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Effect of novel monoclonal antibodies on LIF-induced signaling in chicken blastodermal cells

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Abbreviations:

LIF, leukemia-inhibitory factor; chLIF, chicken LIF; mLIF, mouse LIF; rchLIF, recombinant chLIF; CBC, chicken blastodermal cell; IL, interleukin; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; ALP, alkaline phosphatase; MEK, mitogen-activated protein kinase kinase; CESM, chicken embryonic stem-cell medium; EB, embryoid body.

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Abstract

Leukemia-inhibitory factor (LIF) is indispensable for maintaining the undifferentiated state when propagating mouse embryonic stem (ES) cells. We previously cloned chicken LIF (chLIF) cDNA and demonstrated that it maintained chicken ES cell cultures in an undifferentiated state. Here we developed two monoclonal antibodies, HUL-1 and HUL-2, against chLIF, which specifically recognized recombinant chLIF (rchLIF) produced by *Escherichia coli* and Chinese hamster ovary K1 cells, in enzyme-linked immunosorbent assays and Western blot analysis. In addition, HUL-2 inhibited the phosphorylation of signal transducer and activator of transcription 3 by rchLIF in chicken blastodermal cells (CBCs), but not that of mitogen-activated protein kinase kinase. Furthermore, the addition of HUL-2 to CBC cultures resulted in embryoid bodies forming earlier than in normal cultures. These results indicated that HUL-2 recognized not only rchLIF but also native chLIF, and suggested that CBCs in culture produce LIF, which functions in autocrine signaling.

Keywords: chicken; chicken blastodermal cells; cytokine; ES cells; LIF; mAb; MEK1/2; STAT3

Introduction

Leukemia-inhibitory factor (LIF) is a multifunctional cytokine, which has pleiotropic biological effects on a diverse array of cell types, including hepatocytes, osteoblasts, adipocytes, megakaryocytes, neurons and embryonic stem (ES) cells [1, 2]. *In vitro*, LIF induces differentiation in murine myeloid leukemia (M1) cells [3, 4]. In cultures of growing mouse ES cells, however, the presence of LIF in culture maintains the undifferentiated pluripotent phenotype and its withdrawal leads to their differentiation [5, 6]. As injecting ES cells into blastocysts results in the formation of chimeric mice, and these ES cells can be transfected with a mutant gene, an efficient procedure for producing transgenic mice has become available. LIF has therefore made a large contribution to the development of both stem-cell and molecular biology.

LIF is a member of the interleukin (IL)-6 family, which includes IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin (CT-1) [1, 7-11]. LIF receptors are composed of a low-affinity receptor subunit, LIFRβ, and the gp130 signal-transducing subunit, which is common to all members of the IL-6 family [1]. LIF binds to LIFRβ, which then forms a high-affinity heterodimeric complex with gp130 [7, 8]. There are two major signaling pathways downstream of gp130 in M1 cells: the Jak-signal transducer and activator of transcription (STAT) and Shp2-extracellular signal-regulated kinase (ERK) pathways [12]. Activation of STAT3 by LIF is essential for maintaining the undifferentiated state of mouse ES cells [13, 14]. However, the activation of Shp2 or ERK appears to impair ES cell pluripotency [15]. Thus, the switch between self renewal and differentiation in ES cells generally depends upon the balance of two opposing intracellular signals [16], although recent studies show that the maintenance of

primate ES cells is STAT3 independent [17-19].

In chickens, only a few cytokines have been cloned based on homology, as they have only 20-40% amino-acid sequence identity with their mammalian counterparts. Recently, we cloned chicken LIF (chLIF) cDNA, using mRNA subtraction and rapid amplification of cDNA ends (RACE) methodology, and expressed recombinant chLIF (rchLIF) in bacteria [20]. In chicken ES-like cells, rchLIF enhanced the expression of markers of the undifferentiated state, such as SSEA-1 and alkaline phosphatase (ALP), to a greater extent than mouse leukemia-inhibitory factor (mLIF). Moreover, rchLIF induced the phosphorylation of both STAT3 and mitogen-activated protein kinase kinase (MEK1/2), whereas mLIF only induced the phosphorylation of MEK1/2. These results demonstrated that chLIF is, indeed, more appropriate than mLIF for culturing undifferentiated chicken ES cells, and indicated that chLIF and mLIF bind differently to the chicken heterodimeric receptor (gp130/LIFRβ). However, it remains to be determined whether STAT3 activation is required and is sufficient to maintain the undifferentiated state of chicken ES-like cells, as well as mouse ES cells. In order to investigate this further, we examined the production of the anti-LIF monoclonal antibody (mAb), which inhibits the activation of STAT3.

Previous mutagenesis and structural studies have demonstrated that LIF has three distinct sites, I-III, which are necessary for interactions with LIF receptors [21-23]. Sites I and III mediate the high-affinity binding of LIF to LIFRβ [21-23], with the dominant residues being Phe^{156} and Lys^{159} in Site III. Residues Lys^{170}, Ala^{174} and Val^{175} in Site I, along with two additional residues, Asp^{57} and Lys^{58}, which play more minor roles, make up the putative second binding site for LIFRβ. Site II (Gln^{25}, Ser^{28} and Gln^{32}) participates in direct binding to gp130, and residues Asp^{120}, Ile^{121}, Gly^{124} and Ser^{127} are also

implicated in the interaction with gp130. Although a number of previous studies have examined the interaction of LIF with its receptors, little is known about the relationship between these receptor-binding sites and signal transduction of LIF. In the current study, we hypothesize that examining such factors would contribute to the understanding of LIF-gp130/LIFRβ interactions and describe the use of novel mouse mAbs that are specific for chLIF as additional analytical tools.

Materials and Methods

Cells and cell culture. Fertilized freshly laid unincubated eggs from White Leghorn chickens were purchased from Akita Co. (Fukuyama, Japan). CBCs that were prepared from stage X chicken embryos were incubated and cultured as described previously [24]. The embryonic stages were defined according to Eyal-Giladi and Kochav [25]. CBCs were centrifuged twice at 400 x g and the cell pellet was resuspended at a concentration of 4×10^4 cells/ml in a cytokine-free chicken ES cell medium (CESM), namely Dulbecco's Modified Eagle's Medium, containing high glucose (Invitrogen Corp., CA, USA) with 10% fetal bovine serum (FBS; JRH Bioscience, KS, USA), 2% chicken serