. Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc Natl Acad Sci U S A 1991;88(24):11349-53.
121. O'Neal KD, Yu-Lee LY. The proline-rich motif (PRM): a novel feature of the cytokine/hematopoietin receptor superfamily. Lymphokine Cytokine Res 1993; 12(5):309-12.
122. D'Andrea AD, Yoshimura A, Youssoufian H, Zon LI, Koo JW, Lodish HF. The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. Mol Cell Biol 1991;11(4):1980-7.
123. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y, Nagata S. Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J 1991;10(10):2855-65.
124. He TC, Jiang N, Zhuang H, Quelle DE, Wojchowski DM. The extended box 2 subdomain of erythropoietin receptor is nonessential for Jak2 activation yet critical for efficient mitogenesis in FDC-ER cells. J Biol Chem 1994;269(28):18291-4.
125. Howard OM, Kirken RA, Garcia GG, Hackett RH, Farrar WL. Structural domains of interleukin-2 receptor beta critical for signal transduction: kinase association and nuclear complex-formation. Biochem J 1995;306(Pt 1):217-24.
126. Miura O, Cleveland JL, Ihle JN. Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. Mol Cell Biol 1993;13(3):1788-95.

127. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 1991;252(5006):668-74.
128. Mayer BJ, Jackson PK, Van Etten RA, Baltimore D. Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo. Mol Cell Biol 1992;12(2):609-18.
129. Isfort RJ, Ihle JN. Multiple hematopoietic growth factors signal through tyrosine phosphorylation. Growth Factors 1990;2(2-3):213-20.
130. Kanakura Y, Druker B, Cannistra SA, Furukawa Y, Torimoto Y, Griffin JD. Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors involves tyrosine phosphorylation of a common set of cytoplasmic proteins. Blood 1990;76(4):706-15.
131. Mills GB, May C, McGill M, et al. Interleukin 2-induced tyrosine phosphorylation. Interleukin 2 receptor beta is tyrosine phosphorylated. J Biol Chem 1990; 265(6):3561-7.
132. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. [Review]. Cell 1990;61(2):203-12.
133. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. [Review]. Cell 1994;76(2):253-62.
134. White MF, Shoelson SE, Keutmann H, Kahn CR. A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J Biol Chem 1988;263(6):2969-80.
135. Yin T, Tsang ML, Yang YC. JAK1 kinase forms complexes with interleukin-4 receptor and 4PS/insulin receptor substrate-1-like protein and is activated by interleukin-4 and interleukin-9 in T lymphocytes. J Biol Chem 1994;269(43):26614-7.
136. Russell SM, Johnston JA, Noguchi M, et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science 1994; 266(5187):1042-5.
137. Witthuhn BA, Quelle FW, Silvennoinen O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 1993;74(2):227-36.
138. Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF, Layton JE. Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation. Proc Natl Acad Sci U S A 1994;91(8):2985-8.
139. Quelle FW, Sato N, Witthuhn BA, et al. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol Cell Biol 1994;14(7):4335-41.
140. Rolling C, Treton D, Beckmann P, Galanaud P, Richard Y. JAK3 associates with the human interleukin 4 receptor and is tyrosine phosphorylated following receptor triggering. Oncogene 1995;10(9):1757-61.
141. Narazaki M, Witthuhn BA, Yoshida K, et al. Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc Natl Acad Sci U S A 1994; 91(6):2285-9.
142. Stahl N, Boulton TG, Farruggella T, et al. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science 1994;263(5143):92-5.
143. Tanner JW, Chen W, Young RL, Longmore GD, Shaw AS. The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. J Biol Chem 1995;270(12):6523-30.

144. Goldsmith MA, Xu W, Amaral MC, Kuczek ES, Greene WC. The cytoplasmic domain of the interleukin-2 receptor beta chain contains both unique and functionally redundant signal transduction elements. J Biol Chem 1994;269(20):14698-704.
145. Kuno K, Okamoto S, Hirose K, Murakami S, Matsushima K. Structure and function of the intracellular portion of the mouse interleukin 1 receptor (type I). Determining the essential region for transducing signals to activate the interleukin 8 gene. J Biol Chem 1993;268(18):13510-8.
146. DaSilva L, Howard OM, Rui H, Kirken RA, Farrar WL. Growth signaling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptors. J Biol Chem 1994;269(28):18267-70.
147. Dong F, van Buitenen C, Pouwels K, Hoefsloot L, Löwenberg B, Touw I. Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. Mol Cell Biol 1993;13(12):7774-81.
148. Johnston JA, Kawamura M, Kirken RA, et al. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature 1994;370(6485):151-3.
149. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 1990;61:203-212.
150. Ihle J, Kerr I. Jaks and Stats in signaling by the cytokine receptor superfamily. TIG 1995;11(2):69-74.
151. Ziemiecki A, Harpur A, Wilks A. Jak protein tyrosine kinases: their role in cytokine signaling. Trends in Cell Biology 1994;4:207-212.
152. Darnell J Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. [Review]. Science 1994;264(5164):1415-21.
153. Shual K, Ziemiecki A, Wilks AF, et al. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature 1993;366 (6455):580-3.
154. Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science 1995;267 (5206):1990-4.
155. Wen z. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241-250.
156. Lin JX, Migone TS, Tsang M, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 1995;2(4):331-9.
157. Ihle JN, Witthuhn BA, Quelle FW, et al. Signaling by the cytokine receptor superfamily: JAKs and STATs. [Review]. Trends Biochem Sci 1994;19(5):222-7.
158. Silvennoinen O, Ihle JN, Schlessinger J, Levy DE. Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 1993;366(6455):583-5.
159. Stahl N, Yancopoulos GD. The alphas, betas, and kinases of cytokine receptor complexes. [Review]. Cell 1993;74(4):587-90.
160. Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell J Jr., Yancopoulos GD. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science 1995;267(5202):1349-53.
161. Heim MH, Kerr IM, Stark GR, Darnell J Jr. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. Science 1995;267 (5202):1347-9.

162. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J 1994;13(7):1591-600.
163. Yi T, Zhang J, Miura O, Ihle JN. Hematopoietic cell phosphatase associates with erythropoietin (Epo) receptor after Epo-induced receptor tyrosine phosphorylation: identification of potential binding sites. Blood 1995;85(1):87-95.
164. Klingmuller U, Lorenz U, Cantley LC, Neel BG, Lodish HF. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell 1995;80(5):729-38.
165. Johnston JA, Wang LM, Hanson EP, et al. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases. J Biol Chem 1995;270(48):28527-30.
166. Sharfe N, Dadi H, Roifman C. Jak3 protein tyrosine kinase mediates interleukin-7-induced activation of phosphoinositol-3'kinase. Blood 1995;86(6):2077-2085.
167. Yin T, Keller SR, Quelle FW, et al. Interleukin-9 induces tyrosine phosphorylation of insulin receptor substrate-1 via JAK tyrosine kinases. J Biol Chem 1995;270(35):20497-502.
168. Feig LA. The many roads that lead to Ras [comment]. [Review]. Science 1993;260(5109):767-8.
169. Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J, Bar-Sagi D. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras [see comments]. Nature 1993;363(6424):88-92.
170. Cutler RL, Liu L, Damen JE, Krystal G. Multiple cytokines induce the tyrosine phosphorylation of Shc and its association with Grb2 in hemopoietic cells. J Biol Chem 1993;268(29):21463-5.
171. Damen JE, Liu L, Cutler RL, Krystal G. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. Blood 1993;82(8):2296-303.
172. Satoh T, Nakafuku M, Miyajima A, Kaziro Y. Involvement of ras p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor, but not from interleukin 4. Proc Natl Acad Sci U S A 1991;88(8):3314-8.
173. Dorsch M, Hock H, Diamantstein T. Tyrosine phosphorylation of Shc is induced by IL-3, IL-5 and GM-CSF. Biochem Biophys Res Commun 1994;200(1):562-8.
174. Miura Y, Miura O, Ihle JN, Aoki N. Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. J Biol Chem 1994;269(47):29962-9.
175. Duronio V, Welham MJ, Abraham S, Dryden P, Schrader JW. p21ras activation via hemopoietin receptors and c-kit requires tyrosine kinase activity but not tyrosine phosphorylation of p21ras GTPase-activating protein. Proc Natl Acad Sci U S A 1992;89(5):1587-91.
176. Buday L, Downward J. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 1993;73(3):611-20.
177. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1 [see comments]. Nature 1993;363(6424):83-5.

178. Sasaoka T, Draznin B, Leitner JW, Langlois WJ, Olefsky JM. Shc is the predominant signaling molecule coupling insulin receptors to activation of guanine nucleotide releasing factor and p21ras-GTP formation. J Biol Chem 1994;269(14):10734-8.
179. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation [see comments]. Nature 1993;363(6424):45-51.
180. Li N, Batzer A, Daly R, et al. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling [see comments]. Nature 1993;363(6424):85-8.
181. Lowenstein EJ, Daly RJ, Batzer AG, et al. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 1992;70(3):431-42.
182. Dorsch M, Hock H, Diamantstein T. Gene transfer of the interleukin (IL)-2 receptor beta chain into an IL-7-dependent pre-B cell line permits IL-2-driven proliferation: tyrosine phosphorylation of Shc is induced by IL-2 but not IL-7. Eur J Immunol 1994;24(9):2049-54.
183. Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. Science 1995;268(5207):100-2.
184. Rodriguez-Viciana P, Warne PH, Dhand R, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras [see comments]. Nature 1994;370(6490):527-32.
185. Kodaki T, Woscholski R, Hallberg B, Rodriguez-Viciana P, Downward J, Parker PJ. The activation of phosphatidylinositol 3-kinase by Ras. Curr Biol 1994;4(9):798-806.
186. Hara K, Yonezawa K, Sakaue H, et al. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc Natl Acad Sci U S A 1994;91(16):7415-9.
187. Giorgetti-Peraldi S, Peyrade F, Baron V, Van Obberghen E. Involvement of Janus kinases in the insulin signaling pathway. Eur J Biochem 1995;234(2):656-60.
188. Chauhan D, Kharbanda SM, Ogata A, et al. Oncostatin M induces association of Grb2 with Janus kinase JAK2 in multiple myeloma cells. J Exp Med 1995;182 (6):1801-6.
189. Wang XY, Fuhrer DK, Marshall MS, Yang YC. Interleukin-11 induces complex formation of Grb2, Fyn, and JAK2 in 3T3L1 cells. J Biol Chem 1995;270(47): 27999-8002.
190. Venkitaraman AR, Cowling RJ. Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. Eur J Immunol 1994;24(9):2168-74.
191. Dadi HK, Roifman CM. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. J Clin Invest 1993;92(3):1559-63.
192. Dadi HK, Ke S, Roifman CM. Interleukin 7 receptor mediates the activation of phosphatidylinositol-3 kinase in human B-cell precursors. Biochem Biophys Res Commun 1993;192(2):459-64.
193. Seckinger P, Fougereau M. Activation of src family kinases in human pre-B cells by IL-7. J Immunol 1994;153(1):97-109.
194. Venkitaraman AR, Cowling RJ. Interleukin 7 receptor functions by recruiting the tyrosine kinase p59fyn through a segment of its cytoplasmic tail. Proc Natl Acad Sci U S A 1992;89(24):12083-7.
195. Pleiman CM, Hertz WM, Cambier JC. Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. Science 1994;263(5153):1609-12.

196. Aagaard-Tillery K, Jelinek D. Phosphatidylinositol 3-kinase activation in normal human B lymphocytes. J Immunol 1996;156(12):4543-4554.
197. Karnitz L, Sutor S, Abraham R. The Src-family kinase,Fyn,regulates the activation of phosphatidylinositol 3-kinase in an interleukin 2-responsive T cell line. J Exp Med 1994;179:1799-1808.
198. Prasad K, Janssen O, Kapeller R, Raab M, Cantley L, Rudd C. Src-homology 3 domain of protein kinase p59 fyn mediates binding to phosphatidylinositol 3-kinase in T cells. Proc.Natl.Acad.Sci.USA 1993;90:7366-7370.
199. Stein P, Lee H-M, Rich S, Soriano P. pp59^{fyn} mutant mice display differential signaling in thymocytes and peripheral T cells. Cell 1992;70:741-750.
200. Appleby M, Gross J, Cooke M, Levin S, Qian X, Perlmutter R. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. Cell 1992;70:751-763.
201. Saleem A, Kharbanda S, Yuan ZM, Kufe D. Monocyte colony-stimulating factor stimulates binding of phosphatidylinositol 3-kinase to Grb2.Sos complexes in human monocytes. J Biol Chem 1995;270(18):10380-3.
202. Meisner H, Conway BR, Hartley D, Czech MP. Interactions of Cbl with Grb2 and phosphatidylinositol 3'-kinase in activated Jurkat cells. Mol Cell Biol 1995;15(7):3571-8.
203. Wang J, Auger KR, Jarvis L, Shi Y, Roberts TM. Direct association of Grb2 with the p85 subunit of phosphatidylinositol 3-kinase. J Biol Chem 1995;270(21):12774-80.
204. Satoh T, Fantl W, Escobedo J, Williams L, Kaziro Y. Platelet-derived growth factor receptor mediates activation of ras through different signaling pathways in different cell types. Mol Cell Biol 1993;13(6):3706-3713.
205. Rodriguez-Viciana P, Warne P, Vanhaesebroeck B. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. EMBO J 1996;15(10):2442-2451.
206. Löwenberg B, Putten Wv, Touw I, Delwel R, Santini V. Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. N Engl J Med 1993;328:614-619.

Chapter 2

Heterogeneity of proliferative responses of human B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells to interleukin-7 (IL-7): No correlation with immunoglobulin gene status and expression of IL-7 receptor or IL-2/ IL-4/ IL-7 receptor common γ chain genes

Frans J. Smiers[#,G], Marleen van Paassen[G], Auke Beishuizen[*], Karel Hählen[#], Bob Löwenberg[G,5], Ivo P. Touw[G,5]

[#]Sophia Children's Hospital, [G]Dr.Daniel den Hoed Cancer Center, Erasmus University, [5]Dept. of Hematology, [*]Dept. of Immunology, Rotterdam, The Netherlands

Abstract

Interleukin 7 (IL-7) stimulates proliferation of normal human and murine B cell precursor (BCP) cells in a distinct fashion, depending on the stage of maturation of the cells. For instance, the productive rearrangement of the immunoglobulin heavy chain gene has been demonstrated to be essential for the response of BCP cells to IL-7 as the single proliferation stimulus. IL-7 activates a receptor that consists of the IL-7R protein and the common γ chain (γ_c). BCP acute lymphoblastic leukemia (BCP-ALL) cells variably respond to IL-7. Among 72 cases of BCP-ALL IL-7 activated DNA synthesis in 34. In 4 cases inhibition of DNA synthesis was seen. In the remaining 38 cases IL-7 exerted no effects. We determined whether this heterogeneity in IL-7 response could be correlated with parameters that could influence the IL-7 response. First we show that, in contrast to the murine BCP cells, the IL-7 response of human BCP-ALL cells did not correlate with the status of Ig_H chain gene rearrangement and expression, nor with the rearrangement of Ig_L chain genes. Subsequently, it is demonstrated that IL-7R protein and transcripts as well as γ_c transcripts are equally present in the IL-7 responsive and nonresponsive BCP-ALL samples, indicating that the defective expression of these chains could not be held responsible for IL-7 response failures. Finally, we observed that Kit Ligand (KL), known to synergize with IL-7 in the most primitive stages of normal B cell development, did not enhance the IL-7 responses of BCP-ALL cells.

2.1 Introduction

In the majority of patients with acute lymphoblastic leukemia (ALL), the leukemic cells express features of B cell precursors (BCP) (1). The regulation of proliferation and survival of BCP-ALL cells remains poorly understood. Previous studies in relatively small series of patients have indicated that BCP-ALL cells display largely heterogeneous responses to IL-7, a growth factor for murine and human BCP cells (2,3). Growth stimulation by IL-7 was observed only in a subgroup of patients, whereas in others no or even inhibitory effects of IL-7 on proliferation were seen (4,5). Explanations for this heterogeneity have not been provided.
In normal B cell development in mice, IL-7 responses of BCP have been found to correlate with the status of rearrangement and expression of Ig heavy (Ig_H) and Ig light (Ig_L) chain genes (6,7). Murine pro-B cells with germ line Ig_H genes do not proliferate in response to IL-7 despite the presence of IL-7 receptor proteins on the cell membrane (8,9). During the first maturation step, DHJ_H joining takes place, which may occur in three different reading frames, RF1, RF2 or RF3. BCP cells at this stage of maturation synergistically respond to IL-7 in combination with kit ligand (KL), also known as stem cell factor (SCF) and other stroma derived components (10,11,12,13). DJ_H rearrangement in RF2 may result in the production of DJ_HC_μ protein (14). It has been suggested that DJC_μ protein blocks further maturation by preventing V_H to DJ_H joining (14,15). Upon joining of V_H to DJ_H, BCP cells enter the next maturation stage (6,7,8,16). When VDJ_H rearrangement is productive, cells express the μ protein in the cytoplasm. BCP cells expressing μ have been reported to become responsive to IL-7 as the single growth stimulus, while they have lost responsiveness to KL (6,8,9,16). In agreement with this, Era et al. demonstrated that μ chain expressing transgenic mice show selective expansion of IL-7 dependent BCP (17). Finally, upon the expression of the immunoglobulin κ or λ light chain genes, IL-7 responsiveness is lost (6,17,18). The molecular mechanisms responsible for these distinct IL-7 responses are not yet known.

IL-7 binds to a membrane receptor of the superfamily of hematopoietic growth factor and cytokine receptors (19,20,21). Molecular cloning of the human IL-7 receptor (IL-7R) revealed the existence of 3 different isoforms, one being a soluble protein, resulting from alternative RNA splicing (22). Recently, it has been demonstrated that IL-7R chains form heteromers with the γ chain common to IL-2R and IL-4R (γ_c) and that interaction with γ_c is a prerequisite for high affinity IL-7 binding (23,24).

In the present study, we have investigated to which extent IL-7 responses of BCP-ALL cells from individual patients correlate with biological parameters characteristic of the different stages of normal B cell development. We show that

IL-7 induces a proliferative response in approximately 50% of the BCP-ALL cases. No correlation is apparent between the (lack of) IL-7 response and immunophenotype (Null-ALL, Common-ALL, Pre-B-ALL) and KL does not stimulate DNA synthesis of BCP-ALL cells, neither alone nor in combination with IL-7. In addition it is demonstrated that the ability of BCP-ALL cells to respond to IL-7 as a single growth stimulus is neither determined by Ig gene rearrangement status nor by IL-7R/γ_c expression levels.

2.2 Materials and Methods

BCP-ALL and normal BCP samples

ALL cell samples were isolated from bone marrow or peripheral blood by Ficoll-Hypaque (Nygaard, Oslo, Norway) density gradient centrifugation as described previously (25). T cells were removed from the ALL cell samples by E-rosette formation using 2-aminoethylisothiouronium bromide (AET) treated sheep red blood cells, followed by sedimentation through Ficoll-Hypaque (26). Monocytes were removed by adherence to plastic petri dishes at 37°C for 1 hour. Informed consent was given in all cases. All BCP-ALL cell samples were subjected to routine diagnostic immuno phenotyping. Leukemic cells were subclassified as null-ALL (CD10-, CD19+, CD34+, Cy_μ -), common-ALL (CD10+, CD19+, CD34+/-, Cy_μ-) or pre-B ALL (CD10+, CD19+, CD34+/-, Cy_μ+) (27). For additional analyses, the cells were either used fresh or after cryopreservation using a controlled-freezing apparatus (Planer Biomed, Sunbury-on-Thames, UK)(25).

Nonleukemic BCP were isolated from normal bone marrow using a three-steps purification protocol. First, low density cells were recovered after Ficoll-Hypaque separation. Next, CD3 expressing T lymphocytes and CD14 and CD15 expressing myelomonocytic cells were removed from this fraction by antibody and complement mediated cytolysis as described (28). Finally, CD10 expressing cells, labeled with monoclonal antibody J5 (Becton-Dickinson, Mountain View, CA) and goat-anti-mouse Ig antibodies coupled to fluorescein isothiocyanate (GAM/FITC,Nordic, Tilburg, The Netherlands) were sorted using a FACS 440 cell sorter (Becton Dickinson, Sunnyvale CA).

In vitro culture and growth factors

DNA synthesis was assessed by uptake of ^{3}H-Thymidine (^{3}H-TdR, specific activity 2 Ci/mmol, Amersham International, Amersham, UK) as described (29). In brief,

0.2×10^5 cells were cultured in triplicate in 96 wells dishes (Greiner, Alphen a/d Rijn, the Netherlands) for 3 and 7 days in 100 µL serum-free culture medium (30). Eighteen hours before harvesting on nitrocellulose filters using a Titertek cell harvester (Flow Laboratories, Isrike UK) 0.1 µCi ^{3}H-TdR was added to the cultures. Human recombinant IL-7 and KL (gifts from Immunex Corp., Seattle, WA) were added to the cultures at final concentrations of 100 U/mL and 100 ng/mL, respectively.

Expression of IL-7R and common γ chains

Expression of IL-7R protein was assessed by flow cytometry (FACScan) following labeling of cells with anti human IL7 receptor monoclonal antibody M21 (Immunex Corp., Seattle, WA) and Goat anti mouse immunoglobulin coupled to fluorescent isothiocyanate (GAM/FITC, Nordic Tilburg, The Netherlands). The presence of IL-7R and γ_c chain was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. For this purpose, RNA was isolated from bone marrow samples by guanidine thiocyanate/phenol chloroform extraction (31). Human IL-7R cDNA was PCR amplified with the forward primer CTCCAGAGATCAATAATAGCTC and reverse primer TTGTCGCTCACGGTAAGTTCA. γ_c cDNA was PCR amplified using forward primer GAGCAAGCTTCATGTTGAA-GCCATCATTACC and reverse primer GCCTGAAACCTGAACCCCAAGCTTCTGA. Control reactions on H_2O were performed to exclude possible contamination of PCR reagents and primers with DNA.

Southern and RT-PCR analysis of immunoglobulin genes

DNA was isolated from ALL cells as described previously (32,33) Ten micrograms of DNA were digested with appropriate restriction enzymes. The restriction fragments were size fractionated on agarose gels and transferred to Hybond' nylon membranes (Amersham) as described (32,34). For analysis of the µ gene configuration, DNA was digested with BglII, BamHI and HindIII or SacI and BamHI. The blots were hybridized with J_H probes EcoRI/BglII (2.5 Kb), EcoRI/HindIII (0.9 Kb) of the clone H24 (a gift from Dr. T.Honjo, Kyoto University, Japan) (35) or with the IGHJ6 (1.0 Kb EcoRI/HindIII fragment)(36). For analysis of the κ gene rearrangements, DNA was digested with SacI, Bgl II, or Hind III and hybridized with Igκ probes: HindIII/EcoRI (0.5 Kb), SacI (1.8 Kb) or EcoRI (2.5 Kb), (Dr. Ph. Leder, Harvard Medical School, Boston MA)(37) or with the IGKJ5 probe (0.5 Kb HindIII/SacI fragment)(38). Rearrangement of the lambda light chain gene was analysed by digestion of DNA with EcoRI and hybridization with a BglII/HindIII

fragment (1.4Kb) of the Hu lambda C2 clone (Dr. Ph. Leder)(39). Probes were ^{32}P-labeled by random priming (40). For RT-PCR analysis, three primers were used to amplify transcripts of either DJCμ or VDJCμ rearranged Ig_H genes. One forward primer matched the V_H framework region 3 consensus sequence CTGTCGACA-CGGCCGTGTATTACTG. The second forward primer matched the J_H framework region 4 consensus sequence GGTCACCGTCTCCTCTAGAGT (41). The reverse primer TGCCAGCTGTGTCGGACATGAC was chosen in the fourth exon of the C_μ region. The applicability of these primers was verified on positive (B cell lines) and negative (granulocytes) control samples. In addition, H_2O control reactions were performed to exclude contamination of PCR reagents and primers with DNA.

2.3 Results

IL-7 responses of BCP-ALL cells

IL-7 responses ^{3}H-TdR uptake assay of 72 cases of BCP-ALL are shown in Table 1. In 38 cases, IL-7 failed to induce a proliferative response. In 4 cases of common ALL, IL-7 even inhibited spontaneous ^{3}H-TdR uptake. In 34 cases, IL-7 stimulated DNA synthesis. Stimulation values (IL-7 supplemented versus nonsupplemented controls) were variable and ranged from 1.5 (defined as the lowest level of stimulation) to 37.8 (mean: 4.4, median: 2.2). Cases in which IL-7 induced DNA synthesis were equally distributed over the different immunophenotypical sub-types of BCP-ALL.

Table 1 Proliferative response of BCP-ALL cells to interleukin-7

Immunological Subtype*	Number of patients	Effect of IL-7 on DNA Synthesis in vitro		
		Stimulation	Inhibition	None
Null-all	6	3	0	3
Common-all	48	23	4	21
Pre-B-all	18	8	0	10

* See materials and methods for detailed immunophenotype

Kit-ligand (KL) synergizes with IL-7 in stimulating DNA synthesis in normal but not in leukemic BCP

KL has been shown to synergize with IL-7 in inducing proliferation of BCP in mice (10-12,42). Using highly purified human normal BCP, we obtained comparable results (Table 2). In contrast, in 11 cases of BCP-ALL, no enhancement of IL-7 stimulation by KL was seen (Table 2).

Table 2

	Sample/ Patient	No Factor	IL-7	KL	KL + IL-7	Irrad.[r]
NORMAL	1	138 ± 36[s]	1054 ± 95	546 ± 148	2349 ± 695	42 ± 10
BCP [o]	2	190 ± 91	671 ± 124	1146 ± 196	3359 ± 346	20 ± 3
NULL-ALL	DH	15531 ± 683	14748 ± 191	16704 ± 1052	16381 ± 1212	788 ± 250
	BU	663 ± 59	2692 ± 210	740 ± 47	2717 ± 255	50 ± 12
Common-all	IR	378 ± 43	268 ± 57	482 ± 90	266 ± 34	159 ± 28
	KA	2575 ± 312	2617 ± 194	2407 ± 361	2813 ± 172	33 ± 2
	KE	1644 ± 163	1793 ± 66	1955 ± 102	2007 ± 106	60 ± 14
	PE	250 ± 38	2231 ± 207	196 ± 19	2130 ± 36	89 ± 35
	VW	4397 ± 123	12028 ± 1070	9332 ± 1045	12385 ± 248	34 ± 4
Pre-b-all	LAC	2875 ± 152	2948 ± 121	2817 ± 211	2891 ± 269	105 ± 40
	VS	42 ± 5	568 ± 37	215 ± 18	1512 ± 203	60 ± 15
	KT	282 ± 23	2734 ± 180	274 ± 14	2687 ± 218	36 ± 9
	UR	578 ± 42	7487 ± 389	597 ± 48	9300 ± 1209	52 ± 13
	SP	1967 ± 151	5301 ± 74	1543 ± 66	4653 ± 233	56 ± 19

[o] FACS purified, see Materials and Methods

[s] ^{3}H-TdR uptake; mean cpm ± SD of triplicate cultures

[r] Irradiated (25 Gy) cells

Expression of IL-7R and common γ chains

To examine whether a lack of IL-7 responsiveness of BCP-ALL cells could be explained by the absence of an IL-7R, expression of both IL-7R and γ_c chains was studied. In seven IL-7 nonresponsive cases, IL-7R could be readily detected by flow cytometry using anti-IL-7R MoAb. A representative example of the analysis (Fig 1) shows that the expression levels of IL-7R on IL-7 responsive versus IL-7 nonresponsive BCP-ALL cells does not differ. Further, RT-PCR revealed that the IL-7R wild type form (H20), rather than the truncated (H1) or the soluble (H6) splice variants (22), is predominantly expressed in BCP-ALL cells (Fig 2).

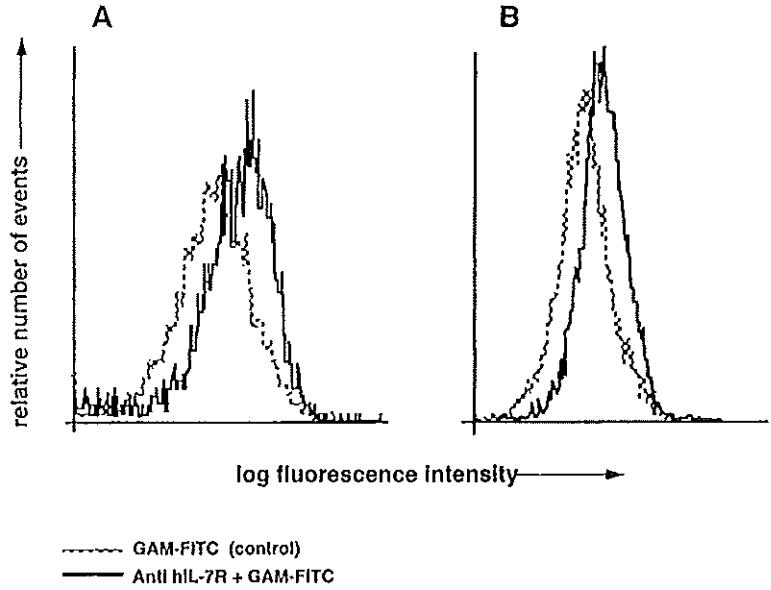

Figure 1 Representative FACS analysis of IL-7 receptor expression in BCP-ALL cells (A) responsive and (B) nonresponsive to IL-7

Transcripts of the γ_c with the expected size of the wild type form were apparent in both IL-7 responsive and nonresponsive BCP-ALL cell samples (Fig 3). Collectively, these findings indicate that the failure of BCP-ALL cells to respond to IL-7 cannot be attributed to the lack of expression of IL-7R and γ_c genes, or the overexpression of nonfunctional splice variants.

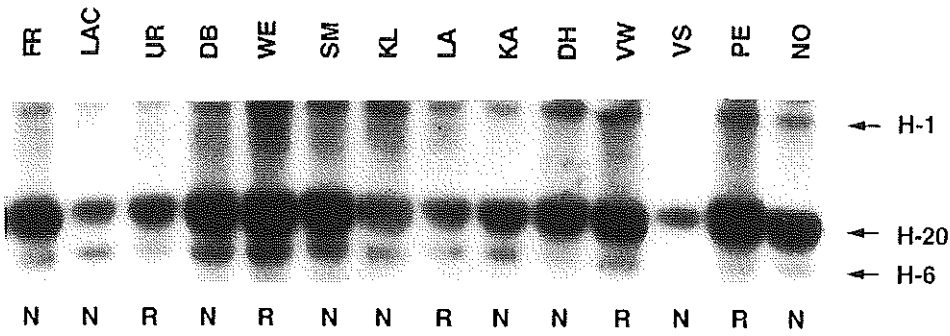

Figure 2 RT-PCR analysis of IL-7R expression in BCP-ALL cells. To visualize lowly expressed splice variants, agarose gel separated products were blotted on Hybond+ membrane, hybridized with a ^{32}P labeled human IL-7R probe and autoradiographed. Results of 15 representative cases are shown either responding (R) (lanes 2,3,8,11 and 13) or nonresponding (N) (lanes 1,4-7,9,10,12 and 14) to stimulation with IL-7

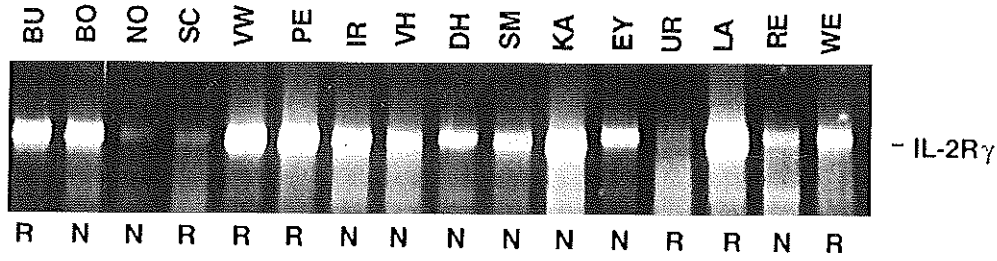

Figure 3 RT-PCR analysis of γ_c chain expression in IL-7 responding (R) (lanes 1,4-6,13,14,16) and nonresponding (N) (lanes 2,3,7-12,15) BCP-ALL cells.

Configuration of immunoglobulin genes

The configuration of the Ig heavy chain and the Ig κ and λ light chain genes was investigated by Southern analysis in 16 IL-7 responsive and 13 IL-7 nonresponsive BCP-ALL cases. In all cases, one or both alleles of the Ig_H gene had been rearranged (Table 3). RT-PCR analysis using a V_H consensus forward primer and a C_H reverse primer showed that in all cases investigated the rearrangement consisted of V_H to DJ_H joining (Fig 4).

Southern blot analysis showed Ig_L chain gene rearrangements in 50% of the BCP-ALL patients analyzed responding to IL-7 and in 62% of those not responding to IL-7 (Table 3).

Table 3

IgH chain gene	Il-7 responsive Cases (n=16)	Il-7 non responsive Cases (n=13)
Germ line: (GG)	0	0
Rearranged: (RR, GR, GD, DR)	16	13
Ig λ and κ chain gene Germ line λ and/or κ: (GG)	8	5
Rearranged λ and/or rearranged or deleted κ: (GR, GD, DR, DD, RR)	8	8

Compilation of data derived from Southern blot analysis. For details see Material and Methods G: germ line, R: rearranged, D: deleted

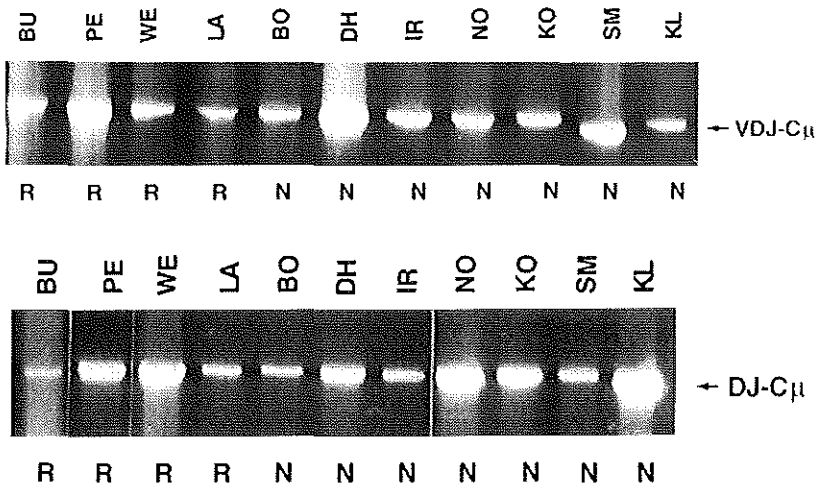

Figure 4 RT-PCR analysis of rearrangement and expression of Ig_H chain genes in BCP-ALL cells. RNA was isolated from IL-7 responding and non responding cases and after cDNA synthesis subjected to PCR. Panel A shows products of RT-PCR with the V_H forward primer and C_μ specific reversed primer. All cases tested showed VDJ rearrangement. Panel B shows products of RT-PCR with the specific J_H forward primer and C_μ primer.

Discussion

Over the years, the understanding of the growth control of BCP-ALL cells has remained very limited. From the increasing list of recombinant growth factors and cytokines that have become available during the last decade, only IL-3 and IL-7 consistently evoke mitogenic responses in BCP-ALL cells, albeit not in all cases (2-5). In this study comprising 72 BCP-ALL cases, including both childhood and adult ALL, we have found that only in about one half of the cases (n=34) a proliferative response to IL-7 is elicited in vitro. In an attempt to explain this heterogeneity, a number of potential mechanisms that might determine or influence the responsiveness of BCP-ALL cells to IL-7 have been investigated. With progression of B cell differentiation, which is tightly correlated with the rearrangement and expression of Ig heavy and light chain genes, BCP have been reported to display distinct response patterns to IL-7 alone or in combination with other stimuli such as KL and insulin-like growth factor-1 (IGF-1) (43,44). We therefore set out to examine whether the differences in responses of BCP-ALL cells to IL-7 and KL (and IGF-1) could also be related to the status of Ig genes. Southern blot analysis showed that μ heavy chain gene rearrangement had occurred in all of 23 cases (12 IL-7 responders, 11 nonresponders), indicating that in neither of these samples the BCP-ALL cells had been arrested at the pro-B cell stage. This would fit with the observation that IGF-1, which has been shown to specifically stimulate proliferation in pro-B cells but not in more advanced stages of B cell development (43,44), failed to induce a proliferative response in BCP-ALL cells in combination with IL-7 (data not shown). Further analysis of the μ gene rearrangement status by RT-PCR using V_H and J_H consensus primers indicated that V_H to DJ_H joining had taken place in all of these BCP-ALL samples. During normal B cell development, BCP lose their responsiveness to KL after VDJ_H joining has occurred (14,15). Therefore, it is not unexpected that BCP-ALL cells also failed to respond to this growth factor (Table 2). A number of studies have indicated that BCP that have undergone successful VDJ_H joining, resulting in the expression cytoplasmic μ proteins, are highly responsive to IL-7 as the single growth stimulus (6,8,16,17). It was suggested that the presence of μ chains but not yet Ig_L chains in the cytoplasm enhances the IL-7 driven proliferation of BCP, and that BCP with nonproductive VDJ_H rearrangements would thus be hampered in their response to IL-7 (7). This hypothetic mechanism could also account for the loss of IL-7 responsiveness following the production of Ig light chains, by assuming that light chain complex formation with the μ chains abolishes the enhancing effects of single μ chains on IL-7 induced proliferation (7). Our observations in BCP-ALL cannot be reconciled with this model, since a) IL-7 responses were seen in cases with null and common ALL, i.e.,

leukemic immunophenotypes characterized by nonproductive VDJ_H rearrangements and b) the rearrangement status (and potential expression) of the Ig light chain genes also failed to show a correlation with IL-7 responsiveness.

Transforming growth factor β (TGF β) has been shown to inhibit IL-7 induced proliferation of normal BCP (6,45). Because normal and leukemic B cells can synthesize TGF β (46,47), we have investigated whether TGF β may have interfered with IL-7 responses of BCP-ALL cells in DNA synthesis assays. However, addition of TGF β neutralizing antibody to the culture medium did not result in an increase of IL-7 induced proliferation in BCP-ALL cells, indicating that IL-7 responses had not been blocked by endogenously produced TGF β (data not shown).

Finally, we have studied whether IL-7 nonresponsive BCP-ALL cells lack functional IL-7 receptors. By FACS analysis using anti IL-7R antibodies, IL-7R protein could be detected on IL-7 nonresponding BCP-ALL cells. Because three IL-7R isoforms exist, including a nonsignaling soluble form (22), we investigated the expression distribution of these splice variants by RT-PCR. No indication was found for overexpression of any of the two nonsignal transducing IL-7R forms in these cells. By a similar RT-PCR based approach, we excluded that a lack of expression of the γ_c could have been responsible for the failure of BCP-ALL cells to stimulation with IL-7.

In conclusion, in contrast to observations in murine BCP, our studies have shown that heterogeneous IL-7 responses of BCP-ALL cells do not correspond to distinct stages of B cell maturation and status of the Ig genes. It is at present not clear whether this lack of correlation is an aberrant characteristic of the leukemic cells or whether it reflects the more complex IL-7 responses of human BCP cells as compared with murine BCP. Irrespective of this and because IL-7 response failures did not correlate with the lack of expression of IL-7R and γ_c, it will now be important to investigate the differences in activation of signaling molecules in IL-7 responsive and nonresponsive BCP-ALL cells.

References

1. Pui C.H, Behm FG, Crist WM. Clinical and biological relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. Blood 1993;82:343-362.
2. Namen AE, Lupton S, Hjerrild, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, GillisS, Cosman D, Goodwin RG. Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 1988;33:571-573.
3. Saeland S, Duvert V, Pandreau D, Caux C, Durand I, Wrighton N, Wideman J, Lee F, Banchereau J. Interleukin-7 induces the proliferation of normal human B-cell precursors. Blood 1991;78: 2229-2238.
4. Skjønsberg C, Erikstein BK, Smeland EB, Lie SO, Funderud S, Beiske K, Blomhoff HK. Interleukin-7 differentiates a subgroup of acute lymphoblastic leukemias. Blood 1991;77:2445-2450.
5. Digel W, Schmid M, Heil G, Conrad P, Gillis S, Porzsolt F. Human interleukin-7 induces proliferation of neoplastic cells from chronic lymphocytic leukemia and acute leukemias. Blood 1991;78:753-759.
6. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-b. J.Immunol. 1989;142:3875-3883.
7. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J.Exp.Med. 1991;173:1213-1225.
8. Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi S-I, Ogawa M, Sakai K, Nishikawa S, Nishikawa S-I. Interleukin 7 production and function in stromal cell-dependent B cell development. J.Exp.Med. 1989;170:333-338
9. Hayashi SI, Kunisada T, Ogawa M, Sudo T, Kodama H, Suda T, Nishikawa S, Nishikawa SI. Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J.Exp.Med. 1990;171:1683-1695.
10. McNiece IK, Langley KE, Zsebo KM. The role of recombinant stem cell factor in early B cell development. J.Immunol. 1991; 146:3785-3790.
11. Funk PE, Varas A, Witte PL. Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. J.Immunol. 1993;150:748-752.
12. Rolink A, Streb M, Nishikawa S, Melchers F. The c-kit encoded tyrosine kinase regulates the proliferation of early pre-B cells. Eur.J.Immunol. 1991;21:2609-2612
13. Palacios R, Nishikawa S. Developmentally regulated cell surface expression and function of c-kit receptor during lymphocyte ontogeny in the embryo and adult mice. Development 1992;115:1133-1147.
14. Gu H, Kitamura D, Rajewsky K, B cell development regulated by gene rearrangement: arrest of maturation by membrane-bound D_{μ}protein and selection of D_H element reading frame. Cell 1991; 65:47-54.
15. Melchers F, Karasuyama H, Haasner D, Bauer S, Kudo A, SakaguchiN, Jameson B, Rolink A. The surrogate light chain in B-cell development. Immunology Today 1993;14:60-68.

16. Faust EA, Saffran DC, Toksoz D, Williams DA, Witte ON. Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from pre-B cells. J.Exp.Med. 1993;177:915-923.
17. Era T, Ogawa M, Nishikawa S-I, Okamoto M, Honjo T, Akagi K, Miyazaki J-I, Yamamura K-I. Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J. 1991;10:337-342.
18. Henderson AJ, Narayanan R, Collins L, Dorshkind K. Status of kL chain gene rearrangement and c-kit and IL-7 receptor expression in stromal cell-dependent pre-B cells.J.Immunol. 1992; 149:1973-1979.
19. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc.Natl.Acad.Sci. 1990;87:6934-6398.
20. Cosman D, Lyman SD, Idzerda RL, Beckman MP, Park LS, Goodwin RG, March CJ. A new cytokine receptor superfamily.Trends Bioch Sci 1990;15:265-70.
21. Cosman D. The hematopoietin superfamily. Cytokine 1993;5:95-106.
22. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March KJ, Namen AE, Park LS. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell 1990;60:941-951.
23. Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S-I, Sugamura K. Functional participation of the IL-2 receptor g chain in IL-7 receptor complexes. Science 1994;263:1453-1454.
24. Noguchi M, Nakamura Y, Rusell SM, Ziegler SF, Tsang M, Cao X, Leonard WJ. Interleukin-2 receptor I chain: a functional component of the interleukin-7 receptor. Science 1993;262: 1877-1880.
25. Touw I, Groot-Loonen J, Broeders L, van Agthoven Th, Hählen K, Hagemeijer A, Löwenberg B. Recombinant hematopoietic growth factors fail to induce a proliferative response in precursor B acute lymphoblastic leukemia. Leukemia 1989;3:356-362.
26. Madsen M, Johnsen HE, Wendelboe HP, Christiansen SE. Isolation of human T and B lymphocytes by E-rosette gradient centrifugation. Characterization of the isolated subpopulations. J.Immunol.Methods 1980;33:323
27. Dongen JJM van, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33: 298-314.
28. Bot FJ, Eijk L van, Broeders L, Aarden LA, Löwenberg B. Interleukin-6 synergizes with M-CSF in the formation of macrophage colonies from purified human bone marrow progenitor cells. Blood 1989;73:435-437.
29. Delwel R, Salem M, Pellens C, Dorssers L, Wagemaker G, Clark S, Löwenberg B. Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum free culture system. Blood 1988;72:1944-1949.
30. Touw I, Pouwels K, Agthoven T van, Gurp R van, Budel L, Hoogerbrugge H, Delwel R, Goodwin R, Namen A, Löwenberg B. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 1990;11:2097-2101.

31. Chromczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Bioch. 1987;162:156-159.
32. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:3.
33 Dongen J.J.M. van, Wolvers-Tettero I.L.M. Analysis of immunoglobulin and T-cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-91
34. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975;98:503-517.
35. Takahashi N, Nakai S, Honjo T. Cloning of human immunoglobulin m gene and comparison with mouse m gene. Nucleic Acids Res 1980;8:5983-5991.
36. Beishuizen A, Verhoeven MJ, Mol EJ, Breit TM, Wolvers-Tettero ILM, Dongen JJM van. Detection of immunoglobulin heavy-chain gene rearrangements by southern blot analysis: recommandations for optimal results. Leukemia 1993;7:2045-2053.
37. Hieter PA, Maizel JV Jr, Leder P. Evolution of immunoglobulin k J region genes. J Biol Chem 1982;257:1516-1522.
38. Beishuizen A, Verhoeven MJ, Mol EJ, Dongen JJM van. Detection of immunoglobulin Kappa-light-chain gene rearrangement patterns by southern blot analysis. Leukemia 1994;8: in press.
39. Hieter PA, Hollis GF, Korsmeyer SJ, Waldman TA, Leder P. Clustered arrangement of immunoglobulin l constant region genes in man. Nature 1981;294:536-540.
40. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983;132:6-13.
41. Steenbergen EJ, Verhagen OJHM, Leeuwen EF van, Borne AEGKr von dem, Schoot CE van der. Distinct ongoing Ig heavy chain rearrangement processes in childhood B-precursor acute lymphoblastic leukemia. Blood 1993;82:581-589
42. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J,Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA,Johnson MJ, Parker VP, Flores JC, Patel AC, Fisher EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Leslie I, Wen D, Lin C-H, Cupples RL, Zsebo KM. Primary structure and functional expression of rat and human stem cell factor DNAs. Cell 1990;63:203-211.
43. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood 1992;5:1207-1212.
44. Gibson LF, Piktel D, Landreth KS. Insulin-like growth factor-I potentiates expansion of interleukin-7 -dependent pro-B cells. Blood 1993;82:3005-3011.
45. Israels LG, Israels SJ, Begleiter A, Verburg L, Schwartz L, Mowat MRA, Johnston JB. Role of transforming growth factor-_ in chronic lymphocytic leukemia. Leukemia Research 1993;17: 81-87.
46. Kehrl JH, Taylor AS, Delsing GA, Roberts AB, Sporn MB, Fauci AS. Further studies of the role of transforming growth factor-beta in human B cell function. J Immunol. 1989;143:1868-1874.
47. Lagneaux L, Delforge A, Dorval C, Bron D, Stryckmans P. Excessive production of transforming growht factor-beta by bone marrow stromal cells in B-cell chronic lymphocytic leukemia inhibits growth of hematopoietic precursors and interleukin-6 production. Blood 1993;82:2379-2385.

Chapter 3

CD20 and CD40 mediated mitogenic responses in B-lineage acute lymphoblastic leukemia

Frans J. Smiers[#,@], Marleen van Paassen[@], Karel Hählen[#], Bob Löwenberg[@,$], Ivo P. Touw[@,$]

[#]Sophia Children's Hospital, [@]Dr.Daniel den Hoed Cancer Center, Erasmus University, [$]Dept. of Hematology, Rotterdam, The Netherlands.

Abstract

Activation of CD20, a cross-membrane ion channel, induces cell cycle progression from G_0 to G_1 in B lymphocytes. Subsequent activation of CD40, a membrane receptor of the nerve growth factor superfamily, transits the B cells to the S phase. CD40 may also act synergistically in combination with IL-4 (B-lymphocytes) or IL-3/IL-7 (B-cell precursors). We investigated the proliferative responses of B-lineage acute lymphoblastic leukaemia (ALL) cells to CD20/CD40 activation. In 18/56 ALL cases CD20 activation resulted in significant increases in DNA synthesis. Similar, although more moderate, effects were seen of activation of CD40 in 10/44 cases. Responses to CD20 or CD40 activation were independent of co-stimulation with IL-3, IL-4 or IL-7, and various cocktails of the different growth stimuli did not act synergistically.

3.1 Introduction

B cell development is governed by an as yet incomplete characterized series of haematopoietic growth factors and other stimuli. Factors that exert growth stimulatory effects on normal B cell precursors include interleukin (IL)-3 and IL-7 (1-4).

CD20 is a 35/37 kD surface polypeptide expressed exclusively on B cells. Onset of expression of CD20 takes place before the appearance of cytoplasmic μ chains and is sustained during B-cell development until the plasma cell stage (5,6). In normal B lymphocytes, activation of CD20 proteins with the antibody 1F5 induces cell cycle transition from G_0 to G_1 but is not sufficient to initiate DNA synthesis (7-11). Activation of CD20 results in its phosphorylation on serine and threonine residues (12,13) and increases the influx of Ca^{2+} ions into the cells. The structural and functional properties of CD20 indicate that the protein acts as a cross-membrane ion channel (14,15).

CD40 is a receptor of the nerve growth factor receptor superfamily and is expressed on B cells, follicular dendritic cells, normal basal and thymic epithelium and several carcinoma and melanoma cell lines (16-18). During B cell development, the appearance of CD40 on the cell membrane precedes the expression of CD20 and immunoglobulin heavy chains (19). Activation of CD40 by either the CD40 ligand or CD40 activating antibodies, in concert with IL-4, induces proliferation and maturation of B-lymphocytes (20-24). Also, co-activation of CD20 and CD40 results in a full mitogenic response of B lymphocytes (25,26). B cell precursors (BCP) proliferate in response to CD40 activation only when co-stimulated with IL-3, IL-7 or IL-10 (27,28).

Here, we studied the proliferation inducing effects of CD20 and CD40 activation on B-lineage acute lymphoblastic leukemia (ALL) cells, alone and in combination with growth factors (IL-3, IL-4 and IL-7). Activation of CD20 resulted in a proliferative response of ALL cells in 18/58 (31%) cases; activation of CD40 induced DNA synthesis in 10/44 (23%) cases. With the exception of one case, no synergistic effects of the various stimuli were seen. These data show that responses of ALL cells to CD20/CD40 activation are heterogeneous and distinct from those of normal (pre-)B cells.

3.2 Materials and Methods

Isolation of cells

ALL cells were isolated from bone marrow or peripheral blood by Ficoll-Hypaque (Nygaard, Oslo,Norway) density gradient centrifigation as described previously (29). T cells were removed from the ALL cell samples by E-rosette formation using 2-aminoethylisothiouronium bromide (AET) treated sheep erythrocytes, followed by sedimentation through Ficoll-Hypaque (30). Monocytes were removed by adherence to plastic petri dishes at 37° C for 1 h. In all cases, informed consent was obtained. The ALL cell samples were subjected to routine diagnostic immunophenotyping with a panel of monoclonal antibodies (MoAb). Six cases were classified as null-ALL (patients 1-6), 36 as common-ALL (patients 7-41, 58), 11 as pre-B-ALL (patients 42-52) and 5 as B-ALL (patients 53-57) according to established criteria (31). Cells were used either fresh or after cryopreservation using a controlled-freezing apparatus (Planer Biomed, Sunbury-on-Thames,UK) (29). Enriched fractions of normal peripheral blood B-lymphocytes were obtained using the same protocol.

DNA synthesis assay

DNA synthesis was assessed by uptake of ^{3}H-Thymidine (^{3}H-TdR, specific activity 2 Ci/mmol, Amersham International, Amersham, UK) as described (32). In brief, 0.2 x 10^{5} cells were cultured in triplicate in 96 wells dishes (Greiner, Alphen a/d Rijn, The Netherlands) for 3 and 7 days in 100 µL serum free medium (33). Eighteen hours before harvesting on nitrocellulose filters using Titertek cell harvester (Flow Laboratories, Irvine) 0.1 µCi ^{3}H-TdR was added to each well. ^{3}H-TdR incorporation was measured by liquid scintillation counting.

Growth factors and CD20 or CD40 activating antibodies

Human IL-3 (Gist Brocades, Delft, The Netherlands) was used at 100 U/Ml; human IL-4 (Dr S. Clark, Genetics Institute Cambridge, MA) was added to the cultures at a 1:5000 dilution of Cos cell supernatant; human IL-7 (Dr. L. Park, Immunex Corp., Seattle, WA) was used at 100 U/Ml. Anti-CD40 monoclonal antibody (MoAb) 14G7, provided by Dr. R.A.W. van Lier (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) was used at a 1:250 dilution of ascites. The CD20 activating MoAb 1F5 (7, 9)(Dr. E.A. Clark (University of Washington, Seattle, WA) was used at a final concentration of 3 µg/ml.

Analysis of CD20 and CD40 expression

Expression of CD20 or CD40 was assessed by flow cytometry (FACScan) after labelling of cells with either the anti CD20 MoAb 1F5 (9) or the activating anti-CD40 antibody 14G7 and goat anti-mouse immunoglobulin coupled to fluorescin isothiocyanate (GAM/FITC, Nordic Tilburg, The Netherlands).

Table 1 Proliferative responses of ALL cells to activation of CD20 in comparison to responses to IL-3 or IL-7[a].

Pt	ALL subtype	No additive to culture	Anti-CD20	IL-3	IL-7	Irradiated cells[#]
1	Null	1.3±0.6[*]	10.9±1.7	3.7±1.8	2.4±0.4	0.4±0.1
6	Null	10.7±0.2	25.2±6.5	50.8±7.6	18.0±1.2	0.7±0.1
10	Common	18.4±0.6	29.8±0.9	31.8±1.0	22.4±2.0	0.4±0.1
13	Common	8.8±0.2	59.1±1.6	18.4±1.3	17.1±1.7	0.6±0.1
15	Common	13.0±0.8	55.5±5.5	23.3±3.0	20.3±1.0	0.2±0.0
16	Common	13.4±0.9	20.6±3.1	n.d.[$]	17.2±1.0	0.2±0.0
22	Common	3.8±0.1	9.3±2.1	18.4±4.2	6.3±0.5	0.4±0.2
30	Common	5.9±0.2	65.9±11	11.4±2.2	10.1±1.8	0.7±0.2
31	Common	5.9±0.5	78.2±7.2	20.0±2.1	27.3±3.5	0.5±0.2
32	Common	3.1±0.3	8.1±2.2	14.8±4.6	18.2±0.4	0.3±0.1
58	Common	7.9±1.3	27.0±2.7	9.8±2.7	6.4±1.1	0.3±0.1
36	Common	2.6±0.4	13.1±2.3	6.6±2.0	2.3±0.6	0.7±0.1
38	Common	7.1±2.2	14.7±3.3	21.0±6.9	10.3±1.9	1.4±0.2
43	Pre-B	10.5±1.8	23.3±6.0	18.9±5.1	19.1±1.4	1.0±0.0
44	Pre-B	29.6±1.9	87.3±9.7	58.1±10	69.3±3.0	0.3±0.2
45	Pre-B	19.3±1.0	39.0±8.0	27.5±1.4	31.0±3.4	0.3±0.2
50	Pre-B	2.2±0.6	24.9±1.0	6.6±1.4	2.2±0.4	0.4±0.1
57	B	19.4±1.8	32.0±1.0	273±14.7	19.6±6.0	0.9±0.2

[a] Only cases responsive to CD20 activation have been included.In these cases, stimulation values (1F5 supplemented versus non-supplemented controls) were >1.5.

[*] Data are from ^{3}H-Thymidine uptake assays and are expressed as mean counts per minute x 100 ± standard deviation of triplicate cultures.

[#] Cells were irradiated (25Gy) before culture.

[$] Not Determined

3.3 Results and Discussion

CD20 activation of ALL cells

Dose titration experiments performed with ALL cells from four patients indicated that maximal stimulation of DNA synthesis is seen at 1F5 concentrations of 1-10 mg/mL. A representative experiment is shown in Fig 1A. Parallel incubations with control MoAb antibody did not result in mitogenic responses (data not shown). Proliferative response to 1F5 (3 mg/mL) were analysed in 58 cases of ALL. CD20 activation resulted in a significant increase of DNA synthesis in 18 cases (Table 1). The (in)ability of 1F5 to induce DNA synthesis did not correlate with the immunologic subtype of ALL. Therefore differences of maturation or phenotype of ALL did not explain the heterogeneity in responses to CD20 activation. A correlation was neither apparant between responses to CD20 activation nor responses to IL-3 or IL-7 (Fig 2). In fact, the random distribution of the plotted stimulation indices indicates that each of the individual responses is independent from any of the responses to the other growth stimuli. As expected, flow cytometric analysis with anti-CD20 showed that ALL cells of 1F5 responding cases expressed CD20. In 15/27 1F5 nonresponders analysed, the ALL cells expressed CD20, indicating that in these cases the absence of response to 1F5 was not caused by lack of CD20.

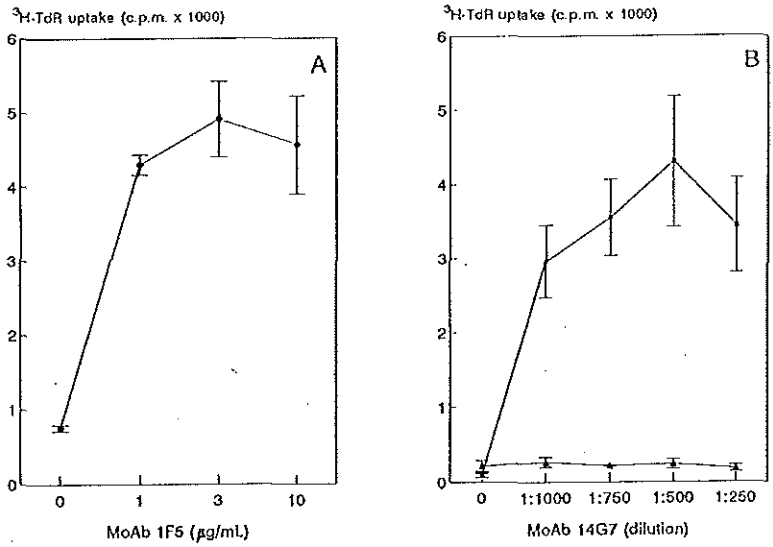

Figure 1 Response of human BCP-ALL cells to titrated dosages of the CD20 activating MoAb 1F5 in a ^{3}H-TdR uptake assay.

Table 2 Proliferative response of ALL cells to activation of CD40 in comparison to responses to IL-3, IL-7 and CD20 activation[@]

Pt	ALL subtype	No additive to culture	Anti-CD40	IL-3	IL-7	Anti-CD20	Irradiated cells[#]
1	Null	1.3±0.6[*]	9.6±2.4	3.7±1.8	2.4±0.5	10.9±1.7	0.4
11	Null	35.4±1.5	70.8±3.1	121±4	33.5±0.2	35.8±1.4	0.4
8	Common	349±22	568±45	341±8	336±12	389±8	0.6
14	Common	5.5±0.9	15.6±3.6	15.0±2.0	5.9±1.6	3.8±1.2	0.4
21	Common	5.6±0.6	32.3±1.3	16.8±1.9	12.2±1.5	8.9±1.2	0.2
26	Common	31.5±1.0	45.5±3.9	35.2±1.6	27.0±1.7	32.9±0.4	1.4
30	Common	5.9±0.2	12.4±1.9	11.4±2.1	10.1±1.8	65.9±11	0.7
48	Pre-B	17.6±3.9	28.9±0.8	25.7±1.1	10.7±4.0	19.8±1.2	0.4
50	Pre-B	2.2±0.6	5.5±0.2	6.5±1.4	2.2±0.4	24.9±1.0	0.3
52	Pre-B	3.2±0.0	8.0±0.6	16.2±2.3	2.0±0.4	3.6±1.3	0.6
55	B	1.8±0.0	5.0±0.3	1.8±0.5	2.0±0.2	1.9±0.3	0.4

[@] Only cases responsive to CD40 activation have been included. In these cases, stimulation values (14G7 supplemented versus non-supplemented controls) were >1.5.

[*] Data are from ^{3}H-Thymidine uptake assays and are expressed as mean counts per minute x 1000 ± standard deviation of triplicate cultures.

[#] Cells were irradiated (25Gy) before culture.

CD40 activation of ALL cells

In dose titration experiments, maximal stimulation of DNA synthesis by CD40 activating antibody 14G7 was reached at dilutions of 1:1000-1:250 (Fig 1B). In 10/44 ALL cases, DNA synthesis was moderately enhanced by 14G7 (1:250 dilution, Table II). Previously, Law *et al*(34) could not demonstrate mitogenic effects of CD40 activation in ALL cells. However, the latter study was based on the analysis of eight cases of ALL only (34). The various immunological subtypes off B -cell ALL were represented among the 10 14G7 responsive cases. No correlation was apparant between responses to anti-CD40 and those to IL-3, IL-4, IL-7 or anti-CD20 (Fig 2). All immunological subtypes of B cell ALL were present among the 10 14G7 responsive cases. No correlation was apparent between responses to anti-CD40 and those to IL-3, IL-7 or anti-CD20.

All 14G7 responders expressed CD40 at levels detectable by flow cytometry. In 6/8 14G7 nonresponders, ALL cells expressed CD40, suggesting that in the majority of cases non-responsiveness of ALL cells following CD40 stimulation is due to ineffective signalling.

Activation of CD20 and CD40 in combination with other stimuli

Certain stimuli act synergistically in inducing proliferative responses in nonleukemic (pre-) B cells. Particularly, this has been demonstrated for the combined activation of CD20 and CD40 (25,26), and for activation of CD40 in combination with IL-4 (20,22-24), IL-3 or IL-7 (28). Proliferative responses of ALL cells to different combinations of stimuli, in the context of activation of CD20 or CD40, are summarized in Table III. Although additive effects (1F5 + IL-3; 1F5 + IL-7; 1F5 + 14G7) were occasionally seen, none of the combinations tested were synergistic, except in a single case of ALL in which both the combinations 14G7 + IL-3 and 14G7 + IL-7 synergistically stimulated DNA synthesis.
The response of ALL cells to activation of CD20 or CD40, with or without the addition of cytokines, differ from those of nonleukaemic (pre-)B cells. Whether this would indicate that ALL cells generally display aberrant responses to CD20 and CD40 activation is not clear. In normal B lymphocytes activation of CD20 induces transition from G0 to G1 (7, 9-11), whereas CD40 activation promotes the transition from G1 to S phase of the cell cycle. Activation of both of these molecules is required for transduction of a full mitogenic signal (25,26,35). The observation that in certain cases ALL cells proliferate in response to either CD20 or CD40 activation as a single growth stimulus could be suggestive of a partial loss of cell cycle control bypassing the necessity of cooperation of signals for a mitogenic response. Further elucidation of signalling properties of CD20 and CD40 in normal and leukaemic (pre-)B cells is needed to clarify these issues.

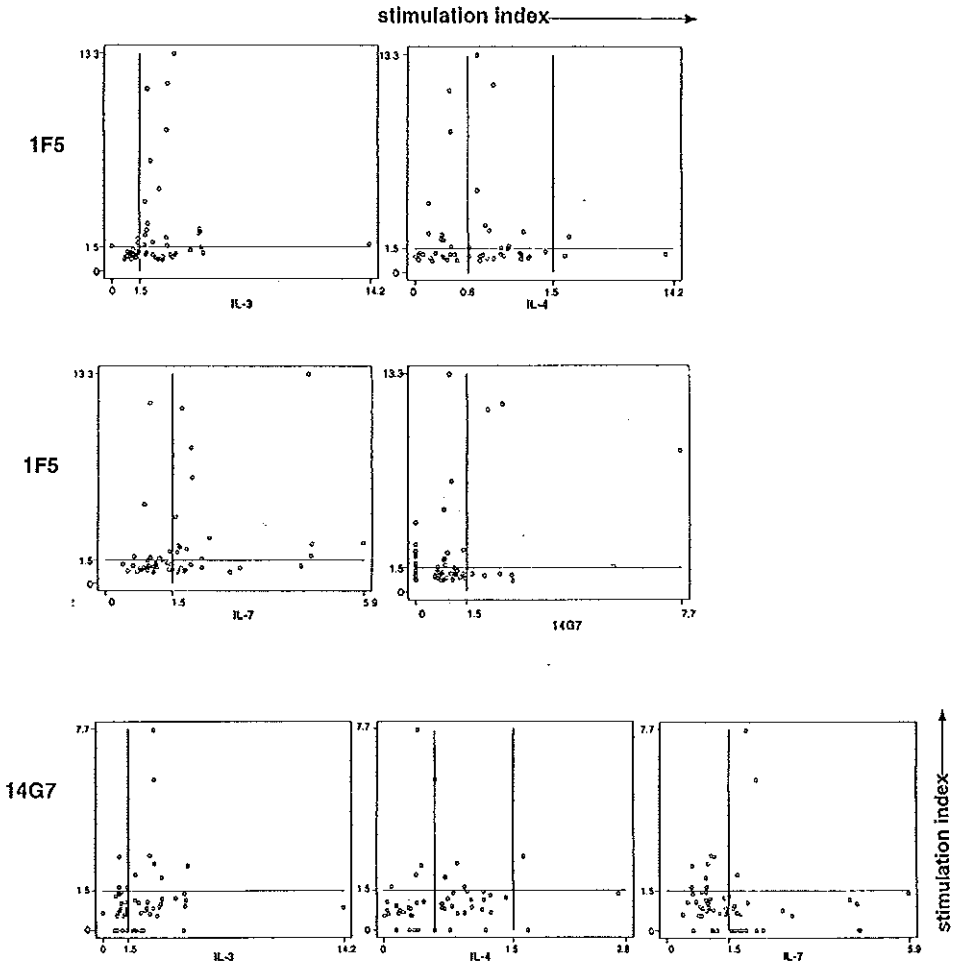

Figure 2 Proliferative responses of BCP-ALL cells in response to different growth stimuli plotted as stimulation indices (SI) in matrix mode. SI of 1F5 (top panel) or 14G7 (lower panel) on the Y-axis are plotted against SI of IL-3, IL-4, IL-7 and 14G7 on the X-axis. SI higher than 1.5 was defined as stimulation, whereas values less than 0.6 were defined as inhibition.

Table 3 Summary of proliferative responses of ALL cells to different stimuli

Stimulus	Frequency of response (%)	Type of response
IL-3	31/58 (53%)	Stimulation
IL-7	24/58 (41%)	Stimulation
IL-4	2/49 (4%)	Stimulation
	22/49 (45%)	Inhibition
MoAb 1F5 (CD20 activation)	18/58 (31%)	Stimulation
Moab 14G7 (CD40 activation)	10/44 (23%)	Stimulation
1F5 + IL-3	22/36 (61%)	Additive responses in 4 cases; no synergy
1F5 + IL-7	25/50 (50%)	Additive responses in 9 cases; no synergy
1F5 + 14G7	22/36 (61%)	Additive response in 5 cases; no synergy
14G7 + IL-3	6/12 (50%)	Synergistic response in 1 case
14G7 + IL-7	5/12 (42%)	Additive response in 2 cases; synergistic response in 1 case
14G7 + IL-4	2/11 (18%)	Stimulation; no additive or synergistic responses
	5/11 (45%)	Inhibition

Acknowledgments:
This work was supported by the Dutch Cancer Society and by the Pediatric Oncology Center Rotterdam. We thank J.W.B. Moll (Dept. of Immunology, Erasmus University Rotterdam, The Netherlands) for assistance with data analysis.

References

1. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J Immunol 1989;142(11):3875.
2. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 1988;333(6173):571.
3. Saeland S, Duvert V, Pandrau D, et al. Interleukin-7 induces the proliferation of normal human B-cell precursors. Blood 1991;78(9):2229.
4. Skjonsberg C, Erikstein BK, Smeland EB, et al. Interleukin-7 differentiates a subgroup of acute lymphoblastic leukemias. Blood 1991;77(11):2445.
5. Rosenthal P, Rimm IJ, Umiel T, et al. Ontogeny of human hematopoietic cells: analysis utilizing monoclonal antibodies. J Immunol 1983;131(1):232.
6. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Expression of cell surface markers after human B lymphocyte activation. Proc Natl Acad Sci U S A 1981;78(6):3848.
7. Clark E, Ledbetter J. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proc Natl Acad Sci U S A 1986;83:4494.
8. Clark EA, Shu G. Activation of human B cell proliferation through surface Bp35 (CD20) polypeptides or immunoglobulin receptors. J Immunol 1987;138(3):720.
9. Clark EA, Shu G, Ledbetter JA. Role of the Bp35 cell surface polypeptide in human B-cell activation. Proc Natl Acad Sci U S A 1985;82(6):1766.
10. Golay JT, Clark EA, Beverley PC. The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle. J Immunol 1985;135(6):3795.
11. Smeland E, Godal T, Ruud E, et al. The specific induction of myc protooncogene expression in normal human B cells is not a sufficient event for acquisition of competence to proliferate. Proc Natl Acad Sci U S A 1985;82(18):6255.
12. Oettgen HC, Bayard PJ, Van Ewijk W, Nadler LM, Terhorst CP. Further biochemical studies of the human B-cell differentiation antigens B1 and B2. Hybridoma 1983;2(1):17.
13. Tedder TF, Schlossman SF. Phosphorylation of the B1 (CD20) molecule by normal and malignant human B lymphocytes. J Biol Chem 1988;263(20):10009.
14. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. J Cell Biol 1993;121(5):1121.
15. Deans JP, Schieven GL, Shu GL, et al. Association of tyrosine and serine kinases with the B cell surface antigen CD20. Induction via CD20 of tyrosine phosphorylation and activation of phospholipase C-gamma 1 and PLC phospholipase C-gamma 2. J Immunol 1993;151(9):4494.
16. Clark E, Yip T, Ledbetter J, et al. CDw40 and BLCa-specific monoclonal antibodies detect two distinct molecules which transmit progression signals to human B lymphocytes. J Immunol 1988;18:451.
17. Paulie S, Rosen A, Ehlin-Henriksson B, et al. The human B lymphocyte and carcinoma antigen CDw40, is a phosphoprotein involved in growth signal transduction. J Immunol 1989;142:590.

18. Young L, Dawson C, Brown K, Rickinson A. Identification of a human epithelial cell surface protein sharing an epitope with the C3d/Epstein-Barr virus receptor molecule of B lymphocytes. International Journal of Cancer 1989;43:786.
19. Uckun F, Gajl-Peczalska K, Myers D, Jaszcz W, Haissig S, Ledbetter J. Temporal association of CD40 antigen expression with discrete stages of human B-cell ontogeny and the efficacy of anti-CD40 immunotoxins against clonogenic B-lineage acute lymphoblastic leukemia as well as B-lineage non-Hodgkin's lymphoma cells. Blood 1990;76:2449.
20. Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science 1991;251(4989):70.
21. Crawford DH, Catovsky D. In vitro activation of leukaemic B cells by interleukin-4 and antibodies to CD40. Immunology 1993;80(1):40.
22. Defrance T, Vanbervliet B, Durand I, Briolay J, Banchereau J. Proliferation and differentiation of human CD5+ and CD5- B cell subsets activated through their antigen receptors or CD40 antigens. Eur J Immunol 1992;22(11):2831.
23. Maliszewski CR, Grabstein K, Fanslow WC, Armitage R, Spriggs MK, Sato TA. Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: cooperative effects of cytokines. Eur J Immunol 1993;23(5):1044.
24. Spriggs MK, Armitage RJ, Strockbine L, et al. Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J Exp Med 1992;176(6):1543.
25. Gordon J, Millsum MJ, Guy GR, Ledbetter JA. Synergistic interaction between interleukin 4 and anti-Bp50 (CDw40) revealed in a novel B cell restimulation assay. Eur J Immunol 1987;17(10):1535.
26. Ledbetter JA, Shu G, Gallagher M, Clark EA. Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen BP50 (CDW40). J Immunol 1987;138(3):788.
27. Larson AW, LeBien TW. Cross-linking CD40 on human B cell precursors inhibits or enhances growth depending on the stage of development and the IL costimulus. J Immunol 1994;153(2):584.
28. Saeland S, Duvert V, Moreau I, Banchereau J. Human B cell precursors proliferate and express CD23 after CD40 ligation. J Exp Med 1993;178(1):113.
29. Touw I, Groot-Loonen J, Broeders L, et al. Recombinant hematopoietic growth factors fail to induce a proliferative response in precursor B acute lymphoblastic leukemia. Leukemia 1989;3(5):356.
30. Madsen M, Johnsen H, Hansen P, Christiansen S. Isolation of human T and B lymphocytes by E rosette gradient centrifugation. Characterization of the isolated subpopulations. J Immnunol Meth 1980;33:323.
31. van Dongen JJ, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33(5-6):298.
32. Delwel R, Salem M, Pellens C, et al. Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum-free culture system. Blood 1988;72:1944.
33. Touw I, Pouwels K, van Agthoven T, et al. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 1990;75(11):2097.
34. Law C, Wormann B, Lebien T. Analysis of expression and function of CD40 on normal and leukemic human B cell precursors. Leukemia 1990;4:732.

35. Golay J, Capucci A, Arsura M, Castellano M, Rizzo V, Introna M. Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. Blood 1991;77:149.

Chapter 4

Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor α chain

Dorien C. van der Plas[=], Frans Smiers[@,=], Karin Pouwels[=], Lies H. Hoefsloot[=], Bob Löwenberg[=] and Ivo P. Touw[=].

[=]Department of Hematology, Dr Daniel den Hoed Cancer Center and Institute of Hematology, Erasmus University, [@]Sophia Children's Hospital, Rotterdam, The Netherlands

Abstract

Interleukin-7 (IL-7) stimulates the proliferation of normal and leukemic B and T cell precursors and T lymphocytes. Activation of the JAK/STAT pathway has been implicated in IL-7R signaling. We investigated which STAT complexes are formed upon stimulation of B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells with IL-7. Gel retardation assays with STAT-binding oligonucleotides showed that IL-7 induces the formation of two major STAT complexes in BCP-ALL cells. Supershifts with anti-STAT antibodies identified these as STAT1 and STAT5 complexes. This pattern of STAT activation was seen in all BCP-ALL cases that respond to IL-7 in proliferation assays.

IL-7 also induced STAT/DNA binding in BCP-ALL cases that failed to proliferate in response to IL-7, suggesting that the ability of IL-7R to activate the JAK/STAT pathway per se is not sufficient for proliferation induction. To determine the contribution of the cytoplasmic domain of the IL-7 receptor α chain (IL-7Rα) to activation of STAT proteins, transfectants of the murine pro-B cell line BAF3 were made that express chimeric receptors consisting of the extracellular domain of human granulocyte colony-stimulating factor receptor (G-CSF-R) and the transmembrane and intracellular domains of human IL-7Rα. Activation of the chimeric G-CSF-R/IL-7Rα with G-CSF resulted in a full proliferative response and induced the phosphorylation of JAK1 but not JAK2. Major STAT complexes activated by G-CSF-R/IL-7Rα contained STAT1 or STAT5, while some formation of STAT3-containing complexes was also seen. These findings establish that STAT1 and STAT5, and possibly STAT3, are activated upon stimulation of precursor B cells with IL-7. The data further indicate that the IL-7Rα chains are directly involved in the activation of JAKs and STATs and have a major role in proliferative signaling in precursor B cells.

4.1 Introduction

Interleukin 7 (IL-7) is a growth factor for B and T cell precursors and mature T-lymphocytes[1]. IL-7 also stimulates the proliferation of leukemic B cell precursors (BCP) in certain cases of acute lymphoblastic leukemia (BCP-ALL)[2,3]. The effects of IL-7 on BCP-ALL cells are diverse. Only in a subgroup of patients, IL-7 induces a proliferative response[3]. This heterogeneity cannot be ascribed to variations in IL-7 receptor expression, nor does it correlate with the maturation status of the ALL cells[4]. These observations suggest that differences in the expression or function of molecules involved in IL-7 signal transduction determine the variation of IL-7 responsiveness among BCP-ALL patients.

The IL-7 receptor (IL-7R) consists of at least two distinct subunits, a unique α chain, and a common γ chain (γ_c) also present in IL-2R, IL-4R, IL-9R, IL-13R and IL-15R complexes[5]. Chemical cross-linking with radioactive IL-4 and IL-7 indicated that the γ_c chain associates with IL-4R and IL-7Rα chains via binding to the respective ligands[6-9]. The cytoplasmic region of the γ_c chain is required for IL-7-induced proliferation[10,11]. After stimulation of T cells or B cell precursors with IL-7, a number of cellular proteins are rapidly phosphorylated on tyrosine residues[12].

Tyrosine kinases of the Janus kinase (JAK) family play a crucial role in mitogenic signaling from a variety of hematopoietic growth factor (HGF) and cytokine receptors[13-18]. JAK proteins are phosphorylated within minutes after binding of HGFs to their respective receptors. The JAKs then phosphorylate proteins known as signal transducers and activators of transcription (STATs). Activated STAT proteins form complexes that translocate to the nucleus, where they interact with specific DNA sequences and control gene expression. IL-7 has been shown to activate both JAK1 and JAK3 in T cells[16]. It was demonstrated that JAK3 is activated via binding to the γ_c chain. By analogy with the IL-2R, it was suggested that JAK1 is activated via binding to the IL-7Rα chain[16]. Zheng et al. have recently reported that STAT1 is tyrosine-phosphorylated upon stimulation of a helper-T cells with IL-7[19]. In contrast, other investigators have shown that STAT3 and STAT5, but not STAT1, are activated by IL-7 in peripheral blood lymphocytes (PBL)[20]. Peptide competition experiments performed by this group further suggested that a domain containing a tyrosine present in the membrane-distal cytoplasmic region of the IL-7Rα protein serves as a docking site for STAT proteins[20]. No information is available regarding the activation of STAT proteins by IL-7 in precursor B cells.

In this study, we have examined which STATs are activated by IL-7 in leukemic B cell precursors. We first show that both STAT1 and STAT5 are activated upon stimulation of human BCP-ALL cells with IL-7. By using transfectants of the murine pro-B cell line BAF3, we then demonstrate that chimeric G-CSF-R/IL-7Rα

proteins, containing the transmembrane and cytoplasmic domains of IL-7Rα, are capable of transducing proliferative signals, activating JAK1, and inducing the formation of STAT1-, STAT5- and possibly also STAT3-containing complexes.

4.2 Materials and methods

Isolation of CP-ALL cells were isolated from bone marrow by Ficoll-Hypaque (Nygaard, Oslo, Norway) density centrifugation. T cells were removed from the ALL cell samples by E-rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells, followed by sedimentation through Ficoll-Hypaque[21]. Monocytes were removed by adherence to plastic petri dishes at 37°C for 1 h. Informed consent was obtained in all cases. Cells were used after cryopreservation using a controlled-freezing apparatus (Planer Biomed, Sunbury-on-Thames, UK).

Generation of IL-7Rα and G-CSF-R/IL7Rα expression constructs. A 1.6 Kb EcoRI cDNA fragment (clone 20) in pBluescript (pBS), encoding full-length human IL-7Rα cDNA, was generously provided by Drs Steven Ziegler and Linda Park (Immunex Corp., Seattle, WA). The 1.6 Kb insert was cut out of pBS with EcoRI, blunted, and ligated into the retroviral expression vector pLNCX, after linearization with HpaI. A chimeric receptor composed of the extracellular part of human G-CSF-R and the transmembrane and intracellular parts of human IL-7Rα was made by recombinant PCR methods essentially as described by Skoda et al[22]. First, sequences encoding nucleotides 129-1675 of the extracellular domain of G-CSF-R were amplified from G-CSF-R cDNA using forward primer GRFR1 (CAAGATCACAAAGCTGGTGAA-CATC) and reverse primer GRRV2 (GAAGATCCTCATAGAGCTGAAAG). The resulting product was cloned into the HincII site of pBS and subsequently excized with HindIII and BglII. In the second PCR, the remainder of the extracellular domain of G-CSF-R (nucleotides 1663-2045) was amplified using forward primer GFFR2 (TGTGATCATCGTGACTCCCTT) that includes a BclI site, and the chimeric G-CSFR/IL-7Rα primer GIL7RRV2 as reverse primer (GGTTAGTAAGATAGGGT-GTAGCTCCGACCC). In the third PCR, sequences encoding the transmembrane and intracellular domains of IL-7Rα were amplified. Human IL-7Rα cDNA was amplified using the following primers: the chimeric G-CSF-R/IL-7Rα primer GIL7RFR2(GGGTCGGAGCTACACCCTATCTTACTAACC) and IL7RRV3 (TTGT-CGCTCACGGTAAGTTCA). The fourth PCR, with primers GRFR2 and IL7RRV3, served to join the PCR products of PCR 2 and 3. The latter PCR product was ligated into the Hi ncII site of pBS and then cut out with BclI and ClaI. Finally, the PCR products of the first PCR (HindIII-BglII fragment) and of the fourth PCR (BclI-ClaI

fragment) were triple ligated into pLNCX (HindIII - ClaI). The resulting product was subjected to nucleotide sequencing to verify in frame ligation of the G-CSF-R and IL-7Rα sequences.

Generation of stable BAF3 transfectants. The BAF3 cells were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS) and an optimal concentration of chinese hamster ovary (CHO) cell-derived recombinant murine IL-3 (provided by Dr. J.N. Ihle, Memphis, TN). pLNCX vectors containing either the full-length IL-7Rα or G-CSF-R/IL-7Rα cDNA were transfected into BAF3 murine pro B cells by electroporation[23]. Stably-transfected clones expressing G-CSF-R/IL-7Rα were first selected on the basis of G418 resistance (1 mg/mL, Gibco-BRL). Subsequently, flow cytometric analysis (FACScan, Becton-Dickinson, Sunnyvale, CA) with antibodies against the extracellular domain of human G-CSF-R[24] was performed to determine the expression of the chimeric receptor proteins on the cell membrane. BAF3 transfectants expressing IL-7Rα were selected for their response to IL-7 in RPMI 1640 medium supplemented with 10% FCS and 100 U/mL human IL-7. Expression of IL-7Rα on BAF3/IL-7Rα cells was verified by FACScan analysis using anti-human IL-7Rα monoclonal antibody M21 (Immunex Corp.).

DNA synthesis and cell proliferation assays. DNA synthesis was assayed by ^{3}H-thymidine (^{3}H-TdR) uptake. Cells (10^4) were incubated in triplicate in 100 µL of 10% FCS-RPMI culture medium supplemented with titrated concentrations of human IL-7, human G-CSF or 10 ng/mL murine IL-3 in 96-well plates for 24 h. Twelve hours before cell harvest, 0.1 µC of ^{3}H-TdR (2 Ci/mM; International Amersham, UK) was added to each well. Radioactivity was measured by liquid scintillation counting. To quantify cell proliferation in suspension culture, 2.5×10^6 cells were incubated in 25-mL culture flasks in 5 mL culture medium supplemented with growth factors at the indicated concentrations, or without factors. Viable cells were counted on the basis of trypan blue exclusion.

JAK1 and JAK2 immunoprecipitations. BAF3 parental cells and transfectants were deprived of serum and factors and incubated in RPMI 1640 medium for 4 hours at 37°C. Then, 2×10^7 cells were incubated for 10 minutes in 4 mL RPMI containing 10 µM sodium orthovanadate (Na_3VO_4) either without factor, or with murine IL-3 (1 µg/mL), human IL-7 (100 ng/mL) or human G-CSF (1 µg/mL). Reactions were stopped by the addition of ice-cold phosphate buffered saline (PBS) containing 100 µM Na_3VO_4. Subsequently, the cells were centrifuged and lysed on ice by the addition of 500 µL lysis buffer (0.5% Triton X-100, 10% glycerol, 50 mM Tris HCl

pH 8.0, 200 mM NaCl, 50 mM NaF, 3 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mM Na_3VO_4, 1 mM Pefabloc (Boehringer, Mannheim, Germany). Cell lysates were cleared by centrifugation for 30 minutes at 10,000 x g and incubated overnight with anti-JAK1 or anti-JAK2 antiserum (gifts from Dr. J.N. Ihle), followed by the addition of Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) for 1 hour at 4°C. Loaded beads were washed five times with ice-cold lysis buffer. Bound proteins were released from the beads by boiling for 5 minutes in sample buffer (10% glycerol, 5% ß mercaptoethanol, 3% SDS, 0.1 M Tris HCl pH 6.8, 0.01% bromphenolblue). After separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels, proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were blocked for 1 hour at 37°C with 0.3% (v/v) Tween-20 in Tris buffered saline (TBS), and incubated overnight at 4°C with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), followed by a 1 hour incubation at room temperature with rabbit-anti-mouse Ig coupled to horseradish peroxidase (RAMPO; DAKO A/S, Glostrup, Denmark). The proteins were visualized by enhanced chemoluminescence (ECL; DuPont, Boston, MA). Subsequently, membranes were stripped in 62.5 mM Tris HCl pH 6.7, 100 mM ß-mercaptoethanol, and 2% SDS for 30 minutes at 50°C, incubated with anti-JAK1 or anti-JAK2 antibody for 1 hour, followed by incubation in peroxidase-conjugated swine-anti-rabbit Ig (SWARPO; DAKO A/S) and subjected to ECL.

Preparation of nuclear extracts. Methods were essentially as described in reference 25. In short: BAF3 parental cells and transfectants were deprived of serum and growth factors for 4 hours. Cells were stimulated for 15 minutes in RPMI without factor or in the presence of murine IL-3 (1 µg/mL), human G-CSF (1 µg/mL) or human IL-7 (100 ng/mL). Stimulation was terminated by the addition of ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. Cells ($50x10^6$) were lysed in 250 µL hypotonic buffer (20 mM Hepes, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 0.125 – µM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.2 % Nonidet P-40, 1 mM DTT, 0.5 µg/mL aprotinin, 0.5 µg/mL leupeptin, 0.5 µg/mL bacitracin, 0.5 µg/mL iodoacetamide, and 1 mM Pefabloc). Nuclei were spun down at 15,000 g for 30 seconds and proteins were extracted by rocking incubation for 30 minutes at 4°C in 75 µL high-salt buffer (hypotonic buffer with 420 mM NaCl, and 20% glycerol). Insoluble materials were removed by centrifugation at 20,000 g at 4°C for 20 minutes.

Nuclear extracts of BCP-ALL cells were prepared from cryopreserved bone marrow or blood samples. First, cells were cultured for 15 hours in serum free medium (10% BSA, 80 mg/mL transferrine, 20 ng/mL sodium-selenite, 2.2 mg/mL linoleic acid, 2.8 mg/mL cholesterol, 7 µL/mL ß-mercaptoethanol, and 10 U/mL

insulin). Cells ($5x10^6$) were then incubated for 15 minutes in serum free medium with human IL-3 (100 ng/mL), with human IL-7 (100 ng/ml), or without factor. Stimulation was stopped by adding ice-cold PBS containing 5 mM NaF and 1 mM Na_3VO_4. Nuclear extracts were prepared as described above for BAF3 cells and transfectants except that the volumina were adapted for lower cell numbers.

Gel retardation assays with STAT binding oligonucleotides. Nuclear extracts were incubated with double stranded g-32ATP end-labeled m67 oligonucleotides (5'CATTTCCCGTAAATC3')26 or ß-casein oligonucleotides (5'AGATTTCTAGGAA TTCAATCC3')27. It has been shown previously that m67 interacts with STAT1 and STAT328, whereas the ß-casein probe specifically binds STAT529. Binding reactions were performed for 30 min at room temperature in 20 mL reaction volume containing 3 mL nuclear extract, 1 mL dIdC (2 mg/mL), 11 mL H2O, 4 ml 5 x binding buffer (50 mM Hepes pH 7.6-7.8, 85 mM NaCl, 15 mM NaMoO4, 4.25 mM DTT, and 25% glycerol)17 and 1 mL labeled double stranded oligonucleotides (10.2 ng/mL). The reaction mixtures were fractionated by electrophoresis on 5% polyacrylamide gels. In supershift experiments, 2 mL of either anti-STAT1, anti-STAT3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or 1 mL anti-STAT5 antibodies (provided by Dr. B. Groner, Freiburg, Germany) were added to the reaction mixture and incubated for 1 hour on ice, before the addition of the radiolabeled oligonucleotides.

4.3 Results

IL-7-induced activation of STAT complexes in BCP-ALL cells. Activation of STATs was analyzed in human BCP-ALL cells from 13 patients. The series included 5 cases in which a proliferative response to IL-7 was observed in ^{3}H-TdR uptake assays, and 8 cases in which IL-7 failed to increase DNA synthesis. In gel retardation experiments using m67 oligonucleotides, IL-7 induced a single DNA-binding complex in all cases (Fig. 1a, Table 1). Supershift assays with anti-STAT1 antibodies demonstrated that this complex contains STAT1 (Fig. 1b). Binding of STAT3 to m67 was not detected. Gel shifts with STAT5- binding ß-casein oligonucleotides showed that IL-7 induced the formation of a single DNA-binding complex (Fig. 2a).

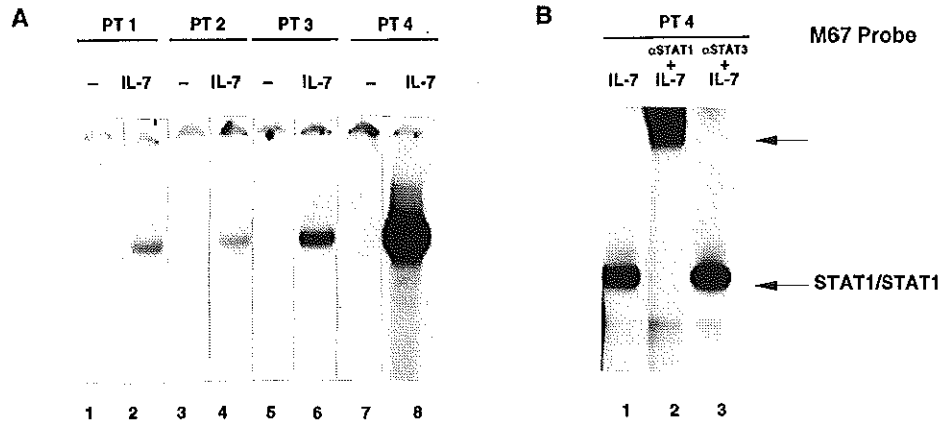

Figure 1 a) Gel retardation analysis of IL-7-induced protein binding to ^{32}P-labeled m67 oligonucleotides in nuclear extracts from 4 BCP-ALL patients (PT1-PT4). After serum and growth factor deprivation, cells were stimulated without factor(-) or with human IL-7; b) Supershift assay of patient 4 showing that the IL-7-induced STAT/m67 complex contains STAT1 but not STAT3. The upper arrow indicates the position of the supershifted STAT1/m67 complex (lane 2).

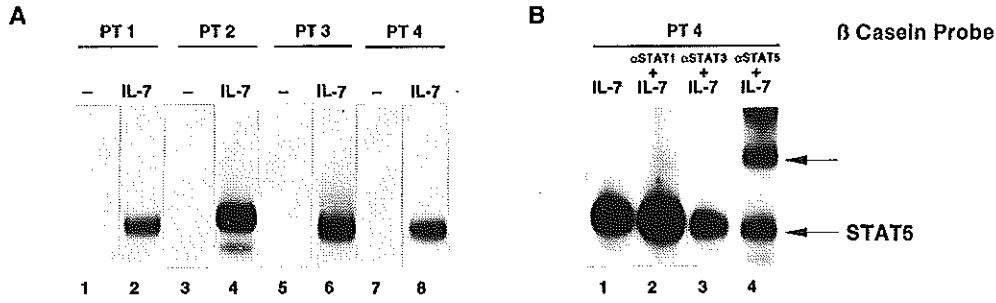

Figure 2 a) Gel retardation analysis of IL-7-induced protein binding to ^{32}P-labeled ß-casein oligonucleotides in nuclear extracts from 4 BCP-ALL patients (PT1-PT4). After serum and growth factor deprivation, cells were stimulated without factor (-) or with human IL-7; b) Supershift assay of patient 4 showing that the IL-7-induced STAT/ß-casein complex contains STAT5. The upper arrow indicates the position of the supershifted STAT5/ß-casein complex (lane 4).

As expected, this complex could be supershifted with anti-STAT5, but not with anti-STAT1 or anti-STAT3 antibodies (Fig. 2b). IL-7 activated STAT5 in all BCP-ALL cases (n=5) in which a proliferative response to IL-7 was observed, and in 4 of the 8 cases that failed to respond to IL-7 in DNA synthesis assays (Table 1).

Table 1 IL-7-induced STAT activation in BCP-ALL cells

BCP-ALL patient no.	Immunologic subtype	IL-7 response (DNA-synthesis assay)	STAT1 activation induced by IL-7*	STAT5 activation induced by IL-7#
1	common ALL	no	+	+
2	null ALL	yes	+	+
3	common ALL	yes	+	+
4	common ALL	yes	+	+
5	common ALL	no	+	-
6	common ALL	no	+	-
7	pre-B ALL	no	+	-
8	pre-B ALL	no	+	+
9.	common ALL	no	+	+
10	common ALL	no	+	+
11	pre-B ALL	no	+	-
12	common ALL	yes	+	+
13	common ALL	yes	+	+

* determined by gel retardation assays using m67 oligonucleotides.

\# determined by gel retardation assays using ß-casein oligonucleotides.

Proliferative responses of BAF3/IL-7R and BAF3/Ch transfectants to IL-7 and G-CSF. First, the abilities of IL-7Rα and chimeric G-CSF-R/IL-7Rα to transduce proliferative signals in BAF3 cells were studied in ^{3}H-TdR uptake assays (Fig. 3). BAF3/IL-7R cells responded to IL-7 (but not to G-CSF) in a dose dependent fashion. Plateau stimulation was reached at concentrations between 100 and 200 ng/mL IL-7. Conversely, BAF3/Ch cells showed a dose dependent response to G-CSF, which reached a plateau at 20-50 ng/mL, but completely lacked responsiveness to IL-7. BAF3 parental cells did neither respond to IL-7 nor to G-CSF. Subsequently, proliferative responses were studied in suspension cultures. BAF3 parental cells died within four days in cultures containing either IL-7 or G-CSF (Fig. 4a). In contrast, BAF3/IL-7R and BAF3/Ch cells survived and proliferated continuously at optimal concentrations of IL-7 and G-CSF, respectively (Fig. 4b and c).

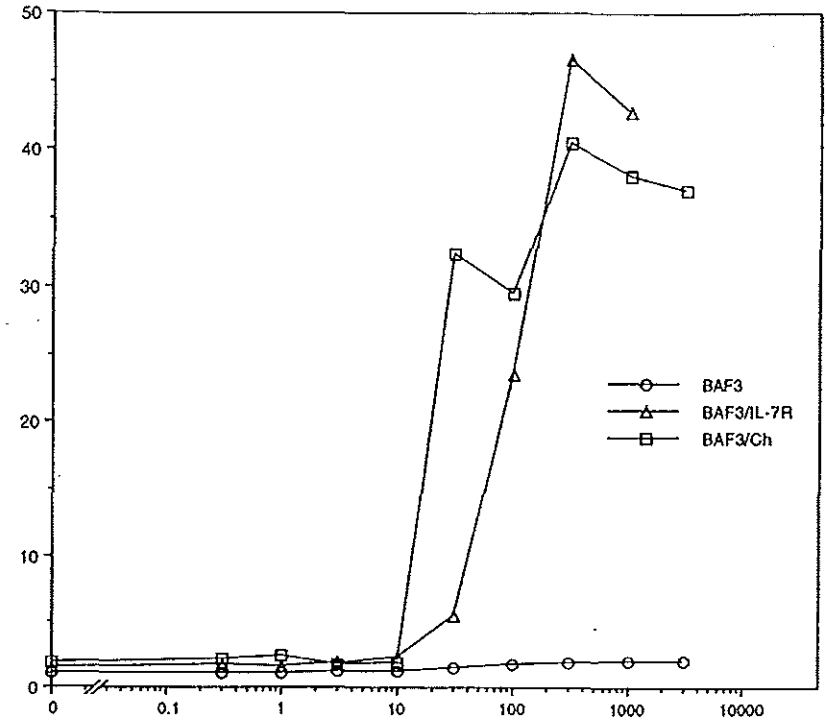

Figure 3 ^{3}H-TdR uptake in BAF3 parental cells BAF3/Ch and BAF3/IL-7R transfectants in response to titrated concentrations of G-CSF or IL-7, respectively. Data are expressed as percentages of maximal stimulation obtained with murine IL-3 for the individual cell clones. Data of BAF3 parental cells are from G-CSF supplemented cultures. Additional control incubations (BAF3 + IL-7; BAF3/Ch + IL-7; BAF3/IL-7R + G-CSF) did not result in increases of ^{3}H-TdR uptake over base-line levels (not shown).

Activation of STAT complexes in BAF3 transfectants. Immunoprecipitations with anti-JAK1 and anti-JAK2 antibodies on cell lysates from BAF3/IL-7R and BAF3/Ch cells are shown in Fig. 5 and Fig. 6. These experiments demonstrate that JAK1 is tyrosine-phosphorylated upon stimulation of BAF3/IL-7R cells with IL-7. In contrast, IL-7 failed to activate JAK2 in these cells. Similarly, stimulation of BAF/Ch cells with G-CSF results in the phosphorylation of JAK1 but not JAK2. Stimulation of the cells with IL-3 induced phosphorylation of JAK1 as well as JAK2. Taken together, these findings indicate that the cytoplasmic domain of IL-7Rα is capable of activation of JAK1 (but not JAK2) without the involvement of the γ_c chain.

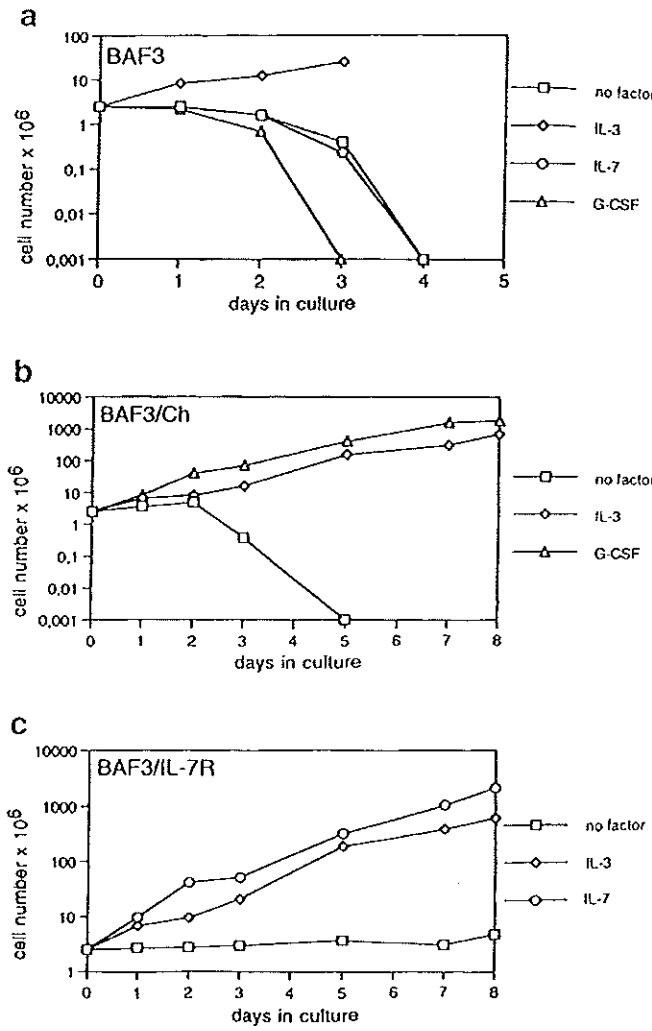

Figure 4 Suspension cultures of BAF3 parental cells (a), BAF3/Ch (b) and BAF3/IL-7R (c) transfectants. Cells were cultured at optimal concentrations of growth factors (IL-7, G-CSF or murine IL-3), or without factor.

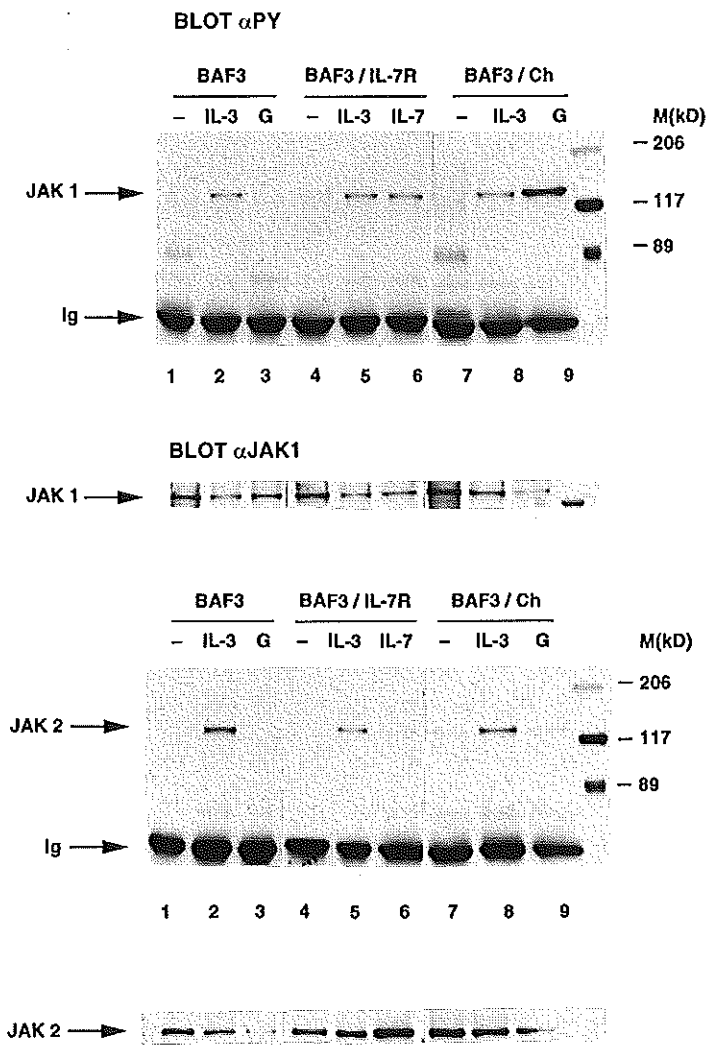

Figure 5 JAK1 immunoprecipitation in BAF3 parental cells and BAF3/IL-7R and BAF3/Ch transfectants. Serum- and growth factor-deprived cells were incubated without factor (-), with murine IL-3, with human G-CSF (G) or with human IL-7. Upper panel: Blot stained with anti-phosphotyrosine antibody 4G10. Lower panel: Same blot was stripped and reprobed with anti-JAK1 antibodies to confirm equal loading of JAK1 proteins.

Figure 6 JAK2 immunoprecipitation in BAF3 parental cells and transfectants. For details of incubation see Fig. 5. Upper panel: Blot stained with antibody 4G10. Lower panel: Same blot was stripped and reprobed with anti-JAK2 antibodies to confirm equal loading of JAK2 proteins.

Activation of STAT complexes in BAF3 transfectants. In gel retardation assays with m67 oligonucleotides, three complexes were visible upon stimulation of the BAF3 transfectants with IL-3 (Fig. 7a). These retarded complexes have previously been identified as STAT1 homodimers, STAT1/STAT3 heterodimers and STAT3 homodimers[30], from bottom to top of the gel. Surprisingly, in view of the observations in BCP-ALL cells, IL-7 consistently failed to induce detectable binding of STATs to m67 oligonucleotides in BAF3/IL-7R cells. Even a 10 fold increase of

the concentration of IL-7 failed to induce STAT/m67 binding in BAF3/IL-7R cells. In contrast, activation of the chimeric G-CSF-R/IL-7Rα resulted in the formation of the three m67-binding complexes, although only marginal amounts of STAT3 homodimeric complexes were detected. Supershift assays confirmed that the retarded bands consisted of STAT1 homodimeric, STAT1/STAT3 heterodimeric and STAT3 homodimeric complexes (Fig. 7b). Gel retardation experiments with ß-casein oligonucleotides showed that both IL-3 and IL-7 stimulation of BAF3 cells expressing the human IL-7Rα or the chimeric receptor induced DNA-binding activity with the double stranded ß-casein oligonucleotide. Supershift assays using anti-STAT5 antibodies demonstrated that this complex contains STAT5 (Fig. 8).

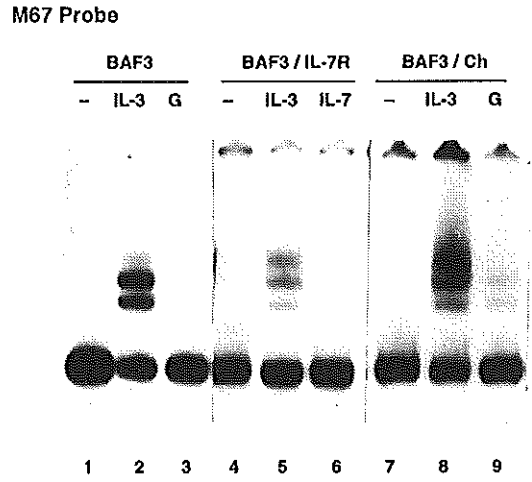

Figure 7A Gel retardation analysis (a) and supershift assays (b) with m67 oligonucleotides in BAF3 cells and transfectants; In the supershift assay anti-STAT1 and anti-STAT3 antibodies were used to detect STAT/m67 complexes in G-CSF-induced BAF3 transfectants. Antibodies against STAT5 and Tyk2 were included as controls.

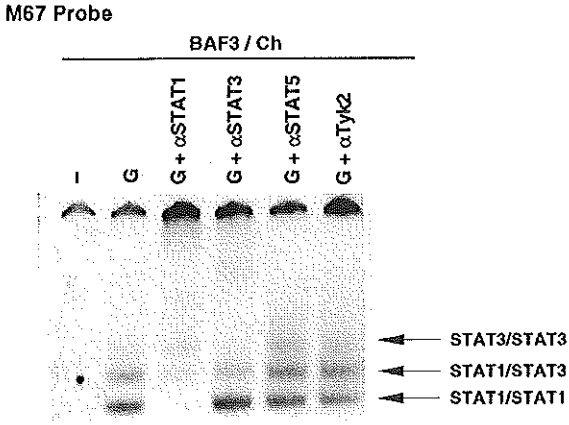

Figure 7b

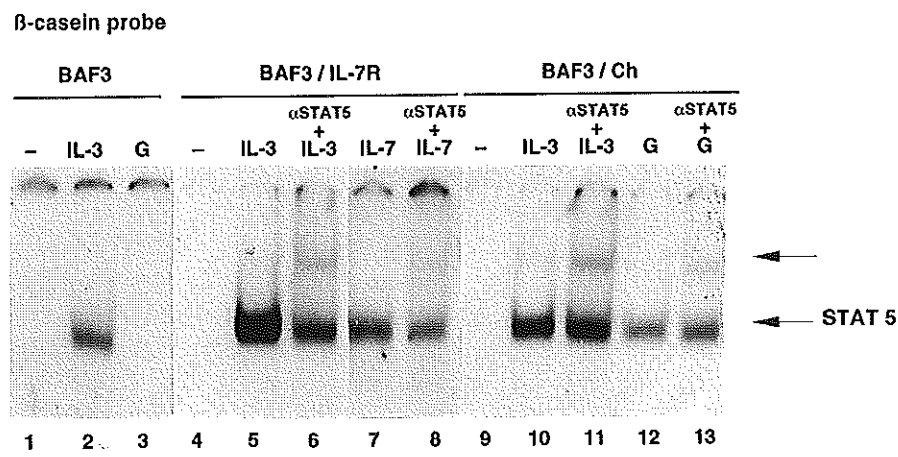

Figure 8 Gel retardation and supershift analysis with ß-casein probe in BAF3 cells and transfectants. Supershift analysis demonstrated that the STAT/ß-casein complexes contain STAT5 (lane 6, 8, 11, and 13). The upper arrow indicates the position of the supershifted STAT5/ß-casein complex.

4.4 Discussion

In this study, we have demonstrated that IL-7 induces the activation of STAT1 and STAT5 in BCP-ALL cells. In most patients, BCP-ALL cells express IL-7Rα and γ_c chains[4]. Yet, IL-7 induces a proliferative response in BCP-ALL cells in about half of the patients only[3,4]. Currently, there is no explanation for this heterogeneous response pattern. In normal B cell precursors, the ability of IL-7 to induce a full mitogenic signal correlates with the rearrangement and expression of the immunoglobulin heavy and light chain (precursor) genes[31]. In contrast, IL-7 responsiveness of BCP-ALL cells does not depend on the immunoglobulin gene status[4]. IL-7 activated STAT1 and STAT5 in all BCP-ALL cases that respond to IL-7 in proliferation assays. However, IL-7 also induced STAT activation in BCP-ALL cases that failed to proliferate in response to IL-7. Thus, activation of STATs appears not sufficient for evoking a mitogenic response in BCP-ALL cells.

IL-2, IL-4, IL-7, IL-13, and IL-15 have overlapping as well as distinct effects on peripheral blood lymphocytes (PBL). Two patterns of STAT activation by these cytokines in PBL have recently been recognized: IL-4 and IL-13 activate a STAT protein known as IL-4NAF, STF-IL-4 and IL-4 STAT, whereas IL-2, IL-7 and IL-15

activate STAT3 and STAT5 but not STAT1[20]. Our findings indicate that the latter pattern cannot simply be extrapolated to other cell types, since in BCP-ALL cells IL-7 activated STAT1 (all cases), STAT5 (most cases) but never STAT3. The reasons for these discrepancies are unknown, but could relate to differential expression of STATs and/or docking molecules involved in the recruitment of STAT proteins to the IL-7R complex. Studies of Kawahara et al. suggested that the cytoplasmic region of the γ_c chain is crucial for IL-7-induced proliferative signaling in F7 cell transfectants[10]. Whether the cytoplasmic domain of IL-7Rα is also required for growth signaling was not investigated in this study. Our experiments with BAF3 transfectants expressing chimeric G-CSF-R/IL-7Rα proteins (BAF3/Ch) indicate that signaling substrates activated via the cytoplasmic domain of IL-7Rα are also fully capable of inducing STAT activation and transducing growth responses. Again, this does not imply that STATs are essential for proliferative signaling and some experiments even directly argue against such a role of STATs. For instance, it was recently demonstrated that the cytoplasmic region of the IL-2R required for the activation of STAT5 is not essential for IL-2-induced cell proliferation[31]. Several other candidate signaling substrates that could play a role in IL-7-induced proliferative signaling have been identified. The src-like protein tyrosine kinases $p59^{fyn}$ and $p53/56^{lyn}$ and the p85 subunit of PI3-kinase are activated by IL-7. Association of $p53/56^{lyn}$ and the p85 subunit of PI3-kinase wiith the IL-7R complex occurs via (phospho-) tyrosine-containing domains located in the membrane-distal cytoplasmic region of the IL-7Rα chain, while $p59^{fyn}$ is associated with the cytoplasmic tail in a phosphotyrosine independent way [33,34]. In view of this, it is noteworthy that truncated IL-7Rα or G-CSF-R/IL-7Rα proteins, that lack this membrane-distal cytoplasmic region, completely fail to transduce proliferation signals in BAF3 cell transfectants (Smiers etal, submitted).

We consistently observed that STAT1- and STAT3-containing complexes were activated by G-CSF in BAF3/Ch cells. In contrast, such complexes were not detected in IL-7-stimulated BAF3/IL-7R cells, although IL-7 activated JAK1 and STAT5, and induced proliferation at levels comparable to those induced by G-CSF in BAF3/Ch cells. We cannot explain this discrepancy. One possibility is that activation of STAT1- and STAT3- containing complexes in the BAF3/IL-7R cells is hampered because complex formation of human IL-7Rα and murine γ_c is limited. This may be due to the very low expression of the γ_c chain[10], which would predictively result in the low activation of JAK3. In agreement with this, we were unable to detect IL-7-induced JAK3 activation in the BAF3/IL-7R cells (data not shown). An alternative more complex hypothesis is based on the assumption that the chimeric G-CSF-R/IL-7Rα complexes activated by G-CSF contain other unknown receptor structures that bind to the G-CSF-R extracellular domain, in

analogy to what has been suggested for the erythropoietin (EPO) receptor[35]. In theory, such an unknown receptor component could then be involved in signaling and, e.g., activate STAT1- and STAT3- containing complexes in the BAF3/Ch cells. However, the heteromeric EPO-R model has remained controversial[36], and for G-CSF-R no evidence supporting the existence of additional receptor chains has been put forward at all.

In summary, our results have established that IL-7 activates STAT1 and STAT5 in leukemic B cell precursors via the cytoplasmic domain of the IL-7Rα chain but suggest that signaling molecules other than STATs, activated via this receptor domain, are essential for transducing growth signals in these cells. The role of STATs in IL-7- mediated responses in normal and leukemic B cell precursors remains to be established.

References

1. Goodwin RG, Lupton S, Schmierer A, Hjerrild KJ, Jerzy R, Clevenger W, Gillis S, Cosman D, Namen AE: Human interleukin 7: Molecular cloning and growth factor activity on human and murine B-lineage cells. Proc. Natl. Sci. USA 1989; 86:302-306.
2. Touw I, Pouwels K, van Agthoven T, van Gurp R, Budel L, Hoogerbrugge H, Delwel R, Goodwin R, Namen A, Lowenberg B: Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 1990; 75: 2097-2101.
3. Skjonsberg C, Erikstein BK, Smeland EB, Lie SO, Funderud S, Beiske K, Blomhoff HK: Interleukin-7 differentiates a subgroup of acute lymphoblastic leukemias. Blood 1991; 77: 2445-2450.
4. Smiers FJ, van Paassen M, Pouwels K, Beishuizen A, Hählen K, Löwenberg B, Touw IP: Heterogeneity of proliferative responses of human B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells to interleukin-7 (IL-7): no correlation with immunoglobulin gene status and expression of IL-7 receptor or IL-2/IL-4/IL-7 receptor common g chain genes. Leukemia 1995; 9: 1039-1045.
5. Taniguchi T: Cytokine signaling through nonreceptor protein tyrosine kinases. Science 1995; 268: 251-255.
6. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, Leonard WJ: Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science 1993; 262: 1877-1883.
7. Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S, Sugamura K: Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. Science 1994; 263: 1453-1454.
8. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri RK, Paul WE, Leonard WJ: Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science 1993; 262: 1880-1883.
9. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K, Sugamura K: Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. Science 1993; 262: 1874-1877.
10. Kawahara A, Minami Y, Taniguchi T: Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2, IL-4, and IL-7 signaling. Mol and Cell Biol 1994; 14: 5433-5440.
11. Nakamura Y, Russell SM, Mess SA, Friedmann M, Erdos M, Francois C, Jacques Y, Adelstein S, Leonard WJ: Heterodimerization of the IL-2 receptor beta- andgamma-chain cytoplasmic domains is required for signaling. Nature 1994; 369: 330-333.
12. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, Goldman AS, Schmalstieg FC, Ihle JN, O'Shea JJ, Leonard WJ: Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science 1994; 266: 1042-1045.
13. Darnell J Jr., Kerr IM, Stark GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994; 264: 1415-1421.
14. Beadling C, Guschin D, Witthuhn BA, Ziemiecki A, Ihle JN, Kerr IM, Cantrell DA: Activation of JAK kinases and STAT proteins by interleukin-2 and interferon alpha, but not the T cell antigen receptor, in human T lymphocytes. EMBO J 1994; 13: 5605-5615.

15. Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvennoinen O: Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem Sci 1994; 19: 222-227.
16. Johnston JA, Kawamura M, Kirken RA, Chen YQ, Blake TB, Shibuya K, Ortaldo JR, McVicar DW, O'Shea JJ: Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature 1994; 370: 151-153.
17. Lamb P, Kessler LV, Suto C, Levy DE, Seidel HM, Stein RB, Rosen J: Rapid activation of proteins that interact with the interferon gamma activation site in response to multiple cytokines. Blood 1994; 83: 2063-2071.
18. Guschin D, Rogers N, Briscoe J, Witthuhn B, Watling D, Horn F, Pellegrini S, Yasukawa K, Heinrich P, Stark GR, Ihle JN, Kerr IM: A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. EMBO J 1995; 14: 1421-1429.
19. Zeng YX, Takahashi H, Shibata M, Hirokawa K: JAK3 Janus kinase is involved in interleukin-7 signal pathway. FEBS Lett 1994; 353: 289-293.
20. Lin J-X, Migone T-S, Tsang M, Friedman M, Weatherbee JA, Zhou L,Yamauchi A, Bloom ET, Mietz J, John S, Leonard WJ: The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 1995; 2: 331-339.
21. Madsen M, Johnsen HE, Hansen PW, Christiansen SE: Isolation of human T and B lymphocytes by E-rosette gradient centrifugation. Characterization of the isolated subpopulations. J Immunol Meth 1980; 33: 323-326.
22. Skoda RC, Seldin DC, Chiang MK, Peichel CL, Vogt TF, Leder P: Murine c-mpl: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. EMBO J 1993; 12: 2645-2653.
23. Palacios R, Steinmetz M: IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 1985; 41: 727-734.
24. Dong F, Brynes RK, Tidow N, Welte K, Löwenberg B, Touw IP: Mutations in the gene for the granulocyte colony-stimulating factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. N Engl J Med 1995; 333: 487-493.
25. Sadowski HB, Gilman MZ: Cell-free activation of a DNA-binding protein by epidermal growth factor. Nature 1993; 362 :79-83.
26. Wagner BJ, Hayes TE, Hoban CJ, Cochran BH: The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J 1990; 9: 4477-4484.
27. Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A: Interleukin-2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J. 1995; 14: 2527-2535.
28. Wen Z, Zhong Z, Darnell JE: Maximal activation of transcription by Stat1 and Stat3 requires both thyrosine and serine phosphorylation. Cell 1995; 82: 241-250.
29. Gouilleux F, Pallard C, Dusanter-Fourt I, Wakao H, Haldosen L-A, Norstedt G, Levy D, Groner B: Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. EMBO J 1995; 14: 2005-2013.
30. de Koning JP, Dong F, Smith L, Schelen AM, Barge RMY, van der Plas DC, Hoefsloot LH, Löwenberg B, Touw IP: The membrane-distal cytoplasmic region of human granulocyte colony-stimulating factor receptor is required for STAT3, but not STAT1 homodimer formation. Blood 1996; 87: 1335-1342.

31. Era T, Ogawa M, Nishikawa S-I, Okamoto M, Homjo T, Akagi K, Miyazaki J-I, Yamamura K-I: Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J 1991; 10: 337-342.
32. Fujii H, Nakagawa Y, Schindler U, Kawahara A, Mori H, Gouilleux F, Groner B, Ihle JN, Minami Y, Miyazaki T, Taniguchi T: Activation of Stat5 by interleukin-2 requires a carboxy-terminal region of the interleukin-2 receptor ß chain but is not essen- tial for the proliferative signal transduction. Proc Natl Acad Sci USA 1995; 92: 5482-5486.
33. Venkitaraman AR, Cowling RJ: Interleukin-7 receptor funcions by recruiting the tyrosine kinase $p59^{fm}$ through a segment of its cyto plasmic tail. Proc Natl Acad Sci USA 1992; 89: 12083-12087.
34. Venkitaraman AR, Cowling RJ: Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the a chain of the interleukin-7 receptor. Eur J Immunol 1994; 24: 2168-2174.
35. Chiba T, Nagata Y, Machide M, Kishi A, Amanuma H, Sugiyama M, Todokoro K: Tyrosine kinase activation through the extracellular domains of cytokine receptors. Nature 1993; 362: 646-648.
36. Yoshimura A, Todokoro K: Second subunit of Epo receptor? Nature 1994; 372: 137-138.

Chapter 5

Both the transmembrane domain and the C-terminal cytoplasmic region of the interleukin-7 receptor α chain are essential for proliferative signaling by G-CSF-R/IL-7Rα chimeras

Frans J. Smiers[1,3], Karin Pouwels[1], Dorien C. van der Plas[1], Bob Löwenberg[1,2] and Ivo P. Touw[1,2].

(1) Department of Hematology, Dr Daniel den Hoed Cancer Center, (2) Institute of Hematology, Erasmus University Rotterdam, (3) Sophia Children's Hospital, Rotterdam, The Netherlands

Abstract

Interleukin (IL-) 7 activates a receptor complex that consists of a specific IL-7Rα chain and a common γ chain (γ_c) shared with the receptors for IL-2, IL-4, IL-9, IL-13 and IL-15.To study which regions in the IL-7Rα are crucial for signaling, we constructed receptor chimeras (Ch) composed of the extracellular domain of the human G-CSF-R and the cytoplasmic domain of human IL-7Rα (Ch1 and Ch2) or mouse IL-7Rα (Ch3 and Ch4). The transmembrane (TM) domain was either from G-CSF-R (Ch1 and Ch3) or IL-7Rα (Ch2 and Ch4). We show that activation of Ch2 and Ch4, induced by G-CSF, induces sustained proliferation of BAF3 transfectants, whereas activation of Ch1 and Ch3 failed to do so, indicating that the IL-7Rα TM domain is of crucial importance for proliferation induction by the chimeric receptors. We then compared the signal transduction abilities of Ch1 and Ch2. Whereas Ch2 activates substrates of the JAK/STAT pathway (JAK1/STAT5), the p21Ras signaling route (SHP-2, ERK1, ERK2) and the PI-3 kinase pathway (p85 and IRS-2), none of these substrates was activated by Ch1, with the notable exception of STAT5. C-terminal truncation of 111 amino acids of Ch2, removing three conserved tyrosines of the cytoplasmic domain, also completely disrupted mitogenic signaling by this chimera. These findings indicate that both the TM domain and the C-terminal region of IL-7Rα play a critical role in signaling and suggest that STAT5 may be activated by IL-7Rα via mechanisms not involving JAKs.

5.1 Introduction

Interleukin (IL-) 7, secreted by stromal cells in the bone marrow and thymus, has profound proliferation inducing effects in both the B and T lymphoid lineages [1-3]. These effects are mediated via a heteromeric receptor complex that consists of an IL-7 specific α chain [4] and a common γ (γ_c) chain, which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 [5-9]. Both the α and γ chains of the IL-7R complex contribute to IL-7-induced intracellular signaling [10]. Recently a number of signaling molecules activated by IL-7R have been identified. These include the JAK kinases JAK1 and JAK3, STAT (signal transducer and activator of transciption) 1 and STAT5, ERK1 and ERK2, phosphatidyl inositol-3 kinase (PI-3K) and the insulin responsive substrate (IRS) 1 and IRS2 [11-29]. It is not yet clear how these molecules are activated by the IL-7R complex.
In the present study, we investigated the signaling function of chimeric receptors containing the extracellular domain of human G-CSF-R and the cytoplasmic domains of human IL-7Rα. Upon activation with G-CSF, these chimeric G-CSF-R/IL-7Rα do not recruit the γ_c chain into the receptor complex, and thus provide tools for studying the specific contribution of the IL-7Rα to IL-7-mediated cellular responses. We show that G-CSF-R/IL-7Rα chimeras are in principle fully capable of activating intracellular mechanisms controlling proliferation, suggesting that the common γ chain is not absolutely required for the transduction of mitogenic signals from IL-7R. In addition, we provide evidence to suggest that the TM domain of IL-7Rα plays a crucial role in IL-7R function. Finally, we show that mitogenic signaling by IL-7Rα is fully dependent on the presence of the membrane distal cytoplasmic region of the receptor. This is in marked contrast to other members of the cytokine receptor family, for instance G-CSF-R, EPO-R, gp130 and the common β chain of GM-CSF/IL-3/IL-5 receptors, of which it has been shown that the membrane proximal region, containing the conserved box1 and box2 sequences, is necessary and sufficient for inducing proliferation in BAF3 cells.

5.2 Materials and Methods

Generation of IL-7Rα and G-CSF-R/IL-7Rα expression constructs

A schematic representation of the receptor constructs is shown in Fig. 1. Four different chimeric receptors composed of the extracellular part of human G-CSF-R, the G-CSF-R transmembrane region (chimera 1 and 3), human- or murine IL-7Rα transmembrane region (respectively chimeras 2 and 4), and the intracellular parts

of the human- or murine IL-7Rα chain (respectively chimeras 1 and 2 and chimeras 3 and 4) were made by recombinant PCR methods essentially as described by Skoda et al [30] and reported for Ch2 in detail in [18]. First, sequences encoding nucleotides 129-1675 of the extracellular domain of G-CSF-R were amplified from G-CSF-R cDNA using forward primer GRFR1 (CAAGATCACAAAGCTGGTGAACATC) and reverse primer GRRV2 (GAAGATCCTCATAGAGCTGAAAG). The resulting product was cloned into the Hinc II site of pBS and subsequently excised with Hind III and Bgl II.

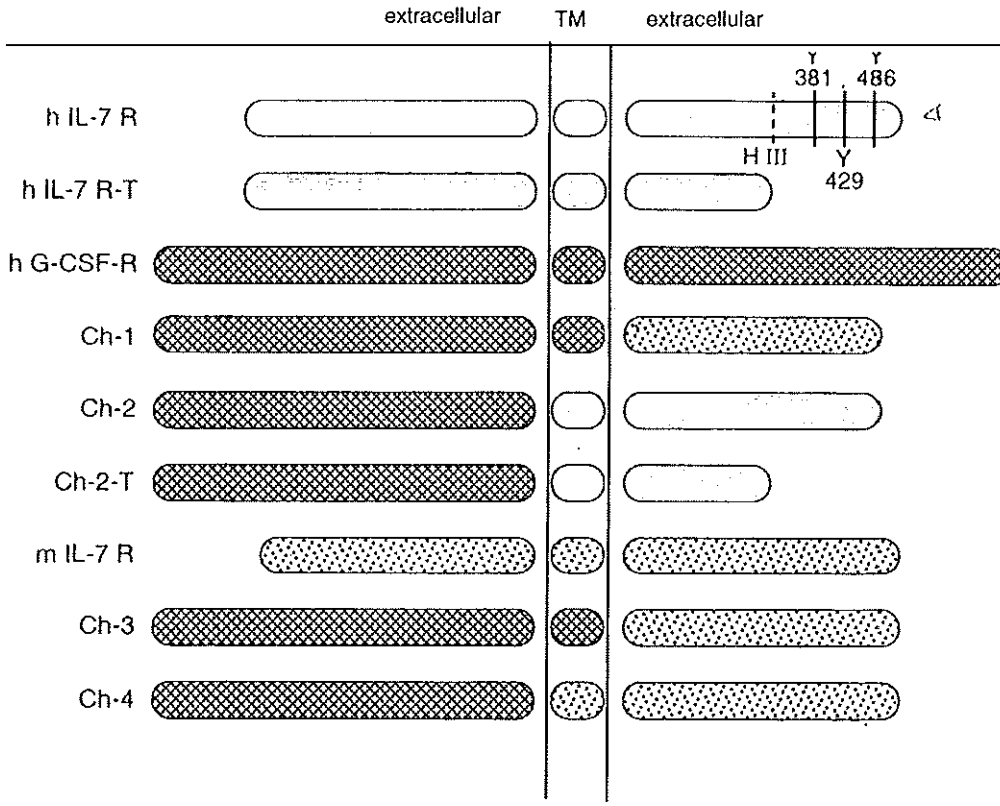

Figure 1 Schematic representation of receptor constructs. TM: transmembrane domain. HIII: HindIII restriction site. hIL-7R and mIL-7R: wild type human and murine IL-7Rα chain; hIL-7R-T: truncated human IL-7Rα mutant; hG-CSF-R: wild type hG-CSF-R; Ch1-4: chimeric G-CSF-R/IL-7Rα receptors as used in these experiments; Ch2-T: truncated form of Ch2. The indicated positions of the tyrosine (Y) residues is according to the nomenclature of Goodwin et al. [4].

In the second PCR, the remainder of the extracellular domain of G-CSF-R (nucleotides 1663-2045) was amplified using forward primer GRFR2 (TGTGATCATCGTGACTCCCTT) that includes a Bcl I site, and the reversed primers GIL-7RV1 (AGGCTTAATCCTTTTTTTGTTGGGGCTGCAACA) for chimeras 1 and 3, GIL7RRV2 (GGTTAGTAAGATAGGGTGTAGCTCCGACCC) for chimera 2, and GIL-7RRV3 (ACTTGGCAAGACAGGGTGTAGCTCCGACCC) for chimera 4.
In the third PCR, sequences encoding the different transmembrane and intracellular domains of human or murine IL-7Ra were amplified. Human IL-7Ra cDNA was amplified using the following primers: GIL-7RFR1 (TGTTGCAGCCCCA ACAAAAAAAGGATTAAGCCT) with IL-7RRV3 (TTGTCCTCACGGTAA GTTCA) for chimera 1 and with IL-7RRV1 (TTGTCGCTCACGTAGTCA) for chimera 3, GIL-7RFR2 (GGGTCGGAGCTACACCCTATCTTACTAACC) and IL-7RRV3 for chimera 2, and GIL-7RFR3 (GGGTCGGAGCTACACCCTGTCTTG CAAGT) with mIL-7RRV1 (TTGGTTGTCGATGGAAGGG) for chimera 4.
The fourth PCR, with primers GRFR2 and IL-7RRV3 (chimeras 1 and 2) and GRFR2 and IL-7RRV1 (chimeras 3 and 4) served to join the PCR products of PCR 2 and 3. The latter PCR product was ligated into the Hinc II site of pBS and then cut out with Bcl I and Cla I. Finally, the PCR products of the first PCR (Hind III-Bgl II fragment) and of the fourth PCR (BclI-ClaI fragment) were triple ligated into pLNCX (Hind III - Cla I). The resulting products were all subjected to nucleotide sequencing to verify in frame ligation of the G-CSF-R and IL-7Rα sequences. To generate Ch2-T and IL-7R-T, the respective plasmids were digested with Hind III and religated, thereby removing the 111 C-terminal amino acids (See Fig. 1) For the construction of the truncated IL-7Rα and chimera 2 receptor the Hind III site in the cytoplasmic part of the IL-7Rα chain was used. The resulting truncated receptor does no longer contain tyrosine residues.

Generation of stable BAF3 transfectants

Interleukin-3 dependent BAF3 cells were maintained in culture medium consisting of RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS) and Chinese hamster ovary (CHO) cell-derived recombinant murine IL-3 (kindly provided by Dr. J.N. Ihle, Memphis, TN). These cells were fully dependent on IL-3 and died within 18 hrs in the absence of growth factor, or in the presence of IL-7 or G-CSF. Expression constructs were transfected into BAF3 cells by electroporation [31]. Stably-transfected clones expressing G-CSF-R/IL-7Rα were first selected on the basis of G418 resistance (1 mg/mL, Gibco-BRL). Subsequently, flow cytometric analysis (FACScan, Becton-Dickinson, Sunnyvale, CA) with antibodies against the extracellular domain of human G-CSF-

R [32] was performed to determine the expression of the chimeric receptor proteins on the cell membrane. BAF3 transfectants expressing IL-7Rα were selected for their response to IL-7 in RPMI 1640 medium supplemented with 10% FCS and 100 U/mL human IL-7. Expression of IL-7Rα on BAF3/IL-7Rα cells was verified by FACScan analysis using anti-human IL-7Rα monoclonal antibody M21 (Immunex Corp.).

DNA synthesis and proliferation assays

DNA synthesis was measured in ^{3}H-thymidine (^{3}H-TdR) uptake assays. Cells (10^4) were incubated in triplicate in 100 µL of RPMI-10% FCS supplemented with titrated concentrations of human IL-7, human G-CSF or 10 ng/mL murine IL-3 in 96-well plates for 24 h. Twelve hours before cell harvest, 0.1 µC of ^{3}H-TdR (2 Ci/mM; International Amersham, UK) was added to each well. Radioactivity was measured by liquid scintillation counting. To quantify cell proliferation in suspension culture, 2.5 x 10^6 cells were incubated in 25-mL culture flasks in 5 mL culture medium supplemented with growth factors at the indicated concentrations, or without factors. Viable cells were counted on the basis of trypan blue exclusion.

Immunoprecipitation and Western blotting

BAF3 parental cells and transfectants were deprived of serum and factors by incubation in RPMI for 4 hours at 37 °C. Then, 2x10^7 cells were incubated for 10 minutes in 4 mL RPMI containing 10 µM sodium orthovanadate (Na_3VO_4) either without factor, or with murine IL-3 (1 µg/mL), human IL-7 (100 ng/mL), human G-CSF (1 µg/mL) or insulin (10 µg/ml). Reactions were stopped by addition of ice-cold phosphate buffered saline (PBS) containing 100 µM Na_3VO_4. For JAK1 immunoprecipitations, cells were centrifuged and lysed on ice by the addition of 500 µL lysis buffer (0.5% Triton X-100, 10% glycerol, 50 mM Tris HCl pH 8.0, 200 mM NaCl, 50 mM NaFcontaining a cocktail of protease inhibitors (3 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mM Na_3VO_4, 1 mM Pefabloc; Boehringer, Mannheim, Germany). Cell lysates were then cleared by centrifugation for 30 minutes at 10,000 x g and incubated overnight at 4 °C with anti-JAK1 antiserum (a gift from Dr. J.N. Ihle). For Grb2 and p85/PI3K immunoprecipitations, cells were lysed in a buffer containing 20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10 mmol/L EDTA, 100 mmol/L NaF, 1% Nonidet P-40, 1 mmol/L DTT, 10% glycerol, 2 mmol/L Na_3VO_4, and 8the cocktail of protease inhibitors mentioned above. Lysates were incubated overnight at 4°C with anti- Grb2 (Santa Cruz Biotechnology Inc.,Santa Cruz, CA) or polyclonal anti-p85PI-3K [33]. Immune complexes were coupled to Pro-

tein A-Sepharose beads (Pharmacia, Uppsala, Sweden) by incubation for 1 hour at 4°C and washed five times with ice-cold lysis buffer. Bound proteins were released from the beads by boiling for 5 min in sample buffer (10% glycerol, 5% ß mercaptoethanol, 3% SDS, 0.1 M Tris HCl pH 6.8, 0.01% bromphenolblue). After separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels, proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were blocked for 1 hour at 37°C with 0.3% (v/v) Tween-20 in Tris buffered saline (TBS), and incubated overnight at 4°C with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), followed by a 1 hour incubation at room temperature with rabbit-anti-mouse Ig coupled to horseradish peroxidase (RAMPO; DAKO A/S, Glostrup, Denmark). The proteins were visualized by enhanced chemoluminescence (ECL; DuPont, Boston, MA). Subsequently, membranes were stripped in 62.5 mM Tris HCl pH 6.7, 100 mM ß-mercaptoethanol, and 2% SDS for 30 minutes at 50°C, incubated with anti-JAK1 antibody, for 1 hour, followed by incubation in peroxidase-conjugated swine-anti-rabbit Ig (SWARPO; DAKO A/S) and subjected to ECL. Whole cell lysates were subjected to Western blotting with anti Erk1/Erk2 antibodies (Santa Cruz) and ECL detection to investigate the activation of p42/p44 MAPK. The activated forms of p42/p44 are retarded in the gel and can be recognized as such.

Preparation of nuclear extracts

Nuclear extracts were prepared as described[34]. In short: BAF3 parental cells and transfectants were deprived of serum and growth factors for 4 hours. Cells were stimulated for 15 minutes in RPMI without factor or in the presence of murine IL-3 (1 µg/mL), human G-CSF (1 µg/mL) or human IL-7 (100 ng/mL). Stimulation was terminated by the addition of ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. Cells ($50x10^6$) were lysed in 250 µL hypotonic buffer (20 mM Hepes, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 0.125 µM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.2 % Nonidet P-40, 1 mM DTT, 0.5 µg/mL aprotinin, 0.5 µg/mL leupeptin, 0.5 µg/mL bacitracin, 0.5 µg/mL iodoacetamide, and 1 mM Pefabloc). Nuclei were spun down at 15,000 g for 30 seconds and proteins were extracted by rocking incubation for 30 minutes at 4°C in 75 µL high-salt buffer (hypotonic buffer with 420 mM NaCl, and 20% glycerol). Insoluble materials were removed by centrifugation at 20,000 g at 4°C for 20 minutes.

Electrophoretic mobility shift assays (EMSA) with STAT binding oligonucleotides

Nuclear extracts were incubated with double stranded γ-^{32}ATP end-labeled ß-casein oligonucleotides (5'AGATTTCTAGGAATTCAATCC3')[31]. It has been shown previously that the ß-casein probe specifically binds STAT5 [35]. Binding reactions were performed for 30 min at room temperature in 20 µL reaction volume containing 3 µL nuclear extract, 1 µL dIdC (2 µg/mL), 11 µL H_2O, 4 µl 5 x binding buffer (50 mM Hepes pH 7.6-7.8, 85 mM NaCl, 15 mM $NaMoO_4$, 4.25 mM DTT, and 25% glycerol) [36] and 1 µL labeled double stranded oligonucleotides (10.2 ng/µL). The reaction mixtures were fractionated by electrophoresis on 5% polyacrylamide gels.

Results and discussion

Proliferation signaling in BAF3 transfectants

First, the proliferative responses of BAF3 transfectants, ectopically expressing IL-7Rα or chimeric G-CSF-R/IL-7Rα proteins, to titrated concentrations of IL-7 of or G-CSF were measured using ^{3}H-TdR uptake assays. BAF/hIL-7Rα and BAF/mIL-7Rα both responded maximally to, respectively, human and murine IL-7 at concentrations of 10 ng/ml (Fig. 2a). Similarly, BAF/Ch2 and BAF/Ch4 cells responded to G-CSF in a dose dependent fashion (Fig 2b). In contrast, the C-terminal truncation mutants of hIL-7Rα (hIL-7R-T) and Ch2 (Ch2-T) completely failed to transduce proliferative signals (Fig. 2 a,b), indicating that mitogenic signaling from IL-7Rα requires the presence of the C-terminal cytoplasmic region of the protein. Strikingly, in view of the fact that they comprised the entire IL-7Rα cytoplasmic domain, Ch1 and Ch3 were also completely unable to deliver proliferative signals, even at a concentration of 100 ng/ml of G-CSF (Fig 2b). Thus, the TM domain of IL-7Rα plays a crucial role in the signaling function of the G-CSF-R/IL7Rα chimeras.

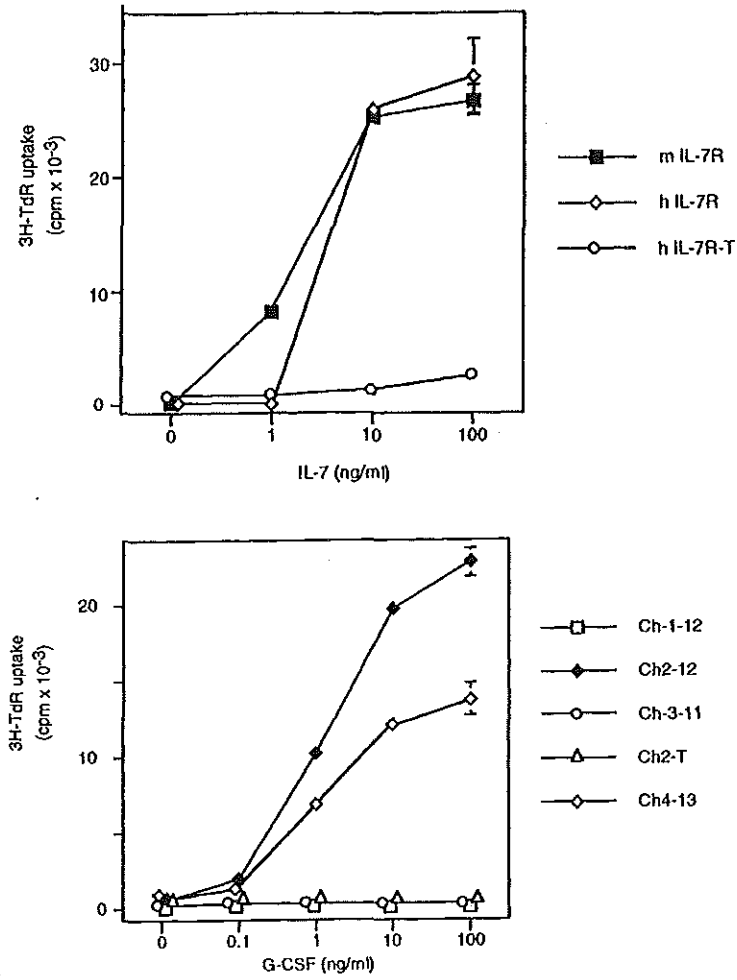

Figure 2 ^{3}H-TdR up2take in BaF3 cells expressing (a) full length forms of human- or murine IL-7Ra, and a C-terminal truncated mutant (hIL-7R-T) in response to increasing concentrations of human IL-7 and (b) expressing various G-CSF-R/IL-7Ra in response to increasing concentrations of human G-CSF.

The IL-7Rα TM domain is required for activation of JAK1, p85(PI-3K), ERK1/ERK2 and SHP-2

Next, we compared the abilities of Ch1 and Ch2 to activate signaling molecules known to be involved in IL-7-mediated cellular responses. Immunoprecipitations with anti-JAK1 followed by Western blot analysis using anti-phosphotyrosine antibodies indicated that JAK1 is activated by Ch2, but not to a detectable extent by Ch1 (Fig. 3). Immunoprecipitations with anti-Grb2 showed that, whereas activation of WT G-CSF-R and IL-3R, induces the formation of complexes of Grb2 with the

p52 Shc and Grb2 with the p70 SHP-2, activation of Ch2 induced the formation of Grb2/SHP-2 but not Grb2/Shc complexes (Fig. 4) [37]. The observation that Ch2 induced ERK1/ERK2 activation without involvement of Shc is in agreement with data from Dorsch et al., who showed that IL-7R does not activate Shc [38]. Ch1 failed to activate the formation of Grb2/SHP-2 complexes. From immunoprecipitations with anti-PI-3K (p85) antibodies, it appeared that both insulin and IL-3 induced complex formation between p85(PI-3K) and the 185 kD protein IRS-2. Co-precipitation of IRS-2 with p85 was also seen upon activation of Ch2 or IL-7R, but not Ch1 (Fig.5). Similarly, whereas Ch2 activated ERK1 and ERK2, Ch1 failed to do this completely (Fig. 6).

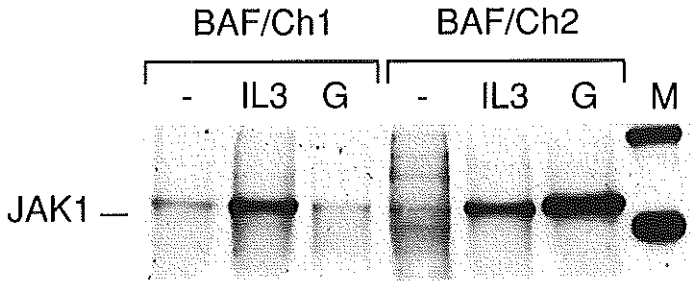

Figure 3 JAK1 immunoprecipitation in BAF/Ch1 and BAF/Ch2 cells. Serum- and growth factor deprived cells were incubated without factor (-), with murine IL-3 or with human G-CSF (G). The blot was stained with anti-phosphotyrosine antibody 4G10.

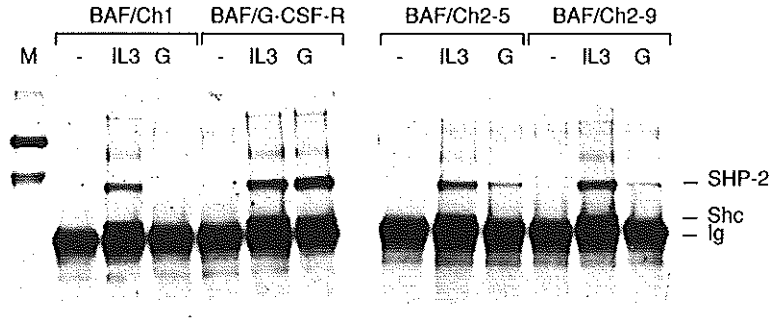

Figure 4 Grb2 immunoprecipitation in BAF/Ch1 and BAF/Ch2 (clones 5 and 9). Blots were stained with anti-phosphotyrosine antibody 4G10. Co-immunoprecipitating proteins Shc and SHP-2 are indicated by arrows.

Although little is known of possible regulatory functions of the transmembrane domain in signal transduction, a number of studies have indicated that alterations in the transmembrane domain may significantly affect the signaling function of growth factor receptors. For instance, a mutation in the transmembrane domain of the receptor encoded by the proto-oncogene neu results in the constitutive activation of its intrinsic tyrosine kinase activity[39, 40]. This mutated neu protein has altered dimerization properties, but these do not explain the constitutive activated state of the receptor, suggesting additional signaling properties of the transmembrane domain[41]. Similarly, mutations in the transmembrane domain of the common β subunit of IL-3/IL-5/GM-CSF receptors renders these receptors constitutively active[42]. Conversely, replacement of the transmembrane region of the PDGF receptor with those of other receptors abolished ligand induced activation of tyrosine kinase activity [43]. Finally, EGFR/c-ros chimeric receptors, differing only in transmembrane domains, were found to have completely opposite signaling effects depending on the transmembrane domain [44]. Our data suggest that the TM domain of IL-7Rα has a function in signaling that cannot be replaced by the G-CSF-R TM domain . Which mechanisms are specifically influenced by this domain is not yet clear. It is possible that the IL-7Rα TM domain contributes to a conformation of the cytoplasmic domain essential for JAK1 binding and activation. Alternatively, full mitogenic signaling from the IL-7R complex may depend on the formation of complexes involving muliple IL-7Rα chains and/or as yet unidentified proteins for which the integrity of the IL-7Rα TM domain is crucial.

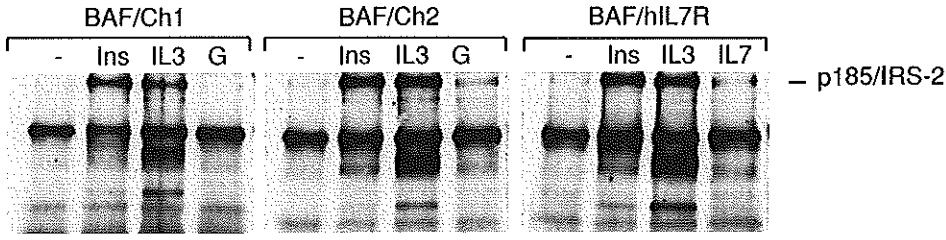

Figure 5 Immunoprecipitation with anti-p85 (PI-3K). Serum- and growth factor deprived cells were incubated without factor (-), insulin (Ins), IL-3 or human G-CSF (G). Western blotting was performed with anti-phosphotyrosine antibody 4G10.

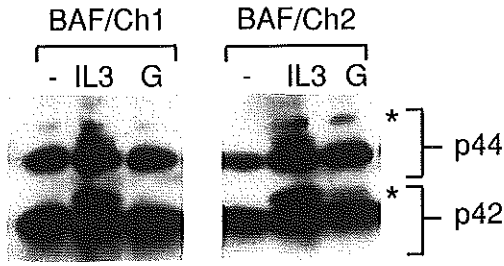

Figure 6 Western blot analysis of activation of ERK1/ERK2 (p44/p42). Activated forms of Erk1/Erk2 are retarded in the gel (marked by asterisks).

STAT5 activation by G-CSF-R/IL-7Rα chimeras fully depends on the presence of the IL-7Rα C-terminus but does not require the IL-7Rα TM domain

The abilities of the receptor mutants to activate STAT5 were analyzed by EMSA with β-casein oligonucleotides [35]. As shown in Fig. 7, activation of STAT5 by IL-7R or Ch2 is completely abolished by truncation of the C-terminus (IL7R-T and Ch2-T). Previously, it has been demonstrated that tyrosine residue (Y429) in the IL-7Rα C-terminus (Fig1) is engaged in the docking of STAT5 to the receptor [17]. Our data suggest that recruitment of STAT5 to the IL-7Rα C-terminus, possibly, but perhaps not exclusively, via Y429, is a prerequisite for IL-7-induced activation of STAT5.

Strikingly, in view of its inability to activate JAK1, Ch1 still induced STAT5 activation, indicating that this chimeric protein is not a completely 'dead' receptor and suggesting that alternative mechanisms not involving JAKs might be involved in the activation of STAT5 mediated via the cytoplasmic domain of IL-7Rα. Because neither JAK2, JAK3 or Tyk2 are activated by IL-7Rα or Ch1 and Ch2 ([18] and data not shown), these data suggest that STAT5 can be activated by IL-7Rα via mechanisms not involving JAKs. It has recently been established that other tyrosine kinases, for instance of the Src family, are also capable of tyrosine phosphorylation of STAT proteins [45-47]. Possibly, the Src-like kinase Fyn, which is constitutively associated to the IL-7Rα C-terminus, is involved in IL-7-induced tyrosine phosphorylation of STAT5 [48].

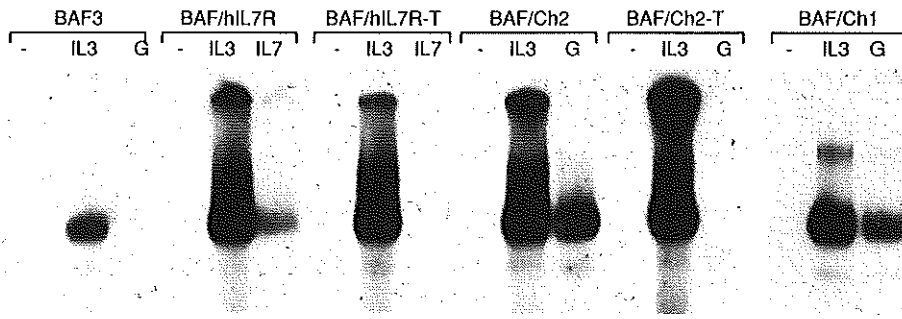

Figure 7 EMSA with β-casein probe on nuclear extracts of BAF3 cells expressing various IL-7Rα and chimeric G-CSF-R/IL-7Rα forms. Cells were deprived of growth factors and serum for 4 hours and then incubated in serum free medium without factor (-), mIL-3 (1 μg/ml), hGCSF (1 μg/ml), or hIL-7 (100 μg/ml).

Acknowledgments

We thank John de Koning for assistance with immunoprecipitations and Karola van Rooyen for graphical work. This work was supported by the Dutch Cancer Society "Koningin Wilhelmina Fonds".

References

1. Murray R, Suda T, Wrighton N, Lee F, and Zlotnik A. IL-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *Int Immunol* 1989; 1(5):526-31.
2. Conlon PJ, Morrissey PJ, Nordan RP, Grabstein KH, Prickett KS, Reed SG, Goodwin R, Cosman D, and Namen AE. Murine thymocytes proliferate in direct response to interleukin-7. *Blood* 1989; 74(4):1368-73.
3. Namen AE, Lupton S, Hjerrild K, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, and Gillis S. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* 1988; 333(6173):571-3.
4. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE, and et a. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell* 1990; 60(6):941-51.
5. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, and Leonard WJ. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor [see comments]. *Science* 1993; 262(5141):1877-80.
6. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri RK, Paul WE, and et a. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor [see comments]. *Science* 1993; 262(5141):1880-3.
7. Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D, and Anderson D. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J* 1994; 13(12):2822-30.
8. Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S-I, and Sugamura K. Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science* 1994; 263:1453-1454.
9. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M, and Sugamura K. Cloning of the gamma chain of the human IL-2 receptor. *Science* 1992; 257(5068):379-82.
10. Kawahara A, Minami Y, and Taniguchi T. Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2, IL-4, and IL-7 signalling. *Mol Cell Biol* 1994; 14(8):5433-40.
11. Johnston JA, Kawamura M, Kirken RA, Chen YQ, Blake TB, Shibuya K, Ortaldo JR, McVicar DW, and O'Shea JJ. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature* 1994; 370(6485):151-3.
12. Foxwell BM, Beadling C, Guschin D, Kerr I, and Cantrell D. Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *Eur J Immunol* 1995; 25(11):3041-6.
13. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, and et a. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science* 1994; 266(5187):1042-5.
14. Tanaka N, Asao H, Ohbo K, Ishii N, Takeshita T, Nakamura M, Sasaki H, and Sugamura K. Physical association of JAK1 and JAK2 tyrosine kinases with the interleukin 2 receptor beta and gamma chains. *Proc Natl Acad Sci U S A* 1994; 91(15):7271-5.

15. Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu ZJ, Oishi I, Silvennoinen O, Witthuhn BA, Ihle JN, and et a. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 1994; 266(5187):1045-7.
16. Zeng YX, Takahashi H, Shibata M, and Hirokawa K. JAK3 Janus kinase is involved in interleukin 7 signal pathway. *Febs Lett* 1994; 353(3):289-93.
17. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S, and et a. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 1995; 2(4):331-9.
18. Plas Dv, Smiers F, Pouwels K, Hoefsloot L, Löwenberg B, and Touw I. Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BaF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor a chain. *Leukemia* 1996; 10:1317-1325.
19. Wang LM, Keegan AD, Li W, Lienhard GE, Pacini S, Gutkind JS, Myers M Jr., Sun XJ, White MF, Aaronson SA, and et a. Common elements in interleukin 4 and insulin signaling pathways in factor-dependent hematopoietic cells. *Proc Natl Acad Sci U S A* 1993; 90(9):4032-6.
20. Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers M Jr., Backer JM, Ullrich A, White MF, and et a. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. *Embo J* 1993; 12(5):1929-36.
21. Tobe K, Matuoka K, Tamemoto H, Ueki K, Kaburagi Y, Asai S, Noguchi T, Matsuda M, Tanaka S, Hattori S, and et a. Insulin stimulates association of insulin receptor substrate-1 with the protein abundant Src homology/growth factor receptor-bound protein 2. *J Biol Chem* 1993; 268(15):11167-71.
22. Kuhné M, Pawson T, Lienhard G, and Feng G-S. The insulin receptor substrate 1 associates with the SH2-containing phophotyrosine phosphatase Syp. *J Biol Chem* 1993; 268:11479-11481.
23. Venkitaraman AR and Cowling RJ. Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. *Eur J Immunol* 1994; 24(9):2168-74.
24. Dadi HK and Roifman CM. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *J Clin Invest* 1993; 92(3):1559-63.
25. Dadi HK, Ke S, and Roifman CM. Interleukin 7 receptor mediates the activation of phosphatidylinositol-3 kinase in human B-cell precursors. *Biochem Biophys Res Commun* 1993; 192(2):459-64.
26. Seckinger P and Fougereau M. Activation of src family kinases in human pre-B cells by IL-7. *J Immunol* 1994; 153(1):97-109.
27. Sharfe N and Roifman C. Differential association of phosphatidylinositol 3-kinase with insulin receptor substrate (IRS)-1 and IRS-2 in human thymocytes in response to IL-7. *J Immunology* 1997; 159(3):1107-1114.
28. Corcoran AE, Smart FM, Cowling RJ, Crompton T, Owen MJ, and Venkitaraman AR. The interleukin-7 receptor alpha chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. *EMBO J* 1996; 15(8):1924-32.

29. Johnston JA, Wang LM, Hanson EP, Sun XJ, White MF, Oakes SA, Pierce JH, and O'Shea JJ. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases. *J Biol Chem* 1995; 270(48):28527-30.
30. Skoda R, Seldin D, Chiang M, Peichel C, Vogt T, and Leder p. Murine c-mpl: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. *Embo J* 1993; 12:2645-2653.
31. Wakao H, Harada N, Kitamura T, Mui A, and Miyajima A. Interleukin-2 and erythropoietin activate STAT5/MGF via distinct pathways. *Embo J* 1995; 14:2527-2535.
32. Dong F, Brynes R, Tidow N, Welte K, Löwenberg B, and Touw I. Mutations in the gene for the granulocyte colony-stimulation factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. *New Engl J Med* 1995; 333:487-493.
33. Ouwens D, Zon Gv, Pronk G, Bos J, Möller W, Cheatham B, Kahn R, and Maassen J. A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and p21 ras-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. *J Biol Chem* 1994; 269:33116-33122.
34. Sadowski H and Gilman M. Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature* 1993; 362:79-83.
35. Gouilleux F, Pallard C, er-Fourt I, Wakao H, Haldosen L, Norstedt G, Levy D, and Groner B. Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-STAT5 DNA binding activity. *EMBO J* 1995; 14:2005-2013.
36. Lamb P, Kessler L, Suto C, Levy D, Seidel H, Stein R, and Rosen J. Rapid activation of proteins that interact with the interferon gamma activation site in response to multiple cytokines. *Blood* 1994; 83:2063-2071.
37. de Koning J, Schelen A, Dong F, van Buitenen C, Burgering B, Bos J, Löwenberg B, and Touw I. Specific involvement of tyrosine 764 of human granulocyte colony-stimulating factor in signal transduction mediated by p145/Shc/Grb2 of p90/Grb2 complexes. *Blood* 1996; 87(1):132-140.
38. Dorsch M, Hock H, and Diamantstein T. Gene transfer of the interleukin (IL)-2 receptor beta chain into an IL-7-dependent pre-B cell line permits IL-2-driven proliferation: tyrosine phosphorylation of Shc is induced by IL-2 but not IL-7. *Eur J Immunol* 1994; 24(9):2049-54.
39. Yarden Y and Ullrich A. Growth factor receptor tyrosine kinases. *Annu.Rev.Biochem.* 1988; 57:443-478.
40. Stern D, Kamps M, and Lao H. Oncogenic activation of $p185^{neu}$ stimulates tyrosine phosphorylation in vivo. *Mol Cell Biol* 1988; 8:3969-3973.
41. Cheng G, Ye ZS, and Baltimore D. Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction. *Proc Natl Acad Sci U S A* 1994; 91(17):8152-5.
42. Jenkins BJ, D'Andrea R, and Gonda TJ. Activating point mutations in the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors suggest the involvement of beta subunit dimerization and cell type-specific molecules in signalling. *EMBO J* 1995; 14(17):4276-87.
43. Williams L. Signal transduction by the platelet derived growth factor receptor. *Science* 1989; 243:1564-1570.
44. Lund TC, Garcia R, Medveczky MM, Jove R, and Medveczky PG. Activation of STAT transcription factors by herpesvirus Saimiri Tip-484 requires p56lck. *J Virol* 1997; 71(9):6677-82.

45. Burke CL, Lemmon MA, Coren BA, Engelman DM, and Stern DF. Dimerization of the p185neu transmembrane domain is necessary but not sufficient for transformation. *Oncogene* 1997; 14(6):687-96.
46. Cao X, Tay A, Guy GR, and Tan YH. Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Mol Cell Biol* 1996; 16(4):1595-603.
47. Chaturvedi P, Sharma S, and Reddy EP. Abrogation of interleukin-3 dependence of myeloid cells by the v-src oncogene requires SH2 and SH3 domains which specify activation of STATs. *Mol Cell Biol* 1997; 17(6):3295-304.
48. Venkitaraman AR and Cowling RJ. Interleukin 7 receptor functions by recruiting the tyrosine kinase p59fyn through a segment of its cytoplasmic tail. *Proc Natl Acad Sci U S A* 1992; 89(24):12083-7

Chapter 6

In vitro proliferative response of acute lymphoblastic leukemia cells related to clinical outcome

F.J. Smiers[@,#], W.L.J. van Putten[*], K. Hählen[@], I. Touw[#,&], B. Löwenberg[#,&]

[@]Sophia Children's Hospital, [*]Dept, of Statistics, [#]Dept. of Hematology, Dr. Daniel den Hoed Cancer Center, [&]Erasmus University, Dept. of Hematology, Rotterdam, The Netherlands.

Summary

Acute lymphoblastic leukemia (ALL) is a highly heterogeneous group of diseases. Recognition of factors that predict outcome permits the identification of patients with different risks and allows for the selection of treatment adjusted to risk. Some progress has been made in the understanding of the role of hematopoietic growth factors (HGFs) that control the proliferation and differentiation of normal and leukemic B- and T-cell progenitors (BCP and BTP). Interleukin 7 is an active growth factor for normal and leukemic precursor B and T cells. IL-4 may inhibit the proliferation of normal and leukemic BCP cells. Sometimes ALL cells show spontaneous proliferative activity in culture, independent of the addition of a growth factor stimulus.

Here we present the results of an analysis in which the predictive value of spontaneous and IL-4 or IL-7 induced DNA synthesis in vitro of ALL cells from 73 patients was investigated with respect to clinical outcome. No significant relationship was found

between spontaneous or IL-7 induced or IL-4 mediated proliferation with clinical outcome.

6.1 Introduction

Acute lymphoblastic leukemia (ALL) is a neoplastic disease with uncontrolled clonal expansion of immature lymphoblastic cells that have lost the capacity for terminal maturation. ALL is a heterogeneous disease and comprises a group of conditions with distinct clinical, pathological, immunological and genetic features. Through the recognition of clinical and hematological risk factors one may adjust treatment to patients with different risks. For instance patient age, white blood cell count (WBC) at diagnosis, immunophenotype and the presence of specific cytogenetic abnormalities of the leukemias express prognostic value and may determine treatment choice.

In normal hematopoiesis hematopoietic growth factors participate in a regulating network that controls the expansion and development of hematopoietic stem cells towards blood cells of the various lineages. Distinct cell stages of maturation respond to specific hematopoietic growth factors. Interleukin 7 (IL-7) is a bone marrow stromal growth factor that may activate normal and leukemic human B and T cell precursors (BCP and BTP) although in leukemic B cell precursor cells considerable heterogeneity of responsiveness has been observed (1,2). In normal B-cell precursor cells IL-7 as single growth stimulus induces proliferation in the pre-B cell stage only. In more immature stages IL-7 synergizes with stromal factors. In mature B cells the response to IL-7 is lost (2). In leukemic BCP cells the maturational stage dependent response pattern no longer exists (2).

IL-4 is a T cell derived growth factor that may potently inhibit the proliferation in normal and leukemic B-cell precursor cells (3,4). In mature B cells IL-4 induces proliferation in synergy with simultaneous B cell receptor activation or CD40 activation. In (im)mature T cells IL-4 induces proliferation.

Proliferation in the absence of exogenous added hematopoietic growth factors (i.e. spontaneous or autonomous proliferation) may be apparent in leukemia cell culture. Spontaneous proliferation in culture has been reported both in ALL and acute myeloid leukemia (AML). In AML the ability of spontaneous proliferation in culture has been demonstrated to correlate with clinical outcome (5). It is not known whether the level of spontaneous proliferation or IL-4 and IL-7 induced proliferation of leukemic cells cells express prognostic value in ALL.

Here we present the results of a study in which in vitro proliferation parameters were related to clinical outcome in a series of pediatric and adult patients with ALL.

6.2 Methods

Patients and treatment

Marrow and blood specimens from 73 patients with pathologically and immunologically confirmed ALL referred to the University Hospital Rotterdam (Daniel den Hoed Cancer Center or the Sophia Children's Hospital) between 1988 and 1994, were analysed.
Patients younger than 15 years at time of diagnosis (n=43) have been treated according protocols of the Dutch Childhood Leukemia Study Group. Adult patients (n=30) have been treated according protocols HOVON-5 and HOVON-18 of the Dutch Hemato-Oncology Cooperative Group. Median follow up for patients still alive is 40 months (range 9-122 months). Allogeneic bone marrow transplantation has been performed in one pediatric and 6 adult patients in remission. Autologous bone marrow reinfusion was done in one adult patient.

Cell sampling

Cells were derived from the bone marrow or peripheral blood taken for diagnostic purposes following informed consent. All cell samples were isolated from bone marrow or peripheral blood by Ficoll-Hypaque (Nygaard,Oslo,Norway) density gradient centrifugation as described previously (6).
T cells were removed from B cell precursor ALL samples by E-rosette formation using 2 aminoethylisothiouronium bromide (AET)-treated sheep red blood cells, followed by sedimentation through Ficoll Hypaque (7). Monocytes were removed by adherance to plastic petri dishes for 1 hour at 37^0 C. All BCP-ALL cell samples were subjected to routine diagnostic immunopheno-typing. Leukemic cells were subclassified as null-ALL (CD10-, CD19+, CD34+, Cytoplasmic$_\mu$ -), common-ALL (CD10+, CD19+, CD34+/-, Cytoplasmic$_\mu$-) or pre-B ALL (CD10+, CD19+, CD34+/-, Cytoplasmic$_\mu$+) (8).
Cytogenetic analysis was performed on bone marrow aspirates and most often also on peririphеral blood, according standard techniques (9,10). Among 43 pediatric patients a t(9;22) was observed in 2 patients, a t(1;19) in 2 patients and translocations involving 11q23 in 5 patients. In 20 patients other cytogenetic aberrations were apparant. Eleven patients did not show cytogenetic abnormalities. In three patients the cytogenetic analysis could not be done. Among 25 adult patients (81%) the cytogenetic analysis revealed a t(9;22) in 6 patients, a translocation involving 11q23 in one patient and diverse other cytogenetic aberrations in 11 patients. In 7 patients no cytogenetic aberrations were seen. In 5 patients no adequate analysis was done.

In vitro culture and growth factors

DNA synthesis was assessed by uptake of ^{3}H-thymidine (^{3}H-TdR, specific activity 2 Ci/mmol;Amersham International, Amersham, UK) as described (11). In brief $0.2x10^5$ cells were cultured in triplicate in 96 well dishes (Greiner, Alphen a/d Rijn, The Netherlands) for 3 and 7 days in 100 µl serum free culture medium (11). Eightteen hours before harvesting on nitrocellulose filters using a Titertek cell harvester (Flow Laboratories, Isrike, UK) 0.1 µCi ^{3}H-TdR was added to the cultures. The amount of desintegrations per minute (dpm) reflects DNA synthesis. Maximum values of DNA synthesis on either day 4 or 7 were used in the analysis. Human recombinant IL-7 (gift from Immunex, Seattle, WA, USA) was added to the cultures at final concentrations of 100 U/ml. Human IL-4 (Dr. S. Clark, Genetics Institute Cambridge, Massachusetts) was added to the cultures at a 1:5000 dilution of COS cell supernatant.

Statistical analysis

IL-4 and IL-7 stimulation indices are expressed as ratios of DNA synthesis values of cultures with IL-4 or IL-7 relative to values of nonstimulated cultures (spontaneous proliferation). For purposes of tabulation and suvival curves the range of IL-4 and IL-7 ratio values were arbitrarily subdivided in 3 categories, labeled as low- (all values below the median), medium- (all values between the median and the third quartile) and high (all values above the third quartile) proliferative activities. Median for spontaneous proli-feration: 1883 desintegrations per minute (dpm). Median IL-4/spontaneous ratio: 0.8; median IL-7/spontaneous ratio: 1.4. Third quartile for IL-4/spontaneous ratio: 1.2; third quartile for IL-7/spontaneous ratio: 2.4. Mean spontaneous proliferation was 3909 dpm (lowest 37 dpm, highest 40337 dpm). Mean IL-7 induced proliferation was 6371 dpm (lowest 147 dpm, highest 51362 dpm). Mean IL-4 induced proliferation was 3676 dpm (lowest 70 dpm, highest 39275 dpm). Overall survival (OS) and event free survival (EFS) were the main endpoints. OS is defined as the time from diagnosis to death, whereas EFS is defined as the time from diagnosis to failure to attain clinical remission (CR)(time set at t_0), death in first CR (at time of death) or relapse after CR (at time of relapse). Correlations with probability to reach CR were not made, as all but 6 patients attained CR.

Patients, alive in continuous CR, were censored at the time of last contact. Survival probabilities were calculated by the method of Kaplan and Meier (12). Cox regression analysis was applied to test for association between factors and OS or EFS (13). The log transforms of spontaneous proliferation values and the ratios of the

IL-4 and IL-7 induced proliferation values with respect to spontaneous proliferation were used in these analysis to test for trend (14). Correlations of the results of proliferation tests with OS and EFS were studied with and without adjustment for age in order to correlate pediatric and adult ALL separately.

Table 1

		0-15 years	≥ 15 years
Number of cases		43	30
Sex	Male	26	21
	Female	17	9
Diagnosis*	null-ALL	2	4
	common-ALL	23	16
	pre-B-ALL	12	2
	T-ALL	6	8
WBC	≤ £50.000/ml	24	18
	>50.000/ml	19	12
	median: range:	38.7 1.70 - 684	36.7 0.96 - 386
Cytogenetic	t(9;22)	2	6
Aberrations	t(1;19)	2	0
	11q23	5	1
	other	20	11
	normal	11	7
	not determined/ no adequate analysis	3	5

* as defined in material and methods

6.3 Results

Table 2 Clinical outcome of patients

	0-15 years	≥ 315 years
Number of cases (n)	43	30
Complete Response (%)	43 (100%)	24 (80%)
Alive in continuous complete remission	29	8
Relapsed (n)	13	12
Dead in complete remission	1	4
Overall Survival[a]	75% ± 8	21% ± 10
Event Free Survival[a]	54% ± 11	19% ± 10

[a]actuarial probability at 5 years ± SE

Patient characteristics

Patient characteristics for age groups 0-15 yrs and ≥15 yrs of age are shown in table 1. In vitro proliferation in the nonstimulated cultures or stimulation of proliferation by IL-7 showed no significant differences between children and adults. Significant inhibition of DNA synthesis by IL-4 was noted in 66% of the cases (p=0.04). IL-4 induced inhibition of proliferation was not significantly different between children and adults (p=0.4).

Clinical Outcome

Table 1 summarizes the treatment results. All patients less than 15 yrs of age reached CR. In adults, CR was attained in 24 of 30 (80%) patients. Actuarial EFS of childhood patients at 5 years is 54% (±11%SE). Five year EFS of adults is estimated at 19% (±10%SE). Five year OS in children is 75% (±8%SE) and in adults 21% (±10% SE). Young age appears a prominent favourable prognostic variable both for EFS and OS (p<0.00001).
High versus low levels of spontaneous proliferation do not express impact on probability of OS or EFS (figure 1, table 3). Inhibition of proliferation by IL-4 correlates with a better OS probability (p=0.03 Cox regression, corrected for

age)(figure 1, table 3). However, no correlation is apparant between IL-4 induced suppression of proliferation and EFS. Induction of proliferation by IL-7 did not correlate with OS or EFS induced proliferation. WBC, percentage lymphoblasts in blood or bone marrow, or percentages of CD10 positive blasts did neither predict for OS or EFS.

Table 3 Overall survival (OS) and Event Free Survival (EFS) ± standard error (SE) in relation to spontaneous DNA synthesis in culture or in vitro proliferation in response to IL-7 or IL-4. In 60 cases, spontaneous-, IL-4 and IL-7 induced DNA synthesis was tested simultaneously. In 5 additional cases, spontaneous and IL-7 induced synthesis was tested simultaneously. In 8 cases spontaneous DNA synthesis only was tested.

		Number of cases	OS ± SE (%)	P-value[a]	EFS ± SE (%)	P-value[a]
Spontaneous proliferation	low[b]	37	45 ± 10	0.16	42 ± 10	0.43
	high	36	58 ± 11		40 ± 10	
IL-7 induced proliferation	low	32	69 ± 9	0.6	49 ± 12	0.8
	medium	17	48 ± 16		26 ± 19	
	high	16	51 ± 14		45 ± 14	
IL-4 induced suppression of proliferation IL-7 induced	low	30	64 ± 12	0.03	42 ± 13	0.16
	medium	15	70 ± 13		56 ± 14	
	high	15	44 ± 14		34 ± 14	

[a] adjusted for age, test for trend based on log transformed values

[b] low, medium and high as defined in materials and methods

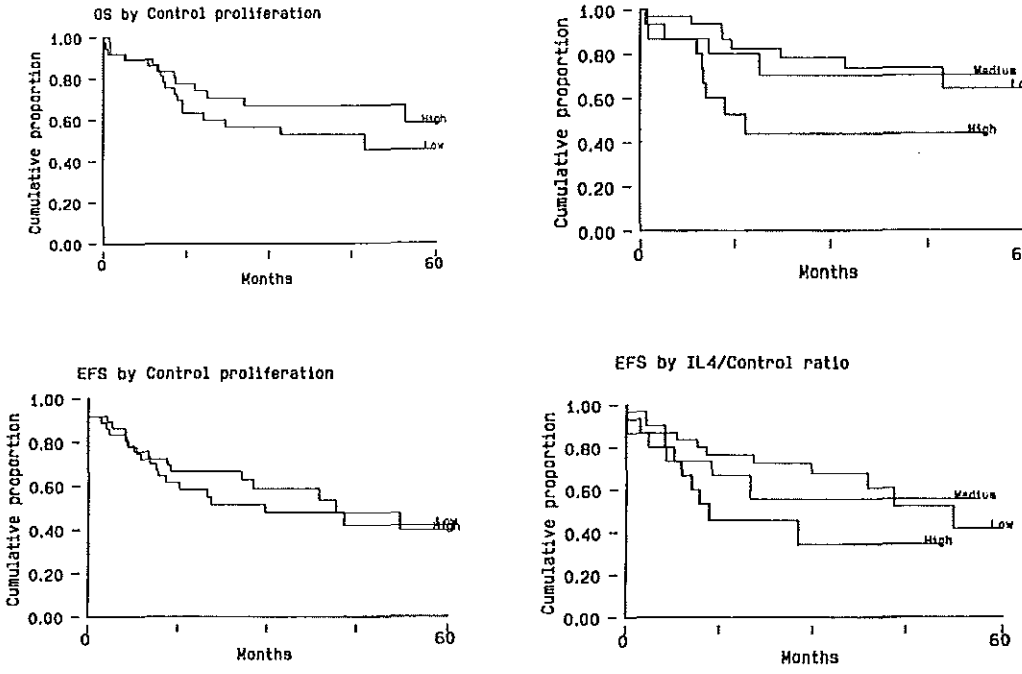

Figure 1 Actuarial overall survival (OS) and event free survival (EFS) according to spontaneous (upper pannel) or IL-4 induced (lower pannel) in vitro proliferation. Curves were constructed by the Kaplan-Meier method.

6.4 Discussion

Hematopoietic growth factors like IL-7 and IL-4 may affect the level of DNA synthesis in normal and leukemic precursor B and T cells in culture (1-4). We analysed whether in vitro responsiveness of ALL cells to these growth factors correlates with clinical outcome. IL-4 suppressed the proliferative activity of ALL cells in vitro. Patients with a high level of impairment of DNA synthesis in cultures with IL-4 had better OS. The significance of the latter relationship remains questionable since suppression of DNA synthesis by IL-4 did not predict for better EFS. Inhibition of proliferation of human lymphoid blast cells by IL-4 has been reported before (3,4,15). The small number of patients included in our analysis did not allow for a multivariate analysis of the IL-4 response and other prognostic factors.

However in univariate analysis in this limited series of cases other generally accepted determinants as WBC did neither express prognostic value. Thus, we cannot rule out that the positive relationship or that the positive relationship between IL-4 response and OS just appeared by chance. The potential difference in outcome predicted by the in vitro proliferative response to IL-4, would require further research.

The responsiveness to IL-7 of ALL cells with different phenotypes is heterogeneous and does not follow the response pattern of normal BCP cells (2). According to the normal IL-7 response pattern, Pre-B-ALL rather than Null- and Common-ALL cells would have been expected to respond to IL-7. In this study we did not analyse the patients according to immunophenotype due to the limited number of patients. We did not find any relation between the IL-7 responsiveness and OS or EFS. A possible relation between clinical outcome and the level of responsiveness to IL-7 in distinct immunophenotype categories of ALL could not be addressed.

Spontaneous in vitro proliferation of acute myeloid leukemia (AML) cells was shown to be a prognostic determinant (5). In ALL however, we do not find a statistically significant relation between the level of spontaneous proliferation and clinical outcome. This might be due to the limited number of patients studied, or indeed reflect a biological difference between AML and ALL.

References

1. Touw I, Pouwels K, Agthoven Tv, et al. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 1990;75:2097.
2. Smiers F, Paassen Mv, Pouwels K, et al. Heterogeneity of proliferative responses of human B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells to interleukin-7(IL-7): no correlation with immunoglobulin gene status and expression of IL-7 receptor of IL-2/IL-4/IL-7 receptor common gamma chain genes. Leukemia 1995;9:1039.
3. Okabe M, Kuni-eda Y, Sugiwura T, et al. Inhibitory effect of interleukin-4 on the in vitro growth of Ph-positive acute lymphoblastic leukemia cells. Blood 1991;:1574.
4. Pandrau D, Saeland S, Duvert V, et al. Interleukin 4 inhibits in vitro proliferation of leukemic and normal human B cell precursors. J Clin Invest 1992;90:1697.
5. Löwenberg B, Putten Wv, Touw I, Delwel R, Santini V. Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. N Engl J Med 1993;328:614.
6. Touw I, Groot-Loonen J, Broeders L, et al. Recombinant hematopoietic growth factors fail to induce a proliferative response in precursor B acute lymphoblastic leukemia. Leukemia 1989;3:356.
7. Madsen M, Johnsen H, Hansen P, Christiansen S. Isolation of human T and B lymphocytes by E rosette gradient centrifugation. Characterization of the isolated subpopulations. J Immnunol Meth 1980;33:323.
8. van Dongen JJ, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33(5-6):298.
9. Hagemeijer A, Smit E, Bootsma D. Improved identification of leukemic cells in methotrexate treated cultures. Cytogen Cell Genet 1979;23:208.
10. Plas vdD, Hählen K, Hagemeijer A. Prognostic significance of karyotype at diagnosis in childhood acute lymphoblastic leukemia. Leukemia 1992;6:176.
11. Delwel R, Salem M, Pellens C, et al. Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum-free culture system. Blood 1988;72:1944.
12. Kaplan E Meier P. Non-parametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457.
13. Young J, Miller R. Incident of malignant tumors in U.S. children. J Pediatrics 1975;86:254.
14. Kalbfleisch J, Prentice R. The statistical analysis of failure time data. 1980;.
15. Taylor C, Grogan T, Salmon S. Effects of interleukin-4 on the in vitro growth of human lymphoid and plasma cell neoplasms. Blood 1990;75:1114.

Chapter 7

Summary and discussion

7.1 Dissociation of IL-7 response and immunophenotype

Based on immunophenotypic analysis acute lymphoblastic leukemia cells (ALL cells) are often considered to represent the neoplastic counterparts of normal B cells at distinct stages of development. Normal B cell development requires specific stimuli from the bone marrow micro environment as well as soluble growth factors. These requirements change with progressive differentiation of B cells.
In Chapter 1 the different steps of normal B cell development are introduced. The genes coding for the Ig heavy and light chain genes are successively rearranged, defining different stages of B cell development. Eventually, proper rearrangement of both $Ig_{H \text{ and } L}$ chain genes results in the synthesis and expression of a functional B cell receptor, defining the stage of the immature B cell. IL-7 induces proliferation and differentiation in precursor B cells during distinct stages of maturation. Depending on the stage of development BCP cells respond to IL-7 either in synergy with other growth stimuli or as single growth factor.
IL-7 binds to a specific membrane expressed IL-7R complex consisting of the IL-7Rα chain and the common γ chain. The IL-7Rα chain belongs to the family of class I cytokine receptors based on structural homologies and does not have an intrinsic tyrosine kinase domain (1). Upon activation of these receptors three important signaling routes can be activated: the JAK/STAT pathway, the MAPK activation pathway or the PI3-kinase pathway. In addition protein tyrosine kinases like the Src kinase Fyn and Src like kinase Btk are activated. These signaling routes interact in several different ways with each other and have been found to play major roles in the control of proliferation and differentiation of hematopoietic cells (2,3). ALL cells somehow escape from the mechanisms that control proliferation and differentiation resulting in the accumulation of functionally defective cells in the bone marrow and peripheral blood. In Chapter 2 we analyse induction of proliferation in response to IL-7 in 72 samples of BCP-ALL and show that, in contrast to their normal counterparts, BCP-ALL cells are highly heterogeneous in their response to IL-7. The response to IL-7 as single growth stimulus during normal murine B cell development is found to be dependent on the functional rearrangement and expression of the Ig heavy chain genes. We show that in BCP-ALL cells, the IL-7 response no longer depends on the rearrangement status of either Ig heavy or light chain genes. Proliferation in response to IL-7 is observed in cells that do not express Ig heavy chains, while absence of IL-7 induced proliferation in cells with functionally rearranged and expressed Ig heavy chain genes is seen as well.
Moreover, we show that in contrast to primitive stages of normal B cell development, BCP-ALL cells lack synergistic responses to IL-7 and KL. This fits with the reported absence of c-kit expression in BCP-ALL cells (4). During differen-

tiation of murine BCP cells the expression of c-kit is lost during transition from pre-B-I to pre-B-II cells (5). Absence of c-kit expression in BCP-ALL cells supports the idea that leukemic human BCP ALL cells represent functionally pre-B II cells.

Three isoforms of the IL-7R α exist, including a nonsignaling soluble and a truncated, membrane bound form. We investigated the expression distribution of these splice variants and excluded overexpression of the nonfunctional isoforms of the IL-7Rα chain. Since the common γ chain is an essential part from the IL-7 receptor we analyzed the expression of this chain and found it present in all cases. Therefore absence of the common γ cannot explain absence of IL-7 response.

Based on immunophenotypic characteristics null- and common-ALL cells are arrested at the pre-B-I maturation stage (figure 2 chapter 1). In normal pre-B-I cells the Ig_H chain genes executed DJ_H joining, but not yet VDJ_H . Their respective Ig_L chain genes still have the germ line configuration. In null- and c-ALL however, VDJ_H rearrangement as well as rearrangements of the Ig_L chain are found. Therefore these cells belong genotypically to the pre-B-II cells.

In murine pre-B-I cells proliferation is induced in response to IL-7 only in synergy with other growth stimuli, while pre-B II cells proliferate to IL-7 as single growth stimulus. The observation that a considerable number of null- and c-ALL cells respond to IL-7 as single growth stimulus, supports the hypothesis that these cells functionally represent pre-B-II cells and not pre-B-I cells. In that case proliferation, but not absence of proliferation in response to IL-7 as single growth stimulus is no longer aberrant, but implies dissociation of normally linked developmental events. Considering the generally low proliferative response to IL-7 of BCP-ALL cells, the highly heterogeneous response pattern to IL-7 in these cells and the absence of effect of IL-7 on cell survival in vitro, it seems unlikely that IL-7 has a major influence in the expansion and accumulation of leukemic BCP cells. Nevertheless in a substantial number of cases the IL-7 response of BCP-ALL cells is aberrant when compared to normal BCP cells in comparable maturational stages.

However, it must be noted that knowledge of the sequential IL-7 responses during normal B cell development is derived from murine models. It cannot be excluded that human and murine B cell development are induced and controlled in different ways as is suggested by several observations.

In murine in vivo models the function of IL-7 has been studied by either knocking out the IL-7 gene, neutralizing IL-7 by antibodies, disruption of the IL-7Rα or common γ chain gene expression. In absence of a functional IL-7R complex or its ligand, both B and T cell development are seriously affected (6-10).

In man no condition is known with complete absence of IL-7 or the IL-7Rα. However, in the X-linked SCID syndrome, the common γ chain is non functional because of a point mutation in the coding gene (11). This syndrome is characterized

by absent or diminished number of T cells and normal or even elevated number of B cells. B cells are not functional due to absent T cells. The presence of normal or even elevated numbers of B cells is a distinct difference with common γ chain lacking mice (10). It remains to be seen whether these B cells are capable of expression of the complete antigen receptor repertoire as B cell in non affected individuals. Recently Pribyl et al (12) showed IL-7 independent differentiation of human BCP cells in an in vitro system. It seems therefore that although IL-7 in B cell development is an important factor for both proliferation and differentiation of BCP cells, its absence in man can be compensated by other regulatory factors.

7.2 Involvement of CD20 and CD40 in the proliferation of BCP-ALL cells

CD20 expression follows CD19 expression early in B cell ontogeny. It is expressed exclusively on B cell lineage cells. Expression of CD20 declines from the stage of mature B cells. Stimulation with IL-7 upregulates the expression of CD20 (13). The function of CD20 is largely unknown. Since no natural ligand (if existing at all) is known for CD20, antibodies against CD20 are used to investigate its function. Following anti CD20 antibody induced activation, tyrosine and serine kinase activity is observed, suggesting association with tyrosine kinases, since CD20 does not have an intrinsic tyrosine kinase domain (14, 15). Indeed association with the Src family tyrosine kinases p56/53lyn, p56lck and p59fyn has been reported (16). CD20 plays a direct role in transmembrane Ca^{++} conductance (17) although the precise mechanism remains to be elucidated. It is unclear whether CD20 forms a Ca^{++} by multimerisation of CD20 molecules or that other transmembrane molecules are involved as well (18). Following activation, CD20 is phosphorylated on multiple serine/threonine residues. In vitro kinases assays revealed that CD20 is a substrate for ubiquitous serine/threonine kinases like PKC, CKII and CaM-KII (19-22). Each kinase is thought to phosphorylate CD20 on specific residues resulting in different functional consequences, like PKC induced phosphorylation of CD20 abrogating the Ca^{++} flux (23). Depending on the phosphorylation on threonine and serine residues, three forms of CD20 can be found with either 33, 35 or 37 kDa. No ligand is known for CD20 and the possibility remains that CD20 does not have an extra cellular ligand, but acts as a Ca^{++} channel in response to cellular activation. Indeed, upon cell activation through the Ig receptor or through CD40 activation, CD20 is activated as well resulting in progression of cell cycle. Activation of CD20 via 1F5 induces cell cycle progression from G0 to G1 but not to S phase (24-26). In Chapter 3 we investigated the effects of activation of B cell

surface antigen CD20. CD20 was activated via binding to the mouse antibody 1F5. In 18 out of 58 cases of BCP-ALL of all immunophenotype, CD20 activation resulted in a significant increase in DNA synthesis. No synergy was observed with other growth factors. Responses or absence of responses were not related to immunophenotype or expression of CD20. Cell-cycle progression from G1 into S phase depends on decreased $[Ca^{++}]_i$ levels(27, 28). Progression to S phase in response to CD20 activation, suggests failure to inhibit drop in $[Ca^{++}]_i$ in leukemic cells. The status of phosphorylation of CD20 in leukemic cells is unknown. Possibly constitutively active phosphorylation on specific serine/threonine residues remains CD20 in the activated state or is PKC induced phosphorylation inhibited, resulting in failure to end the Ca^{++} flux. Progression to S phase in leukemic cells however, may reflect preactivation of proliferative signaling pathways, replacing the extra growth stimulus (like IL-7), needed in normal BCPs to proliferate in response to CD20 activation.

In Chapter 3 we further present the results of CD40 activation in BCP-ALL cells. In mature B cells activation of CD40 induces cell cycle progression into S phase. Only in synergy with other growth stimuli like IL-4 and CD20 activation (29-32) proliferation is induced. In BCP-ALL cells activation of CD40 alone induces DNA synthesis in 10 out of 44 cases. Simultaneous stimulation with other growth factors like IL-3, IL-4 or IL-7 in the majority of cases was not synergistic.

CD40 expression on BCP cells starts early in B cell ontogeny i.e. at the pro-B cell stage (33). Expression is also seen in malignant BCP cells at various stages of maturation (33, 34). Activation of CD40 in normal human BCP cells induces proliferation only in conjunction with other growth stimuli like IL-3, IL-7 or IL-10 (35). The natural ligand for CD40 is expressed on activated T cells (36), but the function of CD40 in BCP remains to be elucidated. Following CD40 activation a number of serine/threonine kinases are activated in addition to activation of CD40 associated tyrosine kinases. In response to CD40 activation, phosphorylation of CD20 was observed in a PKC dependent manner (20), suggesting cross talk between these two molecules. However in our experiments in acute lymphoblastic leukemia cells the mitogenic responses to activation of CD20 or CD40 appeared to be independent, suggesting interruption of connecting signaling routes, thereby losing an important control mechanism over proliferation through these signaling molecules. In table 1 the proliferative responses to various growth stimuli in human, murine and leukemic BCP cells are summarized.

Table 1 Effects of different growth stimuli on human, murine and leukemic BCP cells

Stimulus	Normal BCP cells	Leukemic BCP cells
IL-7	Proliferation of pre-B-II cells.	Proliferation of some but not all Pre-B-II cells.
IL-3	Proliferation of pro-B cells in synergy with FLt3-ligand. Proliferation of Pre-B cells in addition to IL-7 response.	Idem
Kit Ligand[5,52-54]	Proliferation in pro-B cells. Synergistic proliferation of pre-B-I-cells with IL-7.	No effect either as single growth stimulus or in synergy with IL-7.
Flt3 Ligand[55-59]	Proliferation of pro-B and Pre-B-I cells. Synergy with IL-7 in Pre-B-Cells.	No effect either as single growth stimulus or in synergy with IL-7.
IGF 1[59-60]	Proliferation of pro-B cells and possibly pre-B-I cells.	No effect either as single growth stimulus or in synergy with IL-7.
CD20 activation	Cell cycle transition from $G_0 - G_1$ in CD20 expressing B cells. Progression to S phase in synergy with other growth stimuli like CD40 activation.	Cell cycle transition from $G_0 - G_1 - S$ independent from additional growth stimuli in a significant number of BCP ALL samples.
CD40 activation	Cell cycle transition from $G_0 - G_0 S$ phase if additional stimuli like IL-3, IL-7 or IL-10 are present.	Cell cycle transition from G1 – G2 – S in absence of additional stimuli. No synergy with IL–4 or IL–7.

7.3 The JAK/STAT pathway in leukemia

In Chapter 4 results are presented of the investigation of signaling events following activation of the IL-7 receptor in human BCP-ALL cells as compared with signaling through the IL-7Rα chain in a murine cell model.
Upon stimulation of BCP-ALL cells with IL-7 the activation of Jak1, but not Jak2 or Jak3 was observed. In addition gel retardation assays revealed the formation STAT complexes identified by supershifts with anti-STAT antibodies to be STAT1 and STAT5 complexes. Activation of STAT complexes was found in patients with and without a proliferative response to IL-7, suggests other signaling events are needed for proliferative signaling. To study the contribution of the IL-7Rα chain in the signaling events of the IL-7R complex, we constructed a chimeric receptor protein consisting of the extracellular domain of the G-CSF receptor and transmembrane

plus intracellular domain of the IL-7 receptor. Activation of this chimeric receptor by hG-CSF results in dimer complex formation and activation of signaling molecules resulting in G-CSF induced proliferation. Upon transfection and activation of the chimeric receptor in the mouse pro B cell line BaF3, activation of Jak1, but not Jak2 or Jak3 was seen. In addition STAT1 and STAT5 containing complexes were formed, as well as some STAT3 containing complexes.
Interestingly, a similarly constructed chimeric receptor with the transmembrane part derived from the h-G-CSF receptor did not induce proliferation upon transfection despite clear expression of the receptor. This suggests an important role for the IL-7Rα chain transmembrane domain in proliferative signaling. Possibly the transmembrane domain plays a role in the recruitment of membrane bound signaling molecules like for instance the myristylated Src like molecule Fyn. It is however also possible that due to conformational changes, dimerization is hampered resulting in lack of proliferative signaling. Future research is needed to elucicate this matter.
It is generally thought that for proliferative signaling through the IL-7R complex the presence of the common γ chain is necessary (37). The absence of Jak3 activation supports the hypothesis that the common γ chain in BaF3 cells does not associate with the chimeric receptor. Furthermore, binding of the common γ chain occurs through binding to the ligand and the common γ chain does not have affinity for hG-CSF, the ligand of the chimeric receptor. Moreover, expression of the common γ chain in BaF3 cells is undetectably low (38). Data presented here show therefore that the IL-7Rα chain in absence of the common γ chain can elicit a full mitogenic signal.
Recently the JAK-STAT pathway was suggested to be involved in oncogenic transformation. Mutation of the Drosophila JAK analogue Hop-tum, rendering constitutive activation, caused leukemia like hematopoietic defects (39, 40) and in acute myeloid and lymphoid leukemic cells STAT molecules were found to be constitutively activated (41). Inhibition of constitutively activated JAK by tyrphostin, by interference with the kinase domain of JAK, inhibited leukemic cell proliferation, suggesting a possible role for the JAK/STAT pathway in leukemia (42). However, STATs have been implicated in inhibition of proliferation as well. A mutant form of the Epo receptor unable to activate STAT5, exhibited increased proliferation induction upon stimulation (43). Also STAT1 and STAT3 activation have been reported to induce proliferation inhibition (44-46). We did not find constitutive activation of the JAK/STAT pathway in ALL cells. Most likely additional signaling events are involved in regulation of proliferation and in leukemogenesis. The function and effect of activation of STAT molecules in

response to activation of the IL-7R complex in normal and leukemic BCP cells needs further investigation.

