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Review Article

Interleukin-3 receptor as a target for antileukemic drugs

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Abstract

Interleukin-3 (IL-3) is the prototype of a pleiotropic cytokine displaying a key role in the control of the proliferation, survival and differentiation of hematopoietic cells. This cytokine acts via a membrane receptor comprised of an α-chain (IL-3Rα) specific to this receptor and a β-chain (IL-3Rβ) common to IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. Following the interaction with its membrane receptor, a series of biochemical events are triggered, leading to the activation of several transduction pathways that mediate the biological effects of IL-3. IL-3Rα is overexpressed in acute leukemias and in hairy cell leukemias. This observation has prompted the hypothesis that IL-3Rα could represent a target for antileukemia therapy. Therefore, a fusion protein composed of IL-3 and diphtheria toxin was produced and preclinical studies indicated that this compound is preferentially cytotoxic for leukemic cells, including quiescent (G_0) leukemic cells, while sparing normal hematopoietic stem cells. This drug will now be tested in phase I/II studies in leukemic patients relapsing after standard therapy.

Introduction

Approximately 2 x 10^{11} red blood cells and 10 x 10^{10} white blood cells must be replaced each day to maintain adult human hematopoiesis. Mature blood elements are unable to grow further, have a limited life span and must be replaced by new cellular elements generated through the proliferation and differentiation of hematopoietic stem cells (HSCs), followed by hematopoietic progenitor cells (HPCs) and precursors of the various blood lineages. Pluripotent HSCs are able to generate a cell progeny pertaining to all hematopoietic cell lineages, including neutrophils, basophils, eosinophils, monocytes/macrophages, erythrocytes, megakaryocytes, dendritic cells, mast cells, B-, T- and NK lymphocytes (1). Some HSCs possess the capacity to differentiate along both the hematopoietic and endothelial cell lineages and are therefore considered to be hemangioblasts (2).

Two types of HSCs have been defined according to their functional properties: long-term repopulating HSCs and short-term repopulating HSCs. The first type is capable of both sustaining hematopoietic cell production for the entire lifespan (*differentiation*) and generating daughter pluripotent stem cells endowed with the same repopulating and differentiating properties (*self-renewal*). The second type is able to sustain hemato/lymphopoiesis only for short periods of time.

The initial steps of differentiation of HSCs involve the generation of multipotential HPCs which develop large colonies *in vitro* containing hematopoietic cells pertaining to all lineages. Subsequently, these multipotential HPCs differentiate into committed HPCs able to generate the various hematopoietic lineages; these cells are known as colony-forming units and are easily identified using standard clonogenic assays in semisolid medium.

It is important to note that, while the number of HSCs is very low in both bone marrow and peripheral blood, the number of HPCs is markedly higher. Therefore, committed HPCs are responsible in large part for maintaining blood cell production. The subsequent steps of differentiation of committed HPCs consist of their progressive maturation into the morphologically recognizable hematopoietic precursors of the various hematopoietic lineages.

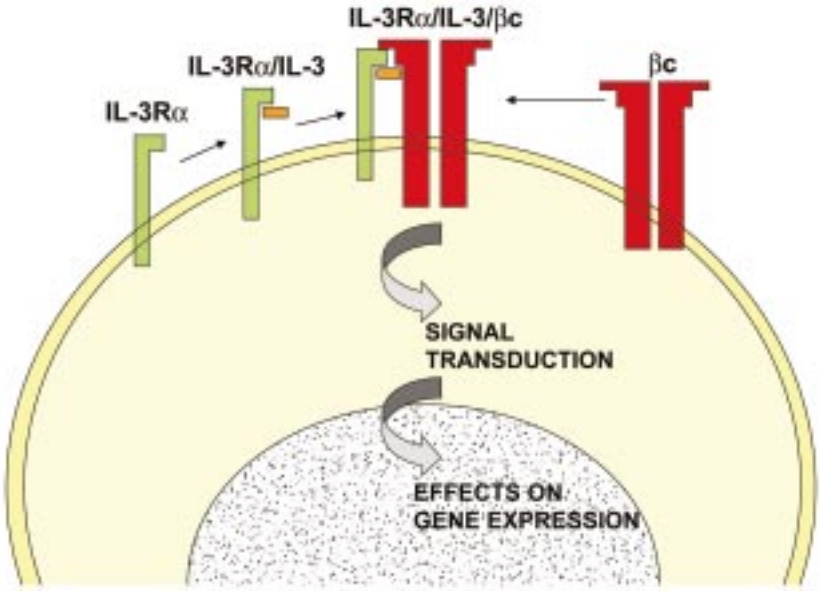

Fig. 1. Molecular assembly of the high-affinity IL-3R. IL-3Rα exists as a monomer on unstimulated cells, while IL-3Rβ is present as a preformed dimer on these cells. IL-3Rα confers receptor specificity; however, the formation of a high-affinity receptor requires the formation of a heterodimer composed of one IL-3Rα chain and one IL-3Rβ homodimer. Following interaction with IL-3, the high-affinity IL-3R transduces activating signals to the target cell, mainly through the IL-3R β-chain.

During all the steps of differentiation/maturation, hematopoietic cells require the presence of growth factors, collectively known as cytokines, to sustain their proliferation and survival. Cytokines are classified into different groups according to their structural features and different cytokine subset families have been identified. Likewise, structural properties allow the identification of cytokine receptor families.

Among the various cytokines, a key role in the control of hematopoietic differentiation, proliferation and survival is played by interleukin-3 (IL-3). IL-3, together with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5, is a member of the short-chain 4α-helical bundle subset of hematopoietic cytokines. Within this cytokine family, IL-3, GM-CSF and IL-5 are distinguished from the others and from a separate subfamily by their ability to bind a shared receptor chain, the common β-receptor (βc), used for receptor signaling (3). The common βc chain is, however, only one of two subunits of a heterodimeric receptor for these cytokines, where the α-receptor chain is specific for each cytokine (IL-3Rα, IL-5Rα and GM-CSFRα) and the βc is common to all three receptors (4). Following cytokine binding to the α-chain of each of these receptors, the βc is recruited in proximity to the Rα and together they form a high-affinity receptor able to initiate receptor activation, intracellular signaling and a series of physiological effects (Fig. 1).

IL-3 and GM-CSF are the prototypes of multipotent cytokines, *i.e.*, cytokines displaying a multiplicity of biological effects, affecting all steps in the program of myeloid differentiation. In fact, both cytokines induce proliferation and differentiation of myeloid progenitors and precursors and promote the survival of mature elements (including neutrophils, eosinophils, basophils and monocytes). Furthermore, IL-3 and GM-CSF synergize with unilineage growth factors, such as G-CSF and M-CSF, to sustain granulocytic and monocytic differentiation, respectively.

Structure of IL-3

Human IL-3 is encoded by a gene comprised of five exons and four introns that spans an area of about 3.2 kb and is located on chromosome 5, near segment 5q31. The full-length human IL-3 mRNA encodes a precursor protein of 157 amino acids, with a 19-amino-acid hydrophobic leader sequence, one interchain disulfide bridge (Cys^{16-84}) and two potential *N*-linked glycosylation sites located at Asn^{15} and Asn^{70}. The cleavage of the signal peptide determines the formation of the mature protein comprised of 133 amino acids.

The functional structure of hematopoietic cytokines is characterized by orientation of their 4α-helices in a so-called “up-up-down-down” antiparallel conformation (5). The structure of IL-3 is similar to that of IL-5 and GM-CSF. It is of interest that the genes encoding these cytokines are all located on human chromosome 5, suggesting a common ancestral relationship.

The domains within the IL-3 molecule responsible for binding to the IL-3R chains have been determined. The IL-3-binding domain of the IL-3R α-chain is located at the

level of its antiparallel first and third loops, while the βc-binding domain is centered around the glutamate residue 22 (6). Other studies based on site mutagenesis and analysis of the three-dimensional structure of IL-3 provided evidence that a class of largely hydrophobic residues is required for the structural integrity of IL-3 and for the formation of the core of a scaffold formed by exposed amino acid residues (7). Particularly, 10 exposed residues are required for the interaction of IL-3 with IL-3Rα: Asp^{21}, Gly^{42}, Glu^{43}, Glu^{45}, Asp^{46}, Met^{49}, Arg^{94}, Pro^{96}, Phe^{113} and Lys^{116}. The interface between IL-3R and IL-3Rα therefore consists of a cluster of hydrophobic residues flanked by electrostatic interactions (7).

The major physiological source of IL-3 is activated T-lymphocytes. Other cell types, such as NK lymphocytes, eosinophils and stromal cells, are also able to produce IL-3 (8). The expression of the IL-3 gene is regulated at both the transcriptional and post-transcriptional level. Transcriptional regulation is regulated by multiple promoter and enhancer elements present in the 5' flanking region of the IL-3 gene. Among the various transcription factors that bind this region of the IL-3 gene, the most relevant for the control of gene expression are activator protein-1 (AP-1) and nuclear factor of activated T-cells (NFAT). The post-transcriptional regulation of the IL-3 gene is mediated by AU-rich elements (AREs), present at the level of the 3' noncoding region.

Structure of IL-3R

All the biological effects of IL-3 are mediated by the interaction of this cytokine with its membrane receptor IL-3R. IL-3R is a member of the gp140 group of the type I cytokine receptor superfamily. This receptor is a heterodimeric receptor comprised of two distinct chains, the α- and β-chains. The α-chain represents the primary binding site for IL-3; however, this receptor α-chain alone, in the absence of the β-chain, binds IL-3 with low affinity (K_d in the range of 50-100 nM) and exhibits rapid dissociation kinetics. The β-chain alone does not display any IL-3-binding capacity; however, this receptor chain through interaction with one α-chain determines the formation of the high-affinity IL-3R (K_d = 100-200 pM) and is mainly responsible for receptor signaling. The IL-3R α-chain (also known as CD123) is a type I transmembrane glycoprotein which belongs to the cytokine receptor superfamily.

IL-3Rα displays 54% and 43% sequence homology with IL-5Rα and GM-CSFRα, respectively. These three receptors together form the IL-3R subgroup of cytokine receptor family.

The IL-3Rα gene encodes a transmembrane glycoprotein of 41.3 kD. Cleavage of the 18-amino-acid signal sequence determines the formation of a mature IL-3Rα protein comprised of 360 amino acids. As for many other membrane receptors, IL-3Rα displays three regions: 1) an extracellular domain comprised of 288 amino acids involved in IL-3 binding; 2) a transmembrane region of 20 amino acids responsible for the anchorage of the receptor to the cell membrane; and 3) a cytoplasmic domain of 52 amino acids involved together with the common β-chain in receptor signaling (Fig. 2). The extracellular domains of the other members of the hematopoietin receptor family are characterized by a 200-amino-acid extracellular homology module containing two fibronectin type III domains with conserved sequence features: 1) an *N*-terminal region of about 100 amino acids that shares significant sequence homology to similar regions of the GM-CSF and IL-5 receptor α-chains; and 2) a cytokine receptor domain containing a membrane-proximal WSXWS motif and four conserved cysteine residues distal to the membrane (Fig. 2) (9). The extracellular domain of the IL-3R α-chain contains six potential glycosylation sites; the presence of *N*-linked oligosaccharides is strictly required for both receptor binding and receptor signaling. Finally, the cytoplasmic tail of the IL-3R α-chain contains a short proline-rich motif similar to Box 1 of the common β-chain.

The common β-chain of IL-3R is shared with IL-5R and GM-CSFR. It has a dual function, allowing the formation of a high-affinity IL-3R and transmitting the signal to the cell originated through receptor activation. The human common β-chain is encoded by a gene located on chromosome 22q12.2-13.1. The analysis of the structure of the cDNA of the human β-chain predicts a 95-kD polypeptide comprised of 880 amino acids, showing three

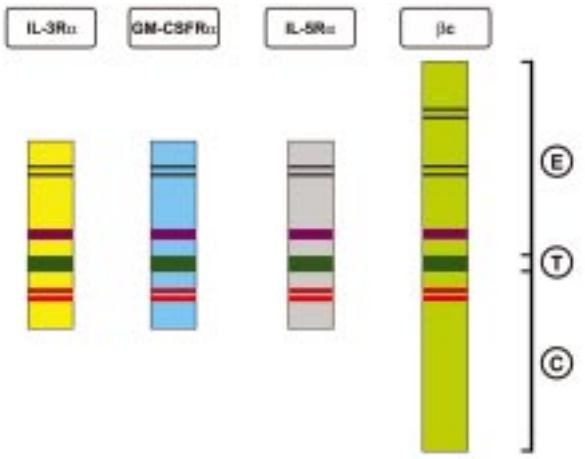

Fig. 2. Structure of the membrane receptors pertaining to the IL-3/GM-CSF/IL-5 cytokine receptor subfamily. The extracellular (E) region of the α-chain contains a homology domain containing two fibronectin type II domains with paired cysteine residues (black lines) and one membrane-proximal WSXWS domain (violet box). The extracellular domain of the common β-chain is similar to the corresponding domains of the α-chains, but it is longer, with two pairs of fibronectin homology domains and two pairs of cysteine residues. The transmembrane (T) region of the receptors is shown in dark green. The α-chains contain a short cytoplasmic (C) tail, while the common β-chain exhibits a much longer cytoplasmic region required for receptor signaling. In this cytoplasmic domain, all cytoplasmic receptor regions possess two conserved boxes (red) involved in JAK binding.

potential *N*-glycosylation sites (10). The receptor β-chain exhibits homology with the corresponding α-chain and shows three receptor regions: 1) an extracellular receptor portion that contains duplicate cytokine receptor modules that represent conserved 200-amino-acid domains of the cytokine receptor superfamily, each domain being characterized by four cysteine residues in the membrane-distal region, a Pro-Pro pair which divides the domain into two 100-amino-acid type III fibronectin subdomains, and a highly conserved WSXWS motif; 2) a short transmembrane domain that associates the receptor with the cell membrane; and 3) a 432-amino-acid cytoplasmic domain, markedly longer that that of the α-chain and responsible for the cell signaling of IL-3R (Fig. 2).

The β-chain is present in two different isoforms: the full-length transcript described above and a truncated transcript lacking a 104-bp segment just 3' of the transmembrane region (11). The deletion of this 104-bp sequence determines the formation of a truncated β-chain (β-chain IT) that conserves only 23 amino acids at the level of the cytoplasmic tail and possesses 23 new amino acids in the *C*-terminal tail (11). As a consequence, the cytoplasmic tail of the truncated β-chain possesses only a short cytoplasmic tail comprised of 46 amino acids, able only to bind the Janus kinase JAK2 and unable to mediate IL-3R signaling. The truncated β-chain not only is unable to transmit receptor signaling, but also acts as an inhibitor of β-chain signaling and therefore as an inhibitor of IL-3R, GM-CSFR and IL-5R. In normal myeloid cells, the truncated β-chain protein corresponds only to a small fraction of the β-chain (about 20%) and its synthesis is reduced by growth factors such as GM-CSF and M-CSF (12). In contrast, in acute myeloid leukemia blasts, the level of truncated β-chain expression is higher than that observed in normal myeloid cells (11).

Although the common β-chain plays a key role in IL-3R signaling, there is evidence that the cytoplasmic domain of IL-3Rα also exerts an important role in receptor signaling. This finding originates from studies focused on determining the structural basis for the specificity of the biological effects of IL-3 compared to GM-CSF. Particularly, in the murine FDP-mix multipotential cell line, IL-3 maintained the multipotential activity of these cells, while GM-CSF induced their differentiation (13). Bioinformatic analysis of IL-3Rα and GM-CSFRα defined a tripeptide sequence adjacent to the proline-rich region of their cytoplasmic domains that affected cytokine-specific function (14).

The dimerization of the α- and common β-chain subunits of IL-3R is recognized as a crucial step in receptor activation; however, the exact composition of the assembled complex, as well as the amino acid residues involved in interchain interactions, are unclear. Some studies have suggested that simple receptor heterodimerization is sufficient to activate IL-3R (15), while cross-linking (16) and dominant-negative (17) studies using surface-expressed receptors suggest that the formation of high-order complexes is required for receptor activation. In this context, dimerization of the common β-chain seems to represent an important and necessary step in receptor activation (18, 19). In line with these observations, the β-chain has been shown to crystallize as a homodimer (20). Experiments carried out with soluble forms of the GM-CSFR α- and β-chains provided evidence that, in the presence of the ligand (GM-CSF), a ternary complex is built up, formed by one β-chain homodimer, a single GM-CSFRα molecule and a single GM-CSF molecule (21). The same seems to apply to IL-3R. Crystallization studies of the β-chain, as well as the development of several appropriate mutants of this receptor chain, allowed the determination of the possible structure of the IL-3-binding domains on the β-chain; this binding structure is formed by domains 1 and 4 of the two different β-chains forming the homodimer (22, 23).

IL-3R signaling

IL-3 mainly induces three types of biological effects in target cells, consisting of the induction of survival, proliferation and cell differentiation. These different biological effects require the activation of several signal transduction pathways, resulting in a reprogrammation of gene expression.

The first event in the mechanism of induction of a cell response to IL-3 is represented by the binding of this cytokine to its specific membrane receptor; the initial binding of IL-3 to the IL-3R α-chain results in the recruitment of a β-chain homodimer with the consequent formation of a high-affinity heterodimeric complex, culminating in initiation of cell signaling.

The intracellular cell signaling originated by activation of IL-3R involves three main signal transduction pathways: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathways and the phosphatidylinositol 3-kinase (PI3-kinase) pathway.

JAK/STAT pathway

The JAK/STAT pathway is used by all cytokines as a signal transduction mechanism for transmitting extracellular signals generated by the activation of their membrane receptors to promoters of target genes (24-26). To date, four mammalian members of the JAK family (JAK1, JAK2, JAK3 and TYK2) have been discovered. JAKs are constitutively associated with membrane-proximal intracellular domains of cytokine receptors and become phosphorylated after ligand binding and dimerization or oligomerization of the respective receptor chains. Activated JAKs constitutively phosphorylate tyrosine residues at the distal part of receptor chains (the deletion of the distal part of the intracytoplasmic segment of βc completely abolishes the response to IL-3 [27]), thereby generating docking sites for STATs and other intracellular signaling molecules (24-26). STATs recruited to specific phosphotyrosine-containing motifs located in the

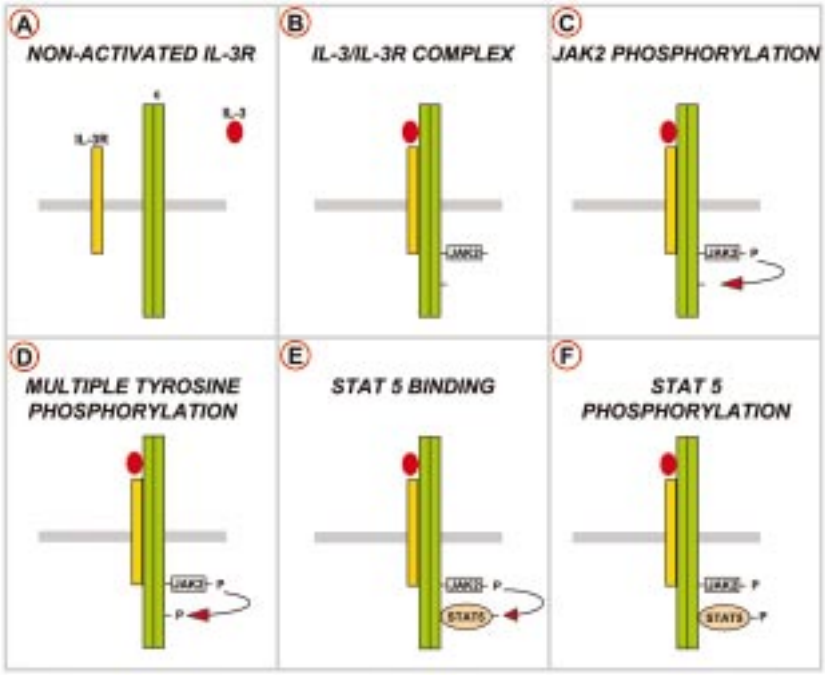

Fig. 3. Mechanism of STAT activation by IL-3. In this schematic model six steps are outlined: A) the IL-3 R is not activated and α- and β-chains do not form a heterodimer; B) the binding of IL-3 to its receptor induces the formation of the heterodimeric high-affinity IL-3R; C) following IL-3R activation, JAK2 is phosphorylated and binds to two tyrosine residues present in the proximal region of the cytoplasmic tail of the common β-chain; D) JAK2 in turn phosphorylates the common β-chain at the level of multiple tyrosine residues that E) act as docking sites for STAT5 binding; and F) STAT5 first binds to the tyrosine residues of the common β-chain and is then phosphorylated. Finally, the phosphorylated STAT5 migrates into the nucleus, where it induces a specific pattern of gene transcription (not shown).

cytoplasmic tails of the receptors are in turn phosphorylated by activated JAKs at distinct tyrosine residues (Fig. 3). After this modification, STATs either homodimerize or heterodimerize, leave the receptor complex, enter the nucleus and bind to response elements of target genes, thus influencing their transcriptional activity (Fig. 3) (24-26).

Gene-targeting experiments have provided clear evidence that JAK2 is strictly required for both the development of the hematopoietic system (28) and for the response to cytokines, including IL-3 (29).

Although JAK2 is the kinase predominantly activated by IL-3, members of the Src family of kinases, such as Fyn, Lyn, Syk, Btk and Hck, have also been reported to be activated by IL-3. JAK activation determines the phosphorylation of the β-chain at the level of six tyrosine residues: Tyr^{577}, Tyr^{612}, Tyr^{695}, Tyr^{750}, Tyr^{806} and Tyr^{866} (30). Three of these tyrosine residues —Tyr^{612}, Tyr^{695} and Tyr^{750}— serve as docking sites for the Src homology 2 (SH2) domains of two members of the STAT family: STAT1 and STAT5. These two STATs are recruited to the receptor complex by their SH2 domains, become tyrosine-phosphorylated on critical residues in their carboxy termini, and then homodimerize or heterodimerize by means of reciprocal SH2 and phosphotyrosine interactions.

The two STATs that play the most relevant role in mediating the biological effects of IL-3/IL-3R are STAT5 and STAT3. As discussed above, STAT5 activation is dependent on the β-chain, while STAT3 activation is mainly dependent on the cytoplasmic tail of the IL-3R α-chain (27). The target genes of STAT3 and STAT5 are represented by a set of genes involved in survival (anti-apoptotic genes), proliferation and differentiation. In this complex, a remarkable activating effect is exerted by STAT5 on retinoic acid receptors (RARs). These receptors regulate the growth and differentiation of primitive myeloid precursors *in vitro,* and knockout mice deficient in RARα/RARγ display a block in granulocytic differentiation (31). IL-3 markedly stimulates RAR expression through a STAT5-dependent mechanism; interestingly, activated STAT5 and RARs physically interact, forming a heterodimer (32).

MAPK pathways

The MAPK pathways include three main signaling cascades: the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38. These pathways are key mediators of a large number of important cellular responses that play a major role in the control of cell growth, survival and proliferation. These

