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Leukemia Inhibitory Factor (LIF)

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Abstract

Leukemia inhibitory factor (LIF) is the most pleiotropic member of the interleukin-6 family of cytokines. It utilises a receptor that consists of the LIF receptor β and gp130 and this receptor complex is also used by ciliary neurotrophic growth factor (CNTF), oncostatin M, cardiotrophin 1 (CT1) and cardiotrophin-like cytokine (CLC). Despite common signal transduction mechanisms (JAK/STAT, MAPK and PI3K) LIF can have paradoxically opposite effects in different cell types including stimulating or inhibiting each of cell proliferation, differentiation and survival. While LIF can act on a wide range of cell types, LIF knockout mice have revealed that many of these actions are not apparent during ordinary development and that they may be the result of induced LIF expression during tissue damage or injury. Nevertheless LIF does appear to have non-redundant actions in maternal receptivity to blastocyst implantation, placental formation and in the development of the nervous system. LIF has also found practical use in the maintenance of self-renewal and totipotency of embryonic stem cells and induced pluripotent stem cells.

Keywords

leukemia inhibitory factor; JAK/STAT/SOCS; LIF receptor; embryonic stem cells; pregnancy; nerve and muscle

Introduction

LIF was first cloned as an inducer of differentiation and inhibitor of proliferation of a myeloid leukemic cell line (M1) [1]. At the same time other groups were purifying activities that suppressed differentiation of embryonic stem cells (DIA) [2], stimulated proliferation of myeloid DA1 cells (HILDA) [3], induced an acute phase response in hepatocytes (HSF) [4], caused neurotransmitter switching in neurons (CNDF) [5] [6] and inhibited adipocyte lipoprotein lipase activity (MLPLI) [7]. Following purification each of these activities was

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Conflict of Interest: NAN is an inventor on patents relating to LIF

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shown to be identical to LIF. In this review we will summarize the molecular features of LIF/receptor interactions and intracellular signalling as well as the highly pleiotropic biological activities of LIF with an emphasis on non-redundant actions.

The LIF glycoprotein

LIF is synthesised as a 202 amino acid precursor that is post-translationally processed into a 20 kDa form by removal of 22 amino acids from its N-terminus. LIF structures have been solved by both X-ray crystallography [8] and nuclear magnetic resonance (NMR) [9] and these studies have shown that, similarly to other IL-6 family cytokines, LIF exists as a compact four-helix bundle in an up-up-down-down configuration. The first helix (helix A) begins at residue Leu44 (residue 22 of the mature chain) and the region N-terminal to this is covalently linked to the C-terminal section of helix 3 via two disulphide bonds (Cys34-Cys156 and Cys40-Cys153). This N-terminal region is important for receptor binding [10]. In addition to the aforementioned disulphide bridges, there is a third disulphide bond that joins helix D to the linker between helices A-B. NMR analyses showed that LIF is an extremely stable molecule as evidenced by extremely slow amide-exchange rates and relaxation parameters.

The LIF Receptor consists of two signalling chains: gp130 and LIFR β

LIF and IL-6 are closely related cytokines that both signal through the shared cytokine receptor gp130 [11]. Despite this, there are dramatic differences between the receptors of these two cytokines, in terms of stoichiometry, composition, architecture and thermodynamics. The signalling competent complex between IL-6 and its receptor is a hexamer that consists of two molecules each of IL-6, gp130 and IL-6 Receptor alpha (IL-6R α) [12, 13]. Hexameric assembly is required because the intracellular domain of IL-6R α does not bind JAK and so signal transduction can only be achieved by transactivation of two JAK molecules bound to the two copies of gp130. This architecture is also shared by IL-11 [14].

On the other hand, the signalling competent complex of LIF with its receptor is a trimer [15] that consists of LIF bound to one molecule of gp130 as well as a second receptor chain called LIFR (LIF receptor-which we will subsequently refer to as LIFR β) [16] that is architecturally similar to gp130 [17]. Like gp130, LIFR β binds JAK via its intracellular domain [18] and hence signal transduction can be initiated by transactivation of the gp130-bound and LIFR β -bound JAK molecules without requiring higher-order association. Notably, there is no “alpha-chain” receptor required for LIF signalling (see Fig. 1).

LIFR β

LIFR β is a single pass transmembrane domain-containing protein and is the tallest of the “tall” cytokine receptors, containing eight distinct domains in its extracellular segment [16]. These eight domains are all of β -sandwich architecture [19] and can be sub-classified as two cytokine binding modules (CBMs, themselves each composed of two individual β -sandwich domains) separated by an immunoglobulin-like domain followed by three fibronectin type III (FnIII) domains at the membrane proximal end. The FnIII domains are sometimes referred to as the “legs” of the tall receptors and ensure the correct placement of the transmembrane

and intracellular domains such that JAK activation, by cross-phosphorylation, can occur after cytokine binding. gp130, which will be more fully discussed elsewhere in this issue, is highly similar, lacking only the first CBM.

The structure of domains 1-5 of LIFR β have been solved by X-ray crystallography, both with [19] and without [15] bound LIF. The two CBM's are structurally similar; both adopt elbow-like structures bent at $\sim 70^\circ$ and overlay with significant similarity [10]. The Ig-like domain that separates these two modules is oriented perpendicular to the first CBM (forming a "T"-helped by a disulphide) and is roughly co-linear with the first β -sandwich domain of the second CBM. Overall, domains 1-5 of the receptor adopt a roughly co-linear "zig-zag"-like conformation, 160 angstroms long x 60 wide. To date there is no structural data on the "legs" of the LIF receptor.

Although termed the LIF-receptor, LIFR β is actually shared between five cytokines: LIF, oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic growth factor (CNTF) and cardiotrophin-like cytokine (CLC). All of these cytokines signal via a gp130:LIFR β heterodimer [20]. The latter three also require an alpha-chain receptor (CNTFR α) to form a quaternary complex in order to initiate signalling. OSM, like LIF, can signal through a simple gp130:LIFR β dimer (Fig. 1).

Interaction between LIF and its receptor

Thermodynamically, LIF engages its receptor in a very different manner to the similar cytokine, IL-6. IL-6 binding to gp130 and IL-6R α is an ordered and co-operative process [12]: IL-6 first recruits its specific alpha receptor chain (IL-6R α) with high affinity ($K_d = 9$ nM) using a surface on one face of the 4-helix bundle known as site I. This IL-6/IL-6R α complex then recruits gp130 using a surface composed of residues from both IL-6 (site II) and IL-6R α . The resulting ternary complex dimerises cooperatively to form the signalling hexamer. Importantly, IL-6 on its own does not display measurable binding affinity for gp130.

LIF, on the other hand, binds with high affinity to both gp130 (via site II) [10] and LIFR β (via site III at one end of the four helix bundle and the Ig-like domain of LIFR β) [10]. Formation of the ternary complex is non-cooperative and is thus unlikely to be ordered. The interaction between LIF with both the receptor chains (gp130 and LIFR β) is entropically driven and there is surprisingly little structural perturbation in either cytokine or receptor upon binding. The interaction of LIF with LIFR β is 80-fold tighter than with gp130, which is perhaps unsurprising given that gp130 interacts with a slightly larger array of cytokines than LIFR β . *In vivo* LIF binds to receptor with high affinity ($K_d = 50$ – 100 pM affinity), consistent with two individual high affinity interactions with the two membrane bound receptor chains [21, 22]. A kinetic analysis showed that interaction of LIF with physiological receptor showed a near diffusion-limited on-rate and an extremely slow off-rate (4×10^{-4} /min suggesting a half-life of more than 24 hours) at 4°C [22].

Using available structures, a model of the LIF:gp130:LIFR β complex can be built (Fig. 2). The full ectodomain structure of gp130 was solved using X-ray crystallography in 2010 [17] and the structure of domains 1-5 of LIFR β in 2007 [19]. Although there is no structural

information regarding the legs of LIFR β , the corresponding region of gp130 shows a pronounced kink between the first and second FnIII domains, which in the case of IL-6 and IL-11, can be modelled into existing cryo-EM data [14, 23]. By assuming the same kink in the FnIII legs of LIFR β , models of the full LIF:LIFR β :gp130 can be built. Interestingly, the models produced are very similar no matter whether the IL-6:gp130:IL-6R α hexameric complex is used as a reference (and LIFR β and LIF aligned to one copy of gp130 and IL-6 respectively) or whether the LIF:LIFR β complex is used as a reference (and the LIF:gp130 structure aligned on LIF). Observation of this model suggests that LIF cross-links its two receptor chains in such a way that they enter the cell membrane in close proximity, potentially important for subsequent activation of intracellular JAKs and agree well with the cryo-EM data of a signalling competent complex of CNTF with its receptor (which also includes gp130 and LIFR β) solved by the Garcia Laboratory [15]. Comparing this model with the well-characterised IL6:IL-6 Receptor system highlights two obvious differences (in addition to their different stoichiometry). The first is the cytokine-sized “gap” between gp130 (Ig-like domain) and LIFR β (CBM2) that, in the IL-6 system, is occupied by a second cytokine molecule. The second is the large extension of CBM1 of LIFR β which begs the obvious question—does it have a function in LIF signalling?

LIF signalling

LIF is a pleiotropic cytokine with a wide range of activities so it is unsurprising that functional LIF receptors are found in a number of different organs including the liver [21], bone [24], uterus [25], kidney and the central nervous system [26]. Quantitative studies have shown that they are found on the surface of ES cells (approximately 100–400 receptors per cell) [22, 27], in particularly high numbers in the liver (several thousand per cell) whilst within the hematopoietic system, LIF receptors primarily exist on monocytes/macrophages (several hundred per cell) [21].

Unlike receptor tyrosine kinases (RTKs), the LIF receptor does not have any intrinsic tyrosine kinase activity within its intracellular domains. Instead both the gp130 and LIFR β chains are constitutively associated with members of the JAK family of tyrosine kinases [28]. These kinases exist in an inactive state under basal conditions but are rapidly activated upon LIF engaging its receptor, via a molecular mechanism that is not yet understood. There are four members of the JAK family (JAK1, JAK2, JAK3, TYK2). Experiments using overexpressed components initially showed that the LIF receptor is capable of binding at least three of the four JAK family members (JAK1, JAK2 and TYK2) [18]. However knockout studies reveal that LIF signalling is highly abrogated in the absence of JAK1 but not JAK2 or TYK2, suggesting it is the dominant JAK family member under physiological conditions [29–31]. In support of this, Ernst and colleagues showed activation of JAK1, 2 and TYK2 following LIF exposure but significantly faster activation kinetics for JAK1, implying again that it is the kinase initially targeted by LIF [32]. Activation of JAK1 occurs by transphosphorylation, the JAK1 molecule on one receptor chain activating the other by phosphorylation of specific tyrosines, particularly Tyr1034. This tyrosine is located within the “activation loop” of the kinase domain and induces a conformational change in this loop such that it moves out of the active site of the kinase to allow substrate and Mg-ATP to dock [33]. Thus LIF engagement of its receptor results in JAK1 becoming catalytically

competent, JAK1 then initiates a cascade of tyrosine phosphorylation that stimulates three distinct signalling pathways: the JAK/STAT [18], MAP-kinase [34] and PI(3) Kinase [35, 36] pathways. Collectively these pathways contribute to differentiation, survival and self-renewal. The contributions, both quantitatively and qualitatively, of the JAK/STAT, MAP-kinase and PI(3) Kinase [35–37] pathways following LIF stimulation are often cell-type specific. As an example, we will next discuss the effects of these pathways on murine Embryonic Stem Cell (ES cell) renewal (see Fig. 3).

LIF signalling in embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of early blastocysts and retain the potential to contribute to all embryonic tissues but not the extra-embryonic structures such as the placenta. Following implantation of the blastocyst the inner cell mass develops into the epiblast from which epiblast stem cells arise with similar properties to ESCs though with somewhat reduced totipotency and capacity for self renewal.

LIF was identified as the differentiation inhibiting activity for mouse ESCs that maintained ESCs in a totipotent state and stimulated self-renewal [27]. In contrast human ESCs do not require LIF for maintenance but rather depend on fibroblast growth factor 2 and activin A. Mouse epiblast stem cells display similar colony morphology, behaviour and cytokine requirements to human ESCs suggesting that in the human, ‘ESCs’ behave more like the more mature epiblast stem cells [38].

(i) STAT3 signalling—Once activated by LIF, receptor-bound JAK1 initially targets tyrosines on the intracellular domains of the LIF receptor for phosphorylation. The phosphorylated receptor chains (both gp130 and LIFR β) then act as scaffolds to recruit a number of signalling entities, perhaps the most important of which is STAT3.

The STAT proteins are a family of transcription factors that are usually present in an inactive state in the cytoplasm [39]. Within the STAT family, STAT-1,3 and 5 are activated by gp130 family cytokines. Within this subgroup STAT3 is seen to be the most important signal transducer following stimulation by LIF and the one which mediates most of the cellular effects [40, 41]. STAT3 docks to phosphorylated tyrosines in both the gp130 and LIFR β chains of the LIF receptor at YxxM motifs (Y767, Y814, Y905 and Y915 in gp130 [42, 43]; Y981, Y1001, Y1028 in LIFR β -human numbering) [44]. Once docked, STAT3 is then itself phosphorylated by JAK1, this activation step allowing it to form a signalling-competent dimer [45] that translocates into the nucleus and up-regulates the transcription of the appropriate cytokine responsive genes.

One direct target of STAT3 is the protein SOCS3 [46–48]. SOCS3 is highly up-regulated following exposure to LIF and it then acts to shut down the JAK/STAT signalling cascade, forming a negative feedback loop [49]. SOCS3 is able to achieve this by binding to both JAK1 and gp130 [50, 51] and both inducing their ubiquitination [52] as well as directly inhibiting JAK’s catalytic activity. Interestingly, the site on gp130 (and potentially LIFR β) to which SOCS3 binds is the phosphotyrosine motif that usually recruits SHP2 (also known as PTNP11) to activate the MAP-kinase pathway (see below) [53]. Therefore, in addition to inhibition of STAT3 signalling, SOCS3 also inhibits MAPK signalling by competing with

SHP2 for its binding site on the LIF receptor. SOCS3 knockout animals die in utero due to defects in placentation caused by overactive LIF signalling [54], a phenotype that can be rescued by knocking out LIFR β [55]

STAT3 is required to maintain ES cells in an undifferentiated state. Overexpression of a dominant-negative STAT3 construct in ES cells leads to both differentiation and a loss of self-renewal [41]. Active STAT3 was in fact shown to be *sufficient* for maintaining ES cells in culture by Matsuda *et al.*, who used a chimera between STAT3 and the estrogen receptor to produce conditionally activated (phosphorylated) STAT3 using Tamoxifen [56]. A number of targets of STAT3-mediated transcription have been linked to its ability to maintain ES cells, most notably myc and nanog.

(ii) PI(3)-kinase signalling—PI(3)-kinase is an enzyme that catalyses the formation of the second messenger molecule, phosphatidylinositol (3,4,5)-triphosphate from phosphatidylinositol (4,5)-diphosphate which in turn activates a number of downstream effectors. Activation of the PI(3)-kinase pathway following LIF stimulation is driven by a cytokine-induced association between JAK1 and the p85 subunit of PI(3)-kinase²². It is unclear whether, like that seen for STAT and MAPK activation, this requires receptor phosphorylation to occur beforehand. Activation of p85 leads to activation of the serine/threonine kinase AKT (protein kinase B) at the cell membrane and stimulation of a number of pathways, such as mTOR, important for cell cycle regulation and metabolism as well as downregulation of wnt signalling by inhibition of GSK3 β (Glycogen Synthase Kinase 3 β).

The importance of PI(3)-kinase signalling in ES cells has been investigated by Paling and colleagues using both pharmacological inhibition and expression of a dominant-negative form of p85. These experiments showed that when the PI(3)-kinase pathway was inhibited, ES cells spontaneously differentiated, even in the presence of LIF [57]. In fact ES cells expressing an activated form of AKT can be maintained in an undifferentiated state in the absence of LIF [58]. Thus, similarly to STAT3, artificially enhanced PI(3)-kinase signalling is sufficient to maintain murine ES cells

(iii) MAPK signalling—Similarly to STAT activation, the MAPK pathway is activated by recruitment of signalling components to the activated LIF receptor [59]. Both chains of the receptor contain sites that, when phosphorylated, can recruit the phosphatase SHP2 [60]. These sites are Y759 in gp130 [44, 61] and Y974 in LIFR β [62]. Activated SHP2 induces Ras/Raf signalling which leads to activation of MAPK and ultimately activation of transcriptional activators such as elk [63]. In many cases this leads to mitogenic and/or differentiation programs being induced. As stated above, SOCS3 also binds pY759 of gp130 and hence its induction by STAT3 leads to inhibition of the MAPK signalling cascade [64].

A comparison of the MAP-kinase pathway to the JAK/STAT and PI(3)K [35, 36] pathways in ES cell renewal highlights some interesting contrasts. Although LIF stimulates activation of all three, inhibition of the MAPK cascade, either pharmacologically or genetically (via deletion of MAPK2) leads to an *increase* in ES cell self-renewal [65, 66]. This implies that MAPK signalling is usually required for differentiation, rather than maintaining

pluripotency and shows that even though MAPK signalling is induced by LIF, it is subservient to STAT3 and PI(3)K signalling in this system.

Indeed SOCS3^{-/-} ESC differentiate to primitive endoderm in the presence of LIF [31, 55, 67], and this appears to be due to overactivity of the MAPK pathway (since the SHP2 pathway activating MAPK is hyperactive in these cells as a result of non-competition with SOCS3 for binding of SHP2 to the receptor, and MEK (MAPK kinase) inhibition restores pluripotency in these ES cells [67]). Thus LIF binding induces competing pathways that have to be balanced in order to achieve pluripotency and self-renewal. Indeed the requirement of ESCs for LIF to maintain totipotency can be overcome by the use of signalling pathway inhibitors. Specifically, these are (i) a MAPK pathway inhibitor (which may mimic the effect of LIF-induced SOCS3, (ii) a GSK3 inhibitor [68] (which may mimic LIF induced activation of the PI(3)K pathway) and (iii) an FGF (Fibroblast Growth Factor) receptor tyrosine kinase inhibitor. In addition, the requirement for LIF can be overcome by the expression of pluripotency genes *nanog*, *Klf2* and mutant *myc* [38]. However, the relationship of LIF signalling to the induction of the pluripotency genes is unclear since only *myc* appears to be a direct transcriptional target of LIF signalling.

Despite the apparent requirement of ESCs for LIF in vitro this does not seem to be essential in vivo because both LIF^{-/-} and LIFR β ^{-/-} blastocysts develop normally in wild-type pseudo-pregnant hosts. Gp130^{-/-} blastocysts do however show a failure to develop after diapause (arrested development of the blastocyst prior to implantation) but it is unclear whether this is due to failed LIF signalling or other cytokines utilising this receptor [69]. These data suggest that while some LIF pathways are directly involved in maintaining pluripotency there may be other pathways that can compensate in the absence of LIF.

LIF also appears to be required during both the induction and maintenance phases of the generation of induced pluripotent stem cells (iPSCs) from mature somatic cells. These cells can be induced by the forced expression of key transcriptional factors (including Oct3/4, SOX2, c-Myc and Klf4) [70] and both the induction of the pluripotency transcriptional network [71] and the survival/self-renewal of iPSCs [72] was dependent on LIF or LIF-like signalling.

Like stem cell factor (SCF), LIF has been shown to stimulate the proliferation of embryonic primordial germ cells (the progenitors of the gametes) in primary cultures on feeder layers [73] and LIF can be used to direct these cells to self-renewing pluripotency (embryonic germ cells) [74]. Since LIF^{-/-} male mice appear fertile and female blastocysts develop normally when transplanted into a normal host, LIF does not seem to play a critical or non-redundant role in the normal development of male or female gametes.

Reproduction

During normal human pregnancy the uterine endometrium is influenced by the altered sex steroid levels of the menstrual cycle to become receptive to the developing blastocyst. Receptivity peaks at day 7 post-fertilization at which time the blastocyst can attach to the uterine epithelial cells and at this time the uterine tissue begins remodelling (decidualisation) to allow the trophectoderm layer of the blastocyst to invade the uterine tissue to help form

the placenta that juxtaposes the fetal and maternal blood systems. The trophoblasts form two layers of the developing placenta (the external syncytiotrophoblast and the inner cytotrophoblast layer) and induce remodeling of the uterine spiral arteries to increase maternal blood flow in apposition to developing blood vessels in the embryonic villi (Fig. 4).

LIF $-/-$ female mice are infertile due to a failure of blastocyst implantation [75]. LIF is highly expressed in the uterine endometrial glands at the time of blastocyst formation and prior to blastocyst implantation in both mice and humans [76] probably as a result of the rise in estrogen levels during the menstrual cycle [77]. LIF receptors are present on the blastocyst as well as the endometrium and trophoblasts but the failure in LIF $-/-$ mice is on the maternal side since both LIF $-/-$ and LIF receptor $-/-$ embryos implant successfully in pseudopregnant wild-type hosts. In addition there is some evidence that LIF and LIF receptor expression may play a role in ectopic pregnancies in the fallopian tubes [78].

LIF secretion levels in uterine flushings and from ex-vivo explants have been reported to be significantly lower at the late proliferative and early secretory phases in women with proven infertility [79] and in rare cases mutations in the LIF gene itself (thought to alter LIF binding to its receptor or to alter LIF expression levels) have been reported in infertile women [80]. This has suggested that LIF might be used to treat infertility in women or conversely that LIF antagonists might be used as contraceptives. Indeed neutralizing antibodies to LIF or an engineered long-lasting LIF antagonist (a modified LIF molecule) have been shown to prevent pregnancies in mice and non-human primates [81, 82].

LIF has been shown to induce differentiation of trophoblast-like choriocarcinoma cell lines and invasiveness of immortalized first trimester trophoblasts [83, 84]. SOCS3 is a major negative regulator of LIF signalling and SOCS3 $-/-$ mice die in utero at E13 from a failure of placentation. The defect is a poorly formed labyrinthine and spongiotrophoblast layer as well as excessive numbers and size of trophoblast giant cells. The use of tetraploid aggregation embryos where the extra-embryonic trophoblast layer is wild type rescued the placentation defect in SOCS3 $-/-$ mice as did crossing to a LIF $-/-$ embryonic background. These data suggest that excessive embryonic LIF acts to differentiate trophoblasts to giant cells and disrupts placental architecture [55, 85].

LIF also appears to play a crucial role in mammary gland involution following weaning. In LIF $-/-$ mice involution is delayed and reduced apoptosis is seen while mammary glands show precocious development during pregnancy and these effects are dependent on STAT3 [86].

Bone Remodelling

Cortical and trabecular bone is continually remodelled to maintain blood calcium levels, restore damaged regions and respond to hormones and diet. Osteoclasts, derived from monocytic precursors, degrade bone while osteoblasts, derived from mesenchymal cells, form the extracellular matrix required for new bone deposition. Osteoblasts can differentiate into osteocytes that are embedded in the bone matrix and send long cellular processes to communicate with osteoblasts at the bone surface. Communication between osteoblasts and

osteoclasts by the secretion of cytokine-like proteins serves as a feedback mechanism to regulate the balance of bone formation and destruction. For example osteoblasts secrete RANKL, a TNF-like cytokine that activates osteoclasts as well as its inhibitor osteoprotegerin, a decoy receptor for RANKL, and osteocytes produce the wnt antagonist sclerostin that is a major inhibitor of bone formation (Fig. 5). Chondrocytes are found at the growth plate of growing bones and in the joints where they produce articular cartilage required for joint movement.

Osteoblasts express high affinity receptors for LIF and LIF enhances the differentiation of bone marrow stromal cells to the osteoblast lineage while also inhibiting differentiation towards adipocytes [87]. While mice injected with a cell line over-expressing LIF showed pronounced sclerotic lesions and elevation of osteoblast numbers [88], mice injected with recombinant LIF or LIF^{-/-} mice did not show dramatic changes in bone formation [89]. On the other hand LIF receptor ^{-/-} mice showed dramatic bone loss [90]. LIF inhibits the expression of sclerostin in osteocytes and sclerostin is a potent inhibitor of bone formation by osteoblasts [87]. Osteoclasts do not express LIF receptor or produce LIF suggesting that LIF effects on these cells at the growth plate are indirectly mediated through osteoblasts and osteocytes via induction of RANKL production [91]. In humans loss of function mutations in the LIF receptor lead to Stuve-Weidemann/Schwartz-Jampel type 2 syndrome exhibiting shortened, bowed bones consistent with decreased bone formation [92].

In human articular chondrocytes LIF expression and secretion was induced by pro-inflammatory cytokines (IL1, IL6, TNFa) and LIF administration itself induced the production of pro-inflammatory cytokines and proteases involved in cartilage degradation. This suggests an important role for LIF in the progression of the inflammatory state and cartilage destruction seen in rheumatoid arthritis [90].

The hypothalamo-pituitary-adrenal axis

This axis refers to the response to stress in which the hypothalamus secretes corticotropin – releasing hormone (CRH) that acts on the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH) that in turn acts on the adrenal cortex to secrete glucocorticoids. The glucocorticoids have multiple effects on the immune system, digestion, energy storage/ utilization and others and also feedback on the pituitary and hypothalamus to inhibit the cycle (Fig. 6).

LIF is expressed in all species of pituitary, mostly in corticotrophs along with ACTH expression. Moreover LIF induces expression of the ACTH precursor (proopiomelanocortin) and secretion of ACTH in primary corticotrophs or cell lines, this effect being synergistic with CRH. This action is mediated by LIFR β /gp130, requires STAT3 activation and is inhibited by SOCS3. Indeed antibodies to LIF or LIFR β reduce the basal rate of ACTH secretion suggesting that autocrine or paracrine LIF production is required for constitutive ACTH secretion.

Other inflammatory cytokines (especially IL1 β) and bacterial products (eg lipopolysaccharide, LPS) also stimulate ACTH secretion and there is some evidence that

part of this response might be via induction of LIF expression. In addition the combination of LIF and IL1 β leads to a synergistic increase in ACTH secretion.

LIF $-/-$ mice do not show reduced basal ACTH levels but fail to increase ACTH in response to prolonged immobilization stress [93].

LIF inhibits cell cycle progression in a corticotroph cell line but increases ACTH secretion suggesting that LIF may act as a differentiation factor for corticotrophs. Indeed transgenic expression of LIF in the pituitary resulted in excess numbers of ACTH-positive corticotroph cells and decreased numbers of somatotroph, lactotroph and gonadotroph cells in the pituitary and these mice suffered dwarfism and hypogonadotropism. These data suggest that LIF signalling may direct differentiation of precursor cells towards the corticotroph and away from other potential lineages [94, 95].

The neuromuscular system and heart

LIF was first described as a factor that could switch neurotransmitter production from catecholamine to acetylcholine in primary cultures of rat sympathetic neurons [6] and subsequently a switch from neuropeptide Y to vasoactive intestinal peptide, calcitonin gene-related peptide and substance P neuropeptides [96]. However LIF $-/-$ mice did not show changes in sweat gland innervation profile but did reveal a failure of neurotransmitter switching after axotomy suggesting that LIF is not involved in regulating neurotransmitter plasticity during development but is involved in this process following neural trauma [97].

LIF also stimulated the development of sensory neurons in cultures of neural crest cells, a process that was augmented by co-treatment with fgf2 [98, 99] and in cultures of developing dorsal root ganglia (DRG), although the latter also required nerve growth factor (NGF) [99]. Similar results were found in nodose ganglion cultures and in all cases the requirement for different cytokines/growth factors depended on development age. It has been suggested that fgf2 may act on the earliest precursors that then become responsive to LIF for survival/differentiation and that differentiated cells then become dependent on NGF for survival. Like NGF, LIF has been shown to be retrograde transported (in a LIF receptor-dependent fashion) from the footpad and gastrocnemius muscle to the sensory neurons in the DRG and this process was dramatically increased after axotomy [100, 101]. An increase in LIF expression in injured nerve [101] again suggests that this action of LIF may not be important in development but rather in injury responses. Similarly LIF was found to promote differentiation of E10 spinal chord motor neurons [102], to be retrograde transported in motor neurons following nerve damage [101] and to reduce motor neuron loss following nerve transection [103]. An additional role of muscle-derived LIF is to control the timing of motor neuron sprouting with LIF $-/-$ mice demonstrating premature withdrawal of synapses from neonatal muscles [104] suggesting a role for LIF in neuromuscular connectivity. Many of the effects of LIF on neural populations are also demonstrated by ciliary neurotrophic factor (CNTF) that shares receptor components with LIF. While there are few neurological defects in LIF $-/-$ mice, there are synergistic losses in motor neuron function in double mutant (LIF $-/-$; CNTF $-/-$) mice [105] as well as in LIF receptor null or CNTFR null mice [106, 107].

In the brain adenoviral LIF delivery into the brains of mice has been shown to increase self-renewal of neural stem cells in the sub-ventricular zone and olfactory bulb at the expense of differentiation into neurons [108].

LIF has additional effects in neural support cells, the glia. It induces differentiation of an astrocyte progenitor cell line into mature GFAP+ astrocytes [109] and induces increased numbers of GFAP+ cells to develop from spinal cord cultures, although serum factors were also required [102]. LIF^{-/-} mice displayed reduced numbers of GFAP+ astrocytes in the hippocampus and dentate gyrus [99] while LIF receptor null mice displayed marked reductions in GFAP+ astrocytes in the brainstem and spinal cord [110]. Similarly LIF induced production, maturation and survival of oligodendrocytes from precursor cell cultures and it has been suggested that glial cell maturation to astrocytes versus oligodendrocytes in the presence of LIF might depend on the presence of other factors such as the extracellular matrix [111]. In vivo LIF administration (via an adenovirus vector into the brain) stimulated the proliferation of oligodendrocyte precursor cells and enhanced oligodendrocyte remyelination of axons in the hippocampus after cuprizone-induced demyelination [112]. In LIF^{-/-} mice there was reduced myelination in regions of the brain [113] and a delay in the generation of oligodendrocytes in the optic nerve [114].

LIF stimulates the proliferation of skeletal muscle precursors (myoblasts) in vitro but inhibits their differentiation into myotubes [115] and, following injury, skeletal muscle regeneration is enhanced by application of LIF in vivo [116]. LIF reduces the atrophy seen in denervated muscle and can stimulate muscle re-innervation in the rat [117]. LIF expression is increased in muscle upon exercise [118] and LIF^{-/-} mice fail to show a hypertrophic response to muscle loading [119] and reduced muscle regeneration following injury [120]. On the other hand developmental muscle defects are not apparent in LIF^{-/-} mice and, as for nerve cells, LIF expression is increased in damaged muscle. Thus, as for the nervous system, the major role of LIF in muscle may be in response to traumatic injury or muscle load rather than in developmental processes (Fig. 7).

LIF has also been demonstrated to have effects on heart muscle, including stimulating the survival and growth of neonatal mouse cardiac ventricular myocytes [121], although it has been difficult to assess whether the effects are due to the use of a common receptor system to cardiotrophin-1. LIF pre-treatment reduced ischemia-reperfusion-induced loss of cardiomyocytes in rabbits [122] and LIF plasmid enhanced recovery in myocardial infarction in mice [123]. In both cases there was evidence that LIF induced protection against reactive oxygen species (eg by induction of superoxide dismutase) and survival pathways in cardiomyocytes (eg Akt/PI3K). It has also been proposed that LIF stimulates bone marrow-derived cardiac stem cells to home to damaged myocardium and stimulates their differentiation into endothelial cells and neovascularization [124].

The hemopoietic system

Despite its identification as a myeloid leukemia-differentiation-inducing factor, LIF has surprisingly few effects on hemopoietic cells. While it has no colony-stimulating activity on its own, it was shown to synergize with interleukin-3 (IL3) in stimulating the proliferation of

human primitive blast cell colonies [125] and in the mouse it synergised with IL3 in stimulating megakaryocyte colony formation and with FLK ligand in stimulating blast colony formation. When injected into mice LIF induced elevated megakaryocyte and platelet numbers and it functionally activated platelets [126]. LIF^{-/-} mice displayed reduced numbers of hemopoietic stem cells (CFU-S) in the bone marrow and spleen but normal numbers of colony-forming cells in the bone marrow and a normal ability to reconstitute irradiated hosts, as well as normal numbers of circulating blood cells [127].

LIF is expressed in the thymic epithelium and is required for normal thymic architecture. LIF ^{-/-} mice show reduced responsiveness of their thymocytes (but not peripheral lymphocytes) to the mitogen concanavalin A and this appears dependent on microenvironmental production of LIF [127]. Regulatory T cells (T regs) in mouse and man were shown to produce high levels of LIF and LIF induced the production of T regs by enhancing the expression of the transcription factor Foxp3 and repressing expression of ROR γ t. In this way LIF appears to be tolerogenic by promoting T reg differentiation and inhibiting pro-inflammatory Th17 cell differentiation while the related cytokine IL6 has the opposite effect [128]. These effects of LIF have been suggested to be important in providing a tolerogenic environment during pregnancy and maintaining tolerance in allotransplantation. While LIF is often up-regulated in inflammatory conditions it again appears to play a protective role by inducing an acute phase response in liver and protecting against endotoxic shock by reducing the production of pro-inflammatory cytokines (TNF and IL6) and increasing the production of anti-inflammatory cytokines like IL10 [129].

Cancer

Early experiments using an injected cell line over-expressing LIF showed a cachexia phenotype with loss of subcutaneous and abdominal fat [88]. Subsequently, Mori et al purified a lipoprotein lipase inhibiting activity from a melanoma cell line that caused cancer cachexia in mice and found that it was identical to LIF [7]. Other cytokines can also cause a cachexic phenotype so the role of LIF in particular tumors is unclear.

LIF (and LIFR β) expression has been noted in many solid tumors including breast, skin colorectal and nasopharyngeal cancers. In the latter case, high circulating LIF levels correlated with tumor recurrence and radioresistance and LIF-induced radioresistance and inhibition of DNA repair was demonstrated on these cells in vitro [130]. Wu et al showed that LIF expression in colorectal cancer cells was induced by low oxygen levels and the transcription factor HIF-2 α and that expression of both proteins was correlated in human specimens [131]. It was also shown that LIF negatively regulated p53 by activating the MDM2 proteolytic pathway in colorectal cancer cells thus providing a rationale for the effects of LIF on increased chemo- and radio-resistance [132]. LIF has been shown to stimulate the growth, inhibit the differentiation and induce metastasis of a variety of tumors in vitro (see eg ref [131]) but it has been difficult to prove a direct role in tumorigenesis.

Summary and outlook

As mentioned by others the name leukemia inhibitory factor is a misnomer because LIF has few effects on myeloid cells and inhibits the growth of few leukemias other than the M1

mouse myeloid leukemic cell line. In contrast LIF receptors are broadly distributed and in principle LIF has a very broad range of activities on almost all organ systems including the hemopoietic, bone remodelling, neural, muscular, endocrine and reproductive systems. Nevertheless LIF knockout mice have a rather restricted set of development defects including loss of female fertility and defects in some neurons and glial populations. Many of the effects of LIF on the other organs appear to be unrelated to development but instead represent a systemic or local response to tissue damage or injury particularly in nerves and muscles. Surprisingly LIF can also have opposite signalling outcomes depending on cell system and developmental stage. For example it is a differentiation inhibitor and maintainer of pluripotency in embryonic stem cells but an inducer of differentiation in M1 leukemia cells, osteoblasts and glia. Similarly it stimulates proliferation of DA1 cells but inhibits proliferation of corticotrophs and it can promote cell survival in some cell types while inducing apoptosis in others. The signalling pathways induced by LIF are similar in most cell types including activation of the JAK1/STAT3, PI3K/Akt and MAPK pathways. The differences in signalling outcome may in part arise from differential levels of activation of these three pathways (for example STAT3 and MAPK seem to have opposing effects on differentiation); different chromatin states that give differential gene activation to the same signal; and/or a different milieu of other cytokines and expression of cytokine receptors on different cells or at different developmental stages.

The biological effects of LIF mentioned above have prompted clinical trials for efficacy in chemotherapy-induced peripheral neuropathy [133], platelet recovery following chemotherapy [134] and infertility in women [135] but none of these studies has produced results promising enough to pursue further. Despite the plethora of possible clinical applications (and potential unwanted effects) LIF remains to find a place in clinical practice. A more sophisticated understanding of biological redundancy, method and timing of delivery will be required to potentially improve on this situation.

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Abbreviations

LIF	leukemia inhibitory factor
CNTF	ciliary neurotrophic growth factor
CT1	cardiotrophin 1
CLC	cardiotrophin-like cytokine
JAK	Janus kinase
STAT	Signal transducer and activator of transcription

MAPK	Mitogen activated protein kinase
PI(3)K	phosphatidyl inositol 3 kinase
IL6	interleukin-6
Gp130	glycoprotein 130
OSM	oncostatin M
CBM	cytokine-binding module
ESC	embryonic stem cells
EM	electron microscopy
NMR	nuclear magnetic resonance
SHP2	SH2-domain-containing phosphatase 2
SOCS	suppressor of cytokine signaling
PTPN11	protein tyrosine phosphatase, non-receptor type 11
MLPLI	Melanoma-derived lipoprotein lipase inhibitor
HILDA	Human interleukin for DA cells
DIA	Differentiation inhibiting activity
HSF	Hepatocyte-stimulating factor
Kd	Equilibrium dissociation constant
FnIII	Fibronectin III domain
CNDF	Cholinergic neuronal differentiation factor

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Biographies

Nicos Nicola obtained his PhD in protein chemistry under Sydney Leach in the Biochemistry Department at the University of Melbourne, Australia. After a short postdoctoral period at Brandeis University, USA with Gerald Fasman he returned to the Walter and Eliza Hall Institute of Medical Research, Australia to work with Tony Burgess and Donald Metcalf on the purification of the colony-stimulating factors. He has remained at that institution for the last 38 years where he is currently co-head of the Division of Cancer and Haematology. His research interests have included cytokines, their receptors and their intracellular signalling pathways in health and disease.