7.4 Signaling through the IL-7R α chain

In chapter 5 we investigated through the construction of chimeric receptors the signaling properties of the IL-7Rα chain when introduced in BaF3 cells. Various constructs were composed of the extracellular domain of the human G-CSF receptor and the complete or truncated intracellular domains of the IL-7Rα chain. IL-7Rα chain mutants missing the carboxy terminus of the IL-7R were unable to transduce proliferative signals. The transmembrane domain was either derived from the G-CSF-R or the IL-7R. Results showed abrogation of mitogenic signaling when the transmembrane domain was derived from the G-CSF-R. The possible role of the transmembrane domain of cytokine receptors in mitogenic signaling is discussed. Induction of proliferation was found to coincide with activated JAK1. Chimeras able to induce proliferation, activated ERK1/ERK2 molecules without activation of the Grb2/Shc complex. This implicates an alternative activation of the p21 Ras pathway. Co-precipitation of IRS-2 with the PI3 kinase subunit p85 in activated cells bearing the proliferation inducing chimeras, suggests involvement of these IRS-2 and PI3 kinase in the p21 Ras pathway. Finally, activation of STAT5 was seen independent of JAK activation. Alternative mechanisms to activate STAT molecules are discussed.

7.5 Clinical relevance of cytokine responses

In Chapter 6 we have investigated the clinical significance of in vitro proliferation of ALL cells in response to IL-4 and IL-7. IL-4 was found to inhibit in vitro proliferation in most cases tested. Responses to IL-7 were highly heterogeneous as described in chapter 2. Inhibition with IL-4 correlated with a better overall survival, but due to the limited number of patients and the hetereogeneous character of ALL, no multivariate analysis could be performed. IL-7 and proliferation in absence of HGFs did not show relation with clinical outcome. The study confirmed age as an important clinical prognostic parameter. Conclusions are difficult to draw, since confidence intervals were large and statistical difference difficult to achieve. These data reflect the heterogeneity of ALL and suggest that responses to IL-7 are not of clinical importance in ALL.

Concluding remarks

Cytokines are involved in control and regulation of hematopoiesis. The cellular response to cytokines depends on cell type and stage of maturation. In acute lymphoblastic leukemia the regulated process of proliferation and differentiation is disturbed and the normal link between immunophenotype and genotype lost. The response to cytokines may help to functionally define leukemia cells. In this thesis we analysed the response of ALL cells to IL-7 and found the response pattern to be highly heterogeneous in all immuno-phenotypic subclasses. We have not been able to identify a general mechanism of dysregulation of the IL-7 response. Development of new in vitro culture techniques is necessary to study the role of IL-7 during the sequential events in B cell development. Differences in responses to IL-7 stimulation cannot be explained by lack of expression of a functional IL-7R. This urges to further investigate the downstream processes following IL-7 stimulation in normal and leukemic BCP cells.

References

1. Bazan JF. A novel family of growth factor receptors: a common binding domain in the growthhormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain.Biochem Biophys Res Commun 1989;164(2):788.
2. Cantley L, Auger K, Carpenter C, et al. Oncogenes and signal transduction. Cell 1991;64:281.
3. White M, Kahn C. The insulin signaling system. J Biol Chem 1994;269(1):1.
4. Buhring H, Ullrich A, Schaudt K, Muller C, Busch F. The product of the proto-oncogene c-kit (p145c-kit) is a human bone marrow surface antigen of hemopoietic precursor cells which is expressed on a subset of acute non-lymphoblastic leukemic cells. Leukemia 1991;5:854.
5. Rolink A, Streb M, Nishikawa S-I, Melchers F. The c-kit-encoded tyrosine kinase regulates the proliferation of early pre-B cells. Eur J Immunol 1991;21:2609.
6. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 1994;180(5):1955.
7. Sudo T, Nishikawa S, Ohno N, et al. Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc Natl Acad Sci U S A 1993;90(19):9125.
8. Maki K, Sunaga S, Komagata Y, et al. Interleukin 7 receptor-deficient mice lack gammadelta T cells. Proc Natl Acad Sci U S A 1996;93(14):7172.
9. He YW, Malek TR. Interleukin-7 receptor alpha is essential for the development of gamma delta + T cells, but not natural killer cells. J Exp Med 1996;184(1):289.
10. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity 1995;2(3):223.
11. Noguchi M, Yi H, Rosenblatt HM, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 1993;73(1):147.
12. Pribyl J, LeBien T. Interleukin 7 independent development of human B cells. Proc Natl Acad Sci USA 1996;93:10348.
13. Oyashiki K, Miyauchi J, Oyashiki J, et al. Interleukin-7 enhances colony growth and induces CD20 antigen of a Ph' acute lymphoblastic leukemia cell line, OM9;22. Leukemia 1993;7(7):1034.
14. Deans J, Schieven G, Shu G, et al. Association of tyrosine and serine kinases with the B cell surface antigen CD20. J Immunol 1993;151(9):4494.
15. Kansas G, Tedder T. Transmembrane signals generated through MHC class II. CD19, CD20, CD39 and CD40 antigens induce LFA-I dependent and independent adhesion in human B cells through a tyrosine kinase-dependent way. J Immunol 1991;147(12):4094.
16. Deans J, Kalt L, Ledbetter J, Schieven G, Dolen J. Association of 75/80-kDa phosphoproteins and the tyrosine kinases Lyn, Fyn and Lck with the B cell molecule CD20. Evidence against involvement of the cytoplasmic regions of CD20. J Biol Chem 1995;270(38):22632.
17. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. J Cell Biol 1993;121(5):1121.

18. Tedder T, Engel P. CD20: a regulator of cell-cycle progression of B lymphocytes. Immunol Today 1994;15(9):450.
19. Tedder TF, Schlossman SF. Phosphorylation of the B1 (CD20) molecule by normal and malignant human B lymphocytes. J Biol Chem 1988;263(20):10009.
20. Valentine M, Meier K, Rossie S, Clark E. Phosphorylation of the CD20 phosphoprotein in resting B lymphocytes. J Biol Chem 1989;264(19):11282.
21. Valentine M, Licciardi K, Clark E, Krebs E, Meier K. Insulin regulates serine/threonine phosphorylation in activated human B lymphocytes. J Immunol 1993;150(1):96.
22. Genot E, Meier K, Licciardi K, et al. Phosphorylation of CD20 in cells from a hairy cell leukemia cell line. Evidence for involvement of calcium/calmodulin-dependent protein kinase II. J Immunol 1993;151(1):71.
23. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. J Cell Biol 1993;121(5):1121.
24. Golay J, Calrk E, Beverley P. The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle. J Immunol 1985;135:3795.
25. Smeland E, Godal T, Ruud E, et al. The specific induction of myc protooncogene expression in normal human B cells is not a sufficient event for acquisition of competence to proliferate. Proc Natl Acad Sci U S A 1985;82(18):6255.
26. Clark EA, Shu G, Ledbetter JA. Role of the Bp35 cell surface polypeptide in human B-cell activation. Proc Natl Acad Sci U S A 1985;82(6):1766.
27. Bubien J, Kirk K, Rado T, Frizzell R. Cell cycle dependence of cloride permea-bility in normal and cystic fibrosis lymphocytes. Science 1990;248:1416.
28. Brent L, Butler J, Woods W, Bubien J. Transmembrane ion conductance in human B lymphocyte activation. J Immunol 1990;145:2381.
29. Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science 1991;251(4989):70.
30. Banchereau J, Rousset F. Growing human B lymphocytes in the CD40 system. Nature 1991;353(6345):678.
31. Clark E, Ledbetter J. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proc Natl Acad Sci USA 1986;83(12):4494.
32. Ledbetter JA, Shu G, Gallagher M, Clark EA. Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen BP50 (CDW40). J Immunol 1987;138(3):788.
33. Uckun F, Gajl-Peczalska K, Myers D, Jaszcz W, Haissig S, Ledbetter J. Temporal association of CD40 antigen expression with discrete stages of human B-cell ontogeny and the efficacy of anti-CD40 immunotoxins against clonogenic B-lineage acute lymphoblastic leukemia as well as B-lineage Non-Hodgkin's lymphoma cells. Blood 1990;76(2449-245612):2449.
34. Uckun F, Schieven G, Dibirdik I, Chandan-Langlie M, Tuel-Ahlgren L, Ledbetter L. Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified serine/threonine-specific protein kinases by the pan-B-cell receptor CD40/Bp50 at discrete developmental stages of human B-cell ontogeny. J Biol Chem 1991;266:17478.

35. Renard N, Duvert V, Blanchard D, Banchereau J, Saeland S. Activated CD4+ T cells induce CD40-dependent proliferation of human B cell precursors. J Immunol 1994;152:1693.
36. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological charac-terization of a murine ligand for CD40. Nature 1992;357(6373):80.
37. Ziegler SE, Morella KK, Anderson D, et al. Reconstitution of a functional interleukin (IL)-7 receptor demonstrates that the IL-2 receptor gamma chain is required for IL-7 signal transduction. Eur J Immunol 1995;25(2):399.
38. Kawahara A, Minami Y, Taniguchi T. Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2,IL-4, and IL-7 signaling. Mol Cell Biol 1994;14:5433.
39. Luo H, Hanratty WP, Dearolf CR. An amino acid substitution in the Drosophila hopTum-l Jak kinase causes leukemia-like hematopoietic defects. EMBO J 1995;14(7):1412.
40. Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N. Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. EMBO J 1995;14(12):2857.
41. Gouilleux-Gruart V, Gouilleux F, Desaint C, et al. STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. Blood 1996;87(5):1692.
42. Meydan N, Grunberger T, Dadi H, et al. Inhibition of acute lymphoblastic leukaemia by Jak-2 inhibitor. Nature 1996;379:645.
43. Chin H, Nakamura N, Kamiyama R, Miyasaka N, Ihle J, Miura O. Physical and functional interactions between STAT5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3. Blood 1996;88(12):4415.
44. Chin Y, Kitagawa M, Su W, You Z, Iwamoto Y, Fu X. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science 1996;272:719.
45. Minami M, Inoue M, Wei S, et al. STAT3 activation is a critical step in gp-130-mediated terminal differentiation and growth arrest of a myeloid cell line. Proc Natl Acad Sci USA 1996;93:3963.
46. Yamanaka Y, Nakajima K, Fukada T, Hibi M, Hirano T. Differentiation and growth arrest signals are generated trough the cytoplasmic region of gp130 that is essential for Stat3 activation. EMBO J 1996;15(7):1557.
47. Funk PE, Varas A, Witte PL. Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. J Immunol 1993;150(3):748.
48. Billips LG, Petitte D, Dorshkind K, Narayanan R, Chiu CP, Landreth KS. Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis. Blood 1992;79(5):1185.
49. McNiece IK, Langley KE, Zsebo KM. The role of recombinant stem cell factor in early B cell development. Synergistic interaction with IL-7. J Immunol 1991;146(11):3785.
50. Namikawa, R MM de Vries JE,Roncarolo M. The FLK2/FLT3 ligand synergizes with interleukin-7 in promoting stromal cell independent expansion and differentation of human fetal pro-b cells in vitro. Blood 1996;87(5):181.
51. Hirayama F, Lyman SD, Clark SC, Ogawa M. The flt3 ligand supports proliferation of lymphohematopoietic progenitors and early B-lymphoid progenitors. Blood 1995;85(7):1762.

52. Eder M, Hemmati P, Kalina U, et al. Effects of Flt3 ligand and interleukin-7 on in vitro growth of acute lymphoblastic leukemia cells. Experimental Hematology 1996;24:371.
53. McKenna H, Smith F, Brasel K, et al. Effects of flt3 ligand on acute myeloid and lymphocytic leukemic blast cells from children. Experimental Hematology 1996;24:378.
54. Gibson LF, Piktel D, Landreth KS. Insulin-like growth factor-1 potentiates expansion of interleukin-7-dependent pro-B cells. Blood 1993;82(10):3005.
55. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood 1992;80(5):1207.

Samenvatting in Nederlands

Hematopoiése of bloedcelvorming is het proces waarbij zich vanuit een pluripotente stamcel de verschillende soorten bloedcellen ontwikkelen. De bloedcellen bestaan uit rode en witte bloedcellen en bloedplaatjes. Witte bloedcellen kunnen worden onderscheiden in granulocyten, lymfocyten en macrofagen en spelen een belangrijke rol bij de afweer tegen lichaamsvreemde stoffen (antigenen). Lymfocyten kunnen worden onderscheiden in T en B lymphocyten, die beiden nauw samenwerken tijdens een afweerreactie. Door verbruik van lymfocyten dient een voortdurende aanmaak van nieuwe cellen plaats te vinden. Dit gebeurt in het beenmerg waar een specifiek milieu zorgt voor de juiste groei en ontwikkelingscondities.
Gedurende het traject van primitieve stamcel tot volledig uigerijpte (gedifferentieerde) lymfocyt, wordt een aantal ontwikkelingsstappen doorlopen. Deze worden besproken in hoofdstuk 1.
Rijpe B cellen brengen op de celmembraan een immunoglobuline (Ig) tot expressie, waaraan antigenen zeer specifieke kunnen binden. Aangezien er zeer veel verschillende soorten antigenen bestaan, moeten er evenveel verschillende Ig's bestaan.
Hiertoe wordt tijdens de ontwikkeling van de B cel voorlopercellen (de B cell precursor cells= BCP) een serie DNA herschikkingen uitgevoerd van de verschillende onderdelen van het de genen die coderen voor het uiteindelijke Ig.
Verschillende stadia van genherschikking definiëren discrete ontwikkelings-stadia van de B cel voorlopercellen: pro-B, pre-B-I en pre-B-II. Niet alleen uitrijping maar ook vermenigvuldiging (proliferatie) van de verschillende ontwikkelingsstadia is van groot belang.
Hematopoietische groeifactoren (HGF's) zijn hormoonachtige stoffen die zowel de proliferatie als differentiatie processen sturen. HGF's oefenen hun werking op cellen uit door middel van activering van zogenaamde receptor structuren die in de celmembraan verankerd zijn. In dit proefschrift is de rol onderzocht van verschillende HGF's in proliferatie en differentiatie van normale en leukemische B cel voorlopercellen. Voor een van deze HGF's, interleukine 7 (IL-7), is onderzocht welke intracellulaire processen plaatsvinden na binding van IL-7 en daarmee activatie van zijn eigen receptor op de celwand van de BCP.
Acute lymfatische leukemie (ALL) is een kwaadaardige ziekte, waarbij de controle over proliferatie en differentiatie van lymfocyten verloren is. Elk type ALL kent ophoping van B of T voorlopercellen in een bepaald stadium van de ontwikkeling. Deze ophoping van onrijpe cellen verstoort de normale aanmaak van bloedcellen, met als gevolg bloedarmoede, stollingsstoornissen, en sterk verminderde afweer.

De respons op IL-7, maar ook op andere groeifactoren, wordt bepaald door het ontwikkelingsstadium waarin de cel verkeert.
In hoofdstuk 2 is onderzocht of de leukemische voorloper B cellen (BCP-ALL cellen) op IL-7 reageren zoals normale voorloper B cellen in een vergelijkbaar ontwikkelingsstadium zouden doen.
Dit bleek niet het geval te zijn. De respons op IL-7 van BCP-ALL cellen viel niet samen met het ontwikkelings stadium van de cel. Onderzocht is of de receptor voor IL-7 compleet tot expressie komt, hetgeen het geval blijkt te zijn. Geconcludeerd kan worden dat BCP-ALL cellen zich ten aanzien van groeifactor respons anders gedragen dan normale BCP cellen en derhalve niet beschouwd kunnen worden als vertegenwoordigers van deze normale ontwikkelingsstadia.
In Hoofdstuk 3 onderzochten wij de respons van BCP-ALL cellen op activatie van CD20 en CD40. CD20 en CD40 zijn eiwitten die tot expressie komen op de celwand van BCP cellen. Van CD20 is de functie niet bekend. De vorm van het eiwit doet vermoeden dat het een receptor is, maar een ligand is niet bekend. Activatie van CD20 in normale BCP cellen leidt tot progressie in de cel cyclus van fase G0 tot G1. Deze fasen gaan vooraf aan de fasen waarin de cel zich vermenigvuldigt. Activatie van CD20 wordt gezien in samenhang met activatie van de cel door signalen van buitenaf.
In dit onderzoek hebben we CD20 kunstmatig geactiveerd met een anti CD20 antilichaam. Gevonden werd dat BCP-ALL cellen in respons op CD20 activatie niet slechts van G0 fase van de celcyclus in de G1 fase overgingen, maar verder gingen tot de S fase, die direct met proliferatie samenhangt. Er werd geen versterking van de respons waargenomen door gelijktijdige stimulatie met andere stimuli, waaronder IL-7.
CD40 is de receptor voor het zogenaamde CD40-ligand (CD40-L). Activatie van CD40 kan plaatsvinden door binding met dit ligand, maar ook door binding van anti CD40 antilichamen. CD40-L wordt tot expressie gebracht door T-cellen en stroma cellen in de thymus en beenmerg. De CD40/CD40-L interactie is belangrijk voor proliferatie en mogelijk ook voor differentiatie. In normale BCP cellen leidt CD40 activatie tot cel cyclus progressie van G1 tot S fase alleen als tegelijkertijd stimulatie plaatsvindt met
IL-3, IL-7, IL-10 of CD20-activatie. In BCP-ALL cellen werd proliferatie door alleen CD40 activatie gezien in een aantal gevallen en was in geen enkel geval versterking van respons gezien door toevoeging van andere groeifactoren. Geconcludeerd wordt dat BCP-ALL cellen zich ten aanzien van de respons op CD20- of CD40-activatie anders gedragen dan normale BCP cellen.
In hoofdstuk 4 en 5 wordt de manier waarop intracellulaire processen worden geactiveerd door IL-7 nader onderzocht. IL-7 bindt aan een specifiek IL-7R

complex bestaande uit een IL-7Rα keten en een common γ keten. Na binding van IL-7 volgt een cascade van activatie van signaalmoleculen uiteindelijk resulterend in verandering van expressie van genen.
In hoofdstuk 4 wordt beschreven dat in BCP-ALL cellen de JAK/STAT signaalweg wordt geactiveerd in respons op IL-7. STAT1 en STAT5 complexen worden gevormd zowel in BCP-ALL cellen die prolifereren na IL-7 stimulatie als in BCP-ALL cellen die dat niet doen. Om te onderzoeken welke signaalwegen door de IL-7Rα keten onafhankelijk van de γ_c keten worden geactiveerd, construeerden wij een kunstmatige receptor uit het extra cellulaire deel van de G-CSF-R en het intracellulaire deel van de IL-7Rα keten (chimere receptor). Identieke formatie van STAT complexen werden gevonden, maar bovendien formatie van STAT complexen waarin STAT3 is betrokken.
In hoofdstuk 5 beschrijven we formatie van STAT5 complexen door een chimere receptor waarin het transmembraan gedeelte van de IL-7Rα keten is vervangen. Activatie van deze receptor leidt niet tot proliferatie. Blijkbaar vervult het transmembraan gedeelte een nog onbekende functie in het doorgeven van proliferatieve signalen. In afwezigheid van het transmembraan gedeelte van de IL-7R vindt geen JAK1 activatie plaats, evenmin als activatie van enkele andere belangrijke signaal moleculen (IRS-2, Syp, MAPK). Daarentegen worden wel STAT5 complexen gevonden. De rol van het transmembraan gedeelte wordt bediscussieerd. Daarnaast wordt geconcludeerd dat STAT5 activatie kan plaatsvinden onafhankelijk van JAK activatie.
In hoofdstuk 6 hebben wij gekeken naar de mogelijke klinische relevantie van groeifactor geïnduceerde groei in vitro van leukemische B- en T- voorlopercellen bij een groep van 73 kinderen en volwassen patienten met ALL. Wij vonden geen aanwijzing dat er een verband bestaat tussen de in vitro groei karakteristieken en het klinisch beloop.

Dankwoord

Een proefschrift, en zo ook dit proefschrift, komt tot stand door samenwerking van vele handen. Het is ondoenlijk het belang van alle individuele handen aan te geven. Enkele handen echter verdienen het in het bijzonder genoemd te worden.

Allereerst mijn begeleider en co-promotor Ivo Touw. Ivo, jij hebt in de afgelopen jaren minstens zo afgezien als ik. Bij de vele tegenslagen die we hebben gehad, zag jij meestal nog wel ergens een lichtpuntje. "De dood of de gladiolen" gold voor jou niet alleen op de fiets, in het werk ging je steeds voor de gladiolen. Zeer bedankt en ik hoop van harte dat we ooit nog eens samen de LBL zullen fietsen.
Mijn promotor Bob Löwenberg. Beste Bob, bedankt voor het vertrouwen dat je me bleef geven om dit proefschrift af te ronden.
De overige leden van de promotiecommissie, Prof. dr H.A. Büller, Prof. dr J.M.J.J. Vossen, Dr. E. Vellenga, dank ik voor het kritisch lezen van het manuscript.

Marleen van Paassen. Marleen, jij was de eerste analyste met wie ik werkte en van jou heb ik cellen leren kweken en DNA leren isoleren. Dat we samen Amsterdamse smartlappen door het lab lieten schallen (tot afschuw van de anderen), heeft mij geholpen te wennen aan het Rotterdamse. Ik hoop nog eens een duet met je te zingen. Bedankt.
Karin Pouwels. Karin, jij was de tweede analyste met wie ik werkte. Jouw inzet, inzicht en gouden handjes hebben een grote bijdrage voor dit proefschrift betekend. Bij jou gaan babbelen en werken hand in hand: een unieke combinatie die je op het lab ook zeer geliefd hebben gemaakt (naast de jaarlijkse oogst van verse asperges natuurlijk).

Alle andere medewerkers en medewerksters van het celkweek lab van de DDHK bedank ik voor hun bijdrage en gezellige samenwerking: Hans Hoogerbrugge, Lianne Broeders, Carin van Buitenen, Anita Scheelen, Leo Budel, Bianca Backx, Dong Fan, Dorien van der Plas, Ruud Delwel. Mijn kamergenoten: John de Koning, Peter Valk (goedesmorgens, het riegen van het vee) en Derrick Louz, veel succes met jullie eigen promoties.
Natuurlijk ook de andere onderdelen van de 13e, waar ik steeds terecht kon voor advies of hulp, bedankt. Karola van Rooyen, onze fotografe, bedankt voor de het verfraaien van vele plaatjes en dia's. De omslag wordt gesierd met een foto van jouw hand. De Wagemakers: Trui, wanneer gaan we nou eindelijk eens samen roeien? Yvonne, dank zij jou kon ik cellen sorteren en toch naar Ajax kijken!!! De Ploemakkers: Adrie, altijd even tijd om een FACS probleempje op te lossen. Carla:

het loopt pas echt lekker na 20 kilometer... De TNOers: mag het wat zachter?!. De Sonnevelders: Jan, bedankt voor je hulp bij het oplossen van computerstoringen en het trouw reorganiseren van mijn bestanden. Jij liet me zien dat een Nederlandse duikinstructeur op kleintjes let die een in het buitenland opgeleide instructeur niet eens ziet. Marry en Peter: altijd goed voor een bak koffie en de laatste nieuwtjes (= roddels). Wim van Putten, statisticus van de Daniël den Hoed, wil ik bedanken voor zijn statistische analyses, zijn altijd vriendelijke adviezen en zijn geduldige lessen in statistiek.

In het Sophia Kinderziekenhuis bedank ik Karel Hählen. Karel, hoewel het onderzoeksonderwerp ver van de kliniek afstond (helaas), heb ik gewaardeerd dat je steeds probeerde te volgen waarmee ik bezig was. Mede dankzij jouw financiële support kon dit onderzoek plaatsvinden en kon ik enkele grote congressen bezoeken en nieuwe ideeën opdoen. Ik hoop in de toekomst veel van jou te leren op het gebied van de kinderoncologie.
Ook de andere (ex) leden van de kinderoncologie Inge Appel, Frederique Hakvoort-Camel, Maarten Egeler en Peter Hoogerbrugge wil ik bedanken voor hun belangstelling en voor het aanleveren van nieuw patienten materiaal. Pas sinds ik zelf in de kliniek werk, begrijp ik hoe moeilijk het is voldoende beenmerg te verkrijgen. Zonder jullie inspanning hiertoe kan veel onderzoek niet plaatsvinden.
Alle medewerkers van het 'oncolab' wil ik bedanken voor hun inzet bij het distribueren van de beenmergen en hun steeds vriendelijke belangstelling.
Ook mijn collega's mede assistenten in het Sophia wil ik danken. Slechts door de "time out" die ik kreeg (en die voor jullie extra diensten betekende), kon ik dit werk afronden. De borrel komt jullie toe.

Thanks should also go to Dr. Menno Verhave and Dr. Steve Krasinski of the Lab. Pediatric GI and Clinical Nutrition, Tufts University in Boston. In your lab at your hand I made the first steps in molecular biology. Both of you are great teachers, with ever lasting enthusiasm and with a great sense of humour. I wish our friendship lasts forever. Thank you for a fantastic time and your friendship.
Prof.Dr Dick Grand and Prof.Dr. Hans Büller I want to thank for facilitating the research fellowship in the New England Medical Center in Boston. It is a great pleasure and honour to have at least one of you in the "promotion committee'.

Wat is een promovendus zonder paranimfen? Wibe, als ik in een dip zat, liet jij me altijd zien dat het nog veel erger kon, waarop wij vervolgens beiden in een nog dieper gat wegzakten. Maar dan stapten we op de fiets, reden ons helemaal leeg en

gingen vol goede moed weer verder. Van jouw liefde voor computers heb ik dankbaar misbruik gemaakt. Ik ben blij dat je mijn paranimf wilt zijn.
Lotus, als Amsterdamse motormuizen tartten wij het lot door in Rotterdam de Mokumse eer te gaan verdedigen. Amsterdamse humor doet het buiten de mooiste stad van Nederland niet zo goed, wat wij een tragisch gemis vinden voor deze buitengewesten (geintje). Natuurlijk moest jij mijn paranimf worden.

Mijn ouders wil ik bedanken voor hun nooit aflatende zorg en liefde. Ik hoop dat mijn kinderen zich wat makkelijker laten sturen dan ik dat deed. Jullie leven stond in het teken van 'de kinderen', ik hoop daar iets voor terug te hebben gegeven.

Lieve Lucilla, in ups en in downs was je er, altijd! Ook al is dit boekje nog zo mooi, het mooiste hebben wij samen: elkaar, Sam, Daan en de toekomst.