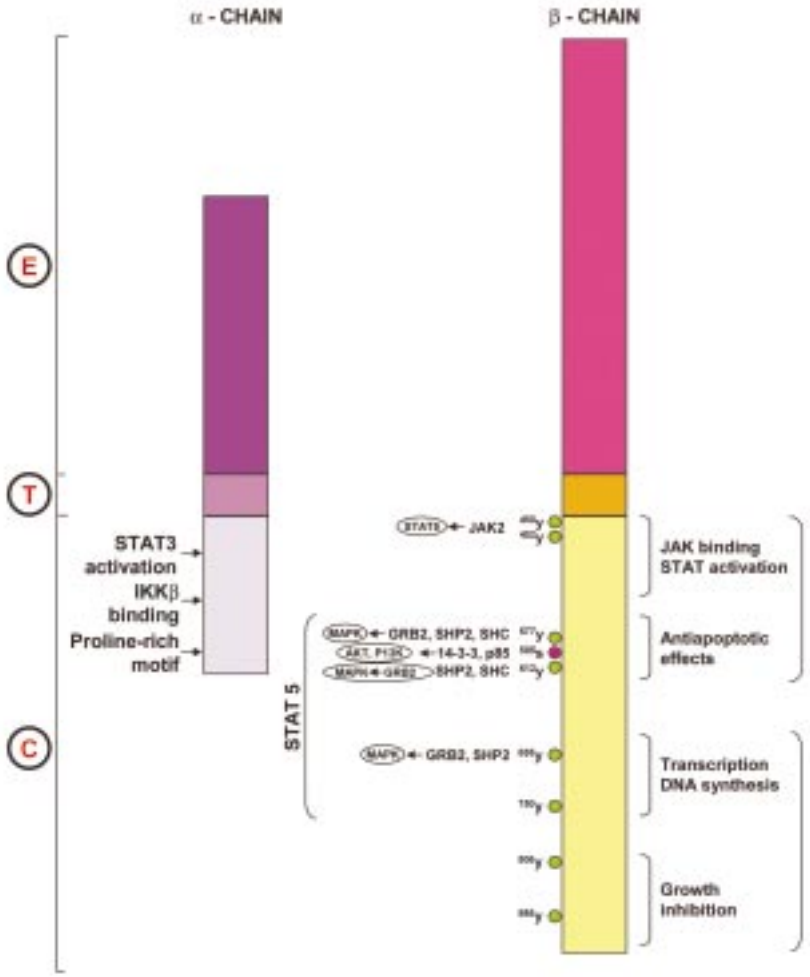

Fig. 4. Main sites of the cytoplasmic tails of the IL-3R α- and β-chains involved in receptor-mediated signal transduction. In the cytoplasmic tail of the common receptor β-chain, the tyrosine (Y) residues and serine (S) residue required for induction of signal transduction are outlined. E = extracellular region; T = transmembrane region; C = cytoplasmic tail.

important signaling pathways are activated in response to many cytokines and growth factors. All three signaling pathways are activated by IL-3.

JNK is a member of the MAPK family and was identified as a stress-activated protein kinase that phosphorylates c-Jun on two sites in the NH_2-terminal activation domain. The upstream pathway leading to JNK activation is complex and involves as an end step the dual phosphorylation of the motif Thr-Pro-Tyr located in the activation loop (33). JNK phosphorylation is mediated by two MAPKs —MAP2K4 and MAP2K7— that cooperatively activate JNK. JNK activation leads to the phosphorylation of a number of transcription factors, most notably the c-Jun component of AP-1, and cellular proteins, mainly those associated with the control of cell apoptosis. This last mechanism in particular is responsible for the antiapoptotic mechanism induced by IL-3 via JNK activation. In fact, JNK is responsible for the phosphorylation and consequent inactivation of the proapoptotic Bcl-2 family protein Bad; phosphorylated Bad becomes unable to bind the antiapoptotic molecule Bcl-X_L (34). IL-3 withdrawal is associated with inactivation of JNK, which leads to dephosphorylation of Bad, which determines its potent activity (34).

The activation of the ERK pathway by IL-3 starts with the phosphorylation of the β-chain at the level of residue Tyr^{577}, induced by JAK2 activation. This tyrosine residue, once phosphorylated, becomes a docking site for the cellular substrate SHC (Src homology and collagen), which then in turn becomes phosphorylated, allowing it to interact with the SH2 domain of the growth factor receptor-bound adaptor protein Grb2 and the nucleotide exchange factor SOS (Fig. 4) (35). The SHC/Grb2 interaction leads to an association with the nucleotide exchange factor for Ras, which in turn leads to the sequential activation of Ras, Raf-1, MEK and ERK. Using β-chain mutants in which a single tyrosine residue is left intact, it was shown that either Tyr^{577}, Tyr^{612} or Tyr^{695} was sufficient to promote SHC phosphorylation, Grb2 association and MAPK activation (Fig. 4) (36).

The downstream targets of activated ERK are represented by genes involved in the control of apoptosis and transcription factors that control the expression of genes involved in regulating proliferation. Concerning apoptosis genes, IL-3 is a stimulator of *Bcl-2* and *Bcl-X_L* expression through an ERK-dependent mechanism (36). In addition to the upregulation of antiapoptotic factors, the ERK pathway also inhibits apoptosis through inactivation of proapoptotic factors, such as Bad, via phosphorylation. Concerning the stimulatory effect on cell proliferation, the ERK pathway induces the transcription factors activating transcription factor 2 (ATF2) and ternary complex factor (TCF), which are capable of activating the proliferation-related genes c-*Fos* and c-*Jun* (37).

The tyrosine phosphatase Src homology protein tyrosine phosphatase 2 (SHP2) appears to be a positive regulator of the cell signaling originated by the common β-chain. In fact, the catalytic activity of SHP2 is strictly required for both JAK2 and ERK activation, but not for PI3-kinase induction (38).

The activation of the MAPK p38 pathway by IL-3 is related to the induction of antiapoptotic mechanisms. In fact, the antiapoptotic *mcl-1* is an immediate-early gene activated by IL-3 through a transcriptional mechanism involving the phosphorylation of PU.1 and subsequent transcriptional activation of the *mcl-1* gene by this phosphorylated transcription factor (39).

PI3-kinase pathway

While β-chain tyrosine phosphorylation is required for the activation of STAT and MAPK signaling cascades, PI3-kinase activation requires phosphorylation of the β-chain at the level of Ser^{585} (Fig. 4). The phosphorylation of Ser^{585} results in recruitment of the adaptor molecule 14-3-3 (40). Mutation at this amino acid position causes a block in PI3-kinase activation by IL-3 (41).

PI3-kinase is a heterodimeric lipid kinase composed of a regulatory subunit (p85) and a catalytic subunit (p110). Following IL-3 stimulation, activated PI3-kinase phosphorylates phosphatidylinositol lipids at the level of the inositol residues present in the cell membrane, thereby allowing the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3), a second messenger that interacts with proteins through pleckstrin homology domains and induces a variety of biological effects (42).

One important target of PIP_3 is the serine/threonine kinase Akt. This kinase plays a key role in the survival signals originated from PI3-kinase activation. Thus, the activation of Akt in response to IL-3 produces phosphorylation of Bad, resulting in 14-3-3 binding and sequestration in the cytoplasm, with consequent cell survival (43). In the absence of IL-3, PI3-kinase and Akt are not activated and Bad remains unphosphorylated, allowing it to translocate to mitochondria where it can bind the antiapoptotic proteins Bcl-2 and Bcl-X_L and induce cell death.

More recently, it was observed that Akt could promote cell survival by acting as an inducer of nuclear factor-κB (NF-κB). Under normal conditions, NF-κB is maintained in an inactive, latent state in the cytoplasm by the inhibitor protein IκB. Akt phosphorylates IκB kinase (IKK), which in turn phosphorylates IκB (44). Phosphorylated IκB is then targeted for degradation by the proteosome, allowing NF-κB to translocate to the nucleus and regulate gene expression. NF-κB is able to regulate the expression of a large set of genes, particularly the antiapoptotic genes *Bcl-2*, *Bcl-X_L* and *A1*. Recent studies provided clear evidence that this pathway is involved in the stimulatory effect exerted by IL-3 on *Bcl-2* gene expression (45).

The PI3-kinase pathway is involved not only in mediating the antiapoptotic effects of IL-3, but also in the control of cell proliferation by IL-3 via induction of the c-Myc protein.

Pim gene

Antiapoptotic mechanisms are induced by IL-3 not only via signal transduction pathways, but also via transcriptional stimulation. This is the case of the Pim-2 protein, a member of a family of serine/threonine kinases consisting of three members (Pim-1, Pim-2 and Pim-3), the expression of which is markedly stimulated by IL-3. In particular, in an IL-3-dependent lymphoid cell line, it was shown that *Pim-2* gene expression was downregulated to the greatest extent following IL-3 deprivation. The activity of Pim-2 as an antiapoptotic factor appears to be related to phosphorylation and consequent inactivation of the proapoptotic proteins Bad and Bim (46). The effects of Pim-2 on cell survival are similar to those displayed by Akt; however, Pim-2 does not appear to act through Akt.

Negative control of IL-3R signaling

Excessively uncontrolled signaling through IL-3R, as well as through other cytokine receptors involved in the stimulation of cell proliferation, could have deleterious effects and lead to uncontrolled cell growth. Therefore, several biochemical mechanisms limit in both extent and time the signaling originating from activation of IL-3R.

One mechanism is based on the activity of the suppressors of cytokine signaling (SOCS) family of small SH2-containing proteins. These proteins inhibit STAT signaling through two different mechanisms: one related to inactivation of JAK2 by blocking the access of STAT5 to receptor binding sites (47), and the other to ubiquitination of JAK2 with subsequent degradation (48). The inhibitory effect of SOCS-1 on STAT5 activity is potentiated by Pim-1, a serine/threonine kinase induced by IL-3. Pim-1 acts by phosphorylating SOCS-1, an event that stabilizes the interaction of this protein with STAT5 (49). It is therefore evident that both Pim-1 and SOCS-1 are components of a negative feedback mechanism that allows STAT5 to attenuate its own activity.

A second mechanism of attenuation of the STAT signaling originating from activation of IL-3R is related to the

activation of cytosolic tyrosine phosphatases that control the level of phosphorylation of cellular substrates of activated tyrosine kinases. The tyrosine phosphatase SHP1 is involved in the negative control of IL-3R signaling, as shown by the observation that its overexpression resulted in a complete loss of IL-3-induced effects. SHP1 interacts directly with the IL-3R common β-chain at the level of Tyr^{612}, after phosphorylation (50).

A third mechanism common to several membrane receptors is represented by receptor internalization, with subsequent degradation. In fact, for the majority of membrane receptors, including IL-3R, ligand binding determines the internalization of the receptor/ligand complex into clathrin-coated vesicles. These vesicles then fuse with early endosomes, where receptors are either resorted for receptor recycling back to the cell membrane or are targeted into late endosomes and finally degraded in lysosomes. Two lines of evidence suggest that this mechanism plays a role in the termination and modulation of IL-3R signaling: 1) prolonged IL-3R signaling is observed in the presence of proteosome inhibitors (51); and 2) biochemical studies provided evidence that during IL-3R internalization, the IL-3R common β-chain, but not the IL-3R α-chain, is proteolytically degraded at the level of the cytoplasmic domain (52).

IL-3R in leukemias

Initial studies based on analysis of the biological responses elicited *in vitro* by IL-3 and on the binding of [^{125}I]-IL-3 to membrane receptors showed that most human acute myeloid leukemia (AML) blasts proliferate in response to IL-3 and express IL-3Rs (53-56). In particular, in more than 85% of AML cases IL-3 elicited a clear stimulation of [^{3}H]-thymidine incorporation and was significantly more active as a single agent than other hematopoietic growth factors (53, 54). Similarly, the analysis of [^{125}I]-IL-3 binding to AML blasts showed that > 80% of AMLs exhibit high-affinity IL-3 receptors (55, 56). In parallel, studies carried out on acute lymphoid leukemias (ALL) showed that the majority of B-cell acute lymphoblastic leukemias (B-ALL), but not T-cell acute lymphoblastic leukemias (T-ALL), are responsive to IL-3. It was shown that IL-3 stimulated the proliferation of B-ALL in the majority of cases (57) and that B-ALL blasts possess functional IL-3Rs (58). Interestingly, the CD40 ligand (CD40L) induces a marked increase in IL-3Rs on B-ALL blasts. In line with this finding, CD40L added together with IL-3 allows sustained *in vitro* growth of B-ALL blasts for up to several weeks (59).

More recently, the availability of antibodies specific for the α- and β-chain of IL-3R allowed us to evaluate in detail the expression of these two receptor chains in leukemias. Antibodies interacting with the *N*-terminal of the IL-3R α-chain and blocking the binding of IL-3 to its receptor, as well as antibodies interacting with other regions of the molecule and failing to inhibit IL-3 binding or function, have been developed (60). In parallel, antibodies specifically interacting with the common IL-3R/GM-CSFR/IL-5R β-chain have been developed, thus allowing the exploration of the expression of both IL-3R chains. The study of normal IL-3Rα during normal hematopoietic differentiation provided evidence that this receptor chain is clearly expressed in $CD34^+$ HPCs and its expression was maintained during all stages of granulocytic and monocytic differentiation, while it was lost after the initial stages of erythroid and megakaryocytic differentiation (61).

Flow cytometry studies using anti-IL3R α-chain monoclonal antibodies have demonstrated expression of this receptor on normal human monocytes (very low expression), eosinophils, basophils, vascular endothelial cells and a subpopulation of B-lymphocytes, but not on neutrophils and T-lymphocytes. The IL-3R/GM-CSFR/IL-5R common β-chain was expressed at low levels in $CD34^+$ cells and its expression increased during the initial stages of granulocytic and monocytic differentiation and was maintained up to the late stages of maturation, while it was transiently expressed only during the early stages of erythroid and megakaryocytic differentiation and then disappeared (62).

Screening of IL-3Rα expression in hemo/lymphopoietic malignancies provided evidence that this receptor chain is expressed in the large majority of B-ALL and AML, usually not expressed in T-ALL or in the majority of lymphomas, and is highly expressed in hairy cell leukemia (63). These findings have been confirmed on a large series of acute leukemias, clearly indicating that an elevated expression of IL-3Rα is mainly observed in AML and B-ALL. We also observed that the IL-3R α-chain is overexpressed (*i.e.*, at levels significantly higher than those observed in normal $CD34^+$ cells, normal cells expressing the highest levels of IL-3R α-chain) in 40% of patients with B-ALL and 45% of patients with AML. The biological consequences of this overexpression pattern were investigated in AMLs, showing that: 1) in all patients the blasts expressing elevated IL-3Rα levels exhibit higher cycling activity and increased resistance to apoptosis triggered by growth factor deprivation; 2) spontaneous STAT5 phosphorylation was frequently observed only in AML patients exhibiting high levels of IL-3Rα expression; and 3) following IL-3 treatment, STAT5 was activated at significantly higher levels in blasts with elevated IL-3Rα expression than in blasts with IL-3Rα levels within the normal range (64).

Given the peculiar properties of leukemic blasts expressing high levels of IL-3Rα, we then explored the possible association between elevated IL-3Rα expression and the response to therapy. In this context, we observed that: 1) there is a good correlation between IL-3Rα levels and the number of leukemia blasts at diagnosis, suggesting a role for IL-3Rα in leukemic blast proliferation/survival; 2) patients with elevated levels of IL-3Rα on their leukemic blasts exhibited a reduced number of complete remissions, a higher relapse rate and a lower survival than patients with normal/low IL-3Rα levels. Overall, these findings suggested that in AML,

deregulated expression of IL-3Rα may contribute to the proliferative advantage of the leukemic blasts and, hence, to a poor prognosis (64).

In addition to these findings, another study extended the analysis of IL-3Rα to subpopulations of AML blasts and, in particular, leukemic stem cells (*i.e.*, the subpopulation of leukemic blasts corresponding to cells that maintain the leukemic process and that seemingly represent the main target of the initial leukemic transformation). The analysis of an AML subpopulation enriched in leukemic stem cells (*i.e.*, $CD34^+/CD38^-$) showed that these cells express high levels of IL-3Rα, while in their normal counterparts (*i.e.*, $CD34^+/CD38^-$ cells isolated from normal controls), low levels of IL-3Rα have been observed, suggesting that IL-3Rα represents a unique marker for AML stem cells (65). These findings showed that the isolation of $CD34^+/CD38^-$/IL-3Rα^{++} cells represents an efficient system for the purification of leukemic stem cells in AML patients (66).

In line with these observations, the study of the immunophenotypic features of AML blasts, differentiated into three different subgroups according to IL-3Rα levels —IL-3R^{high} (IL-3Rα^{+++}), IL-3R^{middle} (IL-3Rα^{++}) and IL-3R^{low} (IL-3Rα^{+})— showed that the group of AML characterized by high expression of IL-3Rα exhibits several interesting and distinctive features compared to the other two subgroups, consisting of: 1) a more elevated expression of progenitor cell markers, such as CD34; 2) a high expression of membrane tyrosine kinase receptors, such as flt3 and c-kit; and 3) a low expression of membrane myeloid maturation markers, such as CD11b and CD15 (67).

Abnormalities of the IL-3/IL-3R system are also involved in the pathogenesis of other hematological malignancies. In chronic myeloid leukemia (CML), there is evidence that the consistent and selective activation of IL-3 production within leukemic (BCR-ABL$^+$) $CD34^+$ cells may act as an autocrine mediator of progenitor CML proliferation (68). Interestingly, IL-3 production is rapidly induced when normal primitive bone marrow progenitors are transduced with BCR-ABL (69). Similarly, in myeloma cells, an IL-3 autocrine pattern was observed, responsible for both stimulation of tumor cell proliferation and osteoclast formation (70).

Development of therapeutic approaches tailored to IL-3R

Targeted therapies are the focus of many reasearch efforts in oncology. The development of biological therapies that target tumor-associated antigens offers hope for improvement in therapy and survival in many cancers, including leukemias (71, 72). Taking advantage of the findings from the studies on IL-3 and IL-3R expression in AML, several investigators have explored the efficacy of drugs based on a diphtheria toxin (DT)-human IL-3 fusion protein. The basic idea is that the fusion protein interacts with the leukemic blasts expressing high levels of IL-3R and is taken up by leukemic cells through receptor endocytosis, following which the toxin portion is released from the endosome into the cytoplasm and the tumor cell is killed by the toxin (73).