Jeff Babon is a Laboratory Head at the Walter and Eliza Hall Institute of Medical Research (Australia) and is a specialist in the field of structural biology and biochemistry. He is a graduate of Melbourne University and obtained his Ph.D at the Murdoch Institute. Jeff Babon undertook postdoctoral training at the National Institute of Medical Research

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Highlights

- Leukemia inhibitory factor is a highly pleiotropic cytokine
- It belongs to the IL6 superfamily characterized by use of the receptor chain gp130
- Despite common signalling pathways it has opposing effects on different cells
- It has non-redundant effects on blastocyst implantation and pregnancy
- It is involved in stress and tissue repair of bone, muscle and nerve

Fig. 1. LIF signals through a heterodimeric receptor

Both chains of the LIF receptor (LIFR β and gp130) are shared by other IL-6 family cytokines. LIF and OSM bind a gp130:LIFR β heterodimer whilst CNTF, CT-1 and CLC bind to the same heterodimer with the help of a specific receptor alpha chain (CNTF α). IL-6 and IL-11 bind a receptor that consists of two gp130 chains and two specific alpha chains (IL-6R α and IL-11R α respectively). Unlike other cytokines represented here, two molecules of IL-6 or IL-11 are required to form the signalling-competent complex, resulting in a hexamer.

Fig. 2. A model of LIF bound to its receptor

Upper panels: Three orthogonal views of LIF (green) bound to the gp130 (blue):LIFR β (red) heterodimer. Left and right panels show orthogonal views of the complex as viewed parallel to the cell-membrane whereas the central panel is viewed looking down towards the cell-membrane. Similar representations of the signalling-competent IL-6:gp130:IL-6R α hexamer structure are shown below. The LIF receptor consists of two cytokine-binding modules (CBMs) separated by an immunoglobulin-like (Ig-like) domain. Three Fibronectin domains (FnIII) make up the “legs” of both receptors. gp130 has a similar architecture to LIFR β but lacks the first CBM. Note the absence of an α -receptor (beige) in the LIF system. The LIF:LIFR β :gp130 model was constructed by overlaying the structure of the LIFR β :LIF (PDB ID: 2Q7N) complex onto the gp130 molecule from the IL-6:gp130:IL-6R α hexamer (PDB IDs: 3L5H, 1P9M) and modelling the “legs” of LIFR β on gp130 (PDB ID: 3L5H).

Fig. 3. LIF signalling

Extracellular LIF stimulates JAK/STAT, MAPK and PI(3)K signalling, forming a 1:1:1 ternary complex. Intracellularly, both chains of the LIF receptor (LIFRβ and gp130) are bound to JAK1, the tyrosine kinase that initiates the signalling cascade. JAK1 phosphorylates five tyrosines on each receptor chain, four of these are docking sites for the transcription factor STAT3 (allowing stimulation of the JAK/STAT signalling pathway) whilst the fifth is a docking site for SHP-2 (which stimulates the MAPK pathway) and SOCS3 (which negatively regulates both the JAK/STAT and MAPK pathways). LIF stimulates a mixture of differentiation, survival and renewal programs, the balance of which determines the cell's fate. In embryonic stem (ES) cells, signalling is skewed towards survival and self-renewal.

Figure 4. LIF actions in reproduction

A. LIF produced by the endometrial glands (and possibly by the blastocyst) acts on the endometrial epithelium to make it receptive to blastocyst attachment and on the stroma to decidualise it ready for implantation and placenta development. LIF also acts on the inner cell mass of the blastocyst to maintain totipotency during diapause and on the trophoblast to induce trophoblast invasion into the endometrium. **B.** LIF is important for the formation of the maternal decidua and balanced LIF activity is essential for formation of trophoblast giant cells as well as the correct architecture of the labyrinth layer in which maternal and fetal blood vessels come into contact.

Fig. 5. LIF actions in bone remodelling

LIF acts on bone stromal cells to enhance differentiation into bone-forming osteoblasts and inhibits the production of osteoblast-inhibitory sclerostin by osteocytes. Osteoblasts produce the TNF-like cytokine RANKL that acts on receptors on macrophages to induce differentiation into multi-nucleate osteoclasts that resorb bone. These linked phenomena allow repair of damaged bone by first clearing the damaged bone and then inducing new bone formation.

Fig. 6. LIF and the hypothalamus-pituitary-adrenal axis

LIF acts on the anterior pituitary to enhance differentiation of precursors into corticotrophs rather than other cell fates and stimulates secretion of ACTH from corticotrophs synergistically with the corticotropin-releasing hormone (CRH) produced by hypothalamic neurons. ACTH then acts on the adrenal cortex to secrete cortisol that has many effector actions in the stress response and also feeds back to inhibit the hypothalamic circuits and the anterior pituitary.

Fig. 7. Neuromuscular effects of LIF

A. LIF acts on neural stem cells to increase self-renewal and can also act to stimulate the production and survival of neurons, GFAP+ astrocytes and oligodendrocytes that sheath neural axons (glial cells). It also acts to switch neurotransmitter choice of sensory neurons (from noradrenalin to acetylcholine). **B.** LIF is produced by skeletal muscle under load or after damage and stimulates proliferation of satellite cells while inhibiting their fusion into myotubes. Increased LIF production in damaged muscle or in neurons can be retrograde transported along axons to the nerve body (shown for motor neurons in the dorsal root ganglion) leading to increased survival.