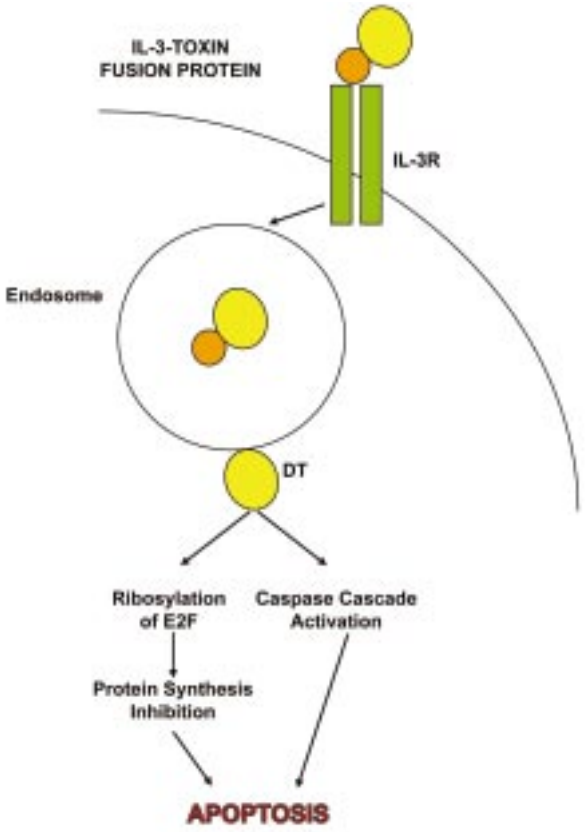

Fig. 5. Mechanism of the cytotoxicity mediated by an IL-3-toxin fusion protein. The fusion protein formed by human IL-3 fused to diphtheria toxin (DT) via a linker first binds to surface IL-3 receptors and is then internalized into endosomes, where it is cleaved, with the consequent release of the catalytic domain of the toxin. The free toxin then diffuses to the cytoplasm, where it mediates its cytotoxic effects through inhibition of protein synthesis.

Recombinant DT is a 535-amino-acid residue protein containing three functional domains: a catalytic domain, connected by an ariginine-rich disulfide loop to a translocation domain, followed by a cell-binding domain. Diphtheria toxin is first transported within the cell by the protein to which it is fused using a receptor-mediated system, and it is then cleaved at the level of endosomes by furin, with the consequent release of the catalytic domain (Fig. 5). This domain translocates to the cytoplasm, where it phosphorylates the ADP-ribosylatable elongation factor 2 (EF-2), thereby inducing inhibition of protein synthesis following by cell death (Fig. 5).

The mechanism through which targeted toxins kill the cells is largely unknown. Recent studies indicate that DT-GM-CSF kills tumor cells through a process involving a death receptor-independent activation of FADD (Fas-associated death domain) and of the caspase machinery. In line with this observation, the killing of leukemic blasts

induced by DT-GM-CSF is blocked by caspase inhibitors (74).

Other studies were initially carried out in murine models showing that a DT-murine IL-3 fusion protein was toxic for murine IL-3-bearing leukemic cell lines, but spared the majority of normal mouse bone marrow-repopulating stem cells (75, 76). The fusion protein used in these studies was composed of the catalytic and translocation domains of DT (DT_{388}) fused via a Met-His linker to human IL-3.

Two subsequent studies evaluated the effect of a DT-human IL-3 fusion protein on AML blasts. In an initial study, preliminary evidence was obtained that the DT-human IL-3 fusion protein was more cytotoxic for AML blasts than for normal bone marrow hematopoietic progenitors (77). In a second study, the issue was studied in more detail, showing that: 1) a high percentage of AML progenitors or AML LTC-IC ("long-term culture-initiating cells") were killed by the IL-3 fusion protein, with most of the surviving progenitors being cytogenetically normal; 2) a significant proportion of normal hematopoietic progenitors were killed, while LTC-ICs were not significantly killed by DT-IL-3; 3) a significant proportion of leukemic but not normal repopulating stem cells, as evaluated by the NOD/SCID assay, were killed by DT-IL-3; and 4) the *in vivo* administration of DT-IL-3 markedly reduced the engraftment of AML cells in NOD/SCID mice (78). Importantly, both DT-IL-3 and DT-GM-CSF displayed the property of killing resting G_0 leukemic blasts, a cell population particularly resistant to cytotoxicity induced by chemotherapeutic or immunological agents commonly used in the therapy of acute leukemias (79).

On the basis of these observations, the same investigators have evaluated the antileukemic activity of DT-human IL-3 in the treatment of immunocompromised mice engrafted with human IL-$3R^+$ AML blasts. The results of these studies showed a substantial killing of malignant progenitors *in vivo* by DT-IL-3, with potency less than that of a standard antileukemic cytotoxic drug such as cytarabine, but comparable to that displayed by other agents for target-specific therapy (such as DT-GM-CSF or anti-CD33-calicheamicin) (80).

Thus, further preclinical development of DT-IL-3 appears warranted. This drug could be useful in the treatment of patients refractory to standard chemotherapy or in association with chemotherapy. Furthermore, DT-IL-3 may have a toxicity profile more favorable than that of DT-GM-CSF. The fusion protein comprised of GM-CSF and DT was developed as a clinical-grade reagent and was used for the treatment of AML patients resistant to standard chemotherapy; of 31 treated patients, 3 remissions were observed. However, DT-GM-CSF produced dose-limiting toxicity due to liver injury related to the binding of GM-CSF receptors present on the surface of liver macrophages (81). Since IL-3Rs are not present on liver macrophages, it is expected that DT-IL-3 may have a better toxicity profile and a wider therapeutic index than DT-GM-CSF. This finding was verified using a clinical-grade preparation of DT_{388}-IL-3 fusion protein, which showed 10 times lower acute toxicity in monkeys compared to the corresponding GM-CSF fusion protein. Furthermore, DT_{388}-IL-13 did not cause any liver toxicity in these animals (82).

In addition to the wild-type IL-3, IL-3 mutants exhibiting a binding affinity for the IL-3R higher than wild-type IL-3 were fused to the toxin. Importantly, these IL-3-DT mutants killed leukemic blasts at a dose 5-fold lower than the standard DT-IL-3 fusion protein (83).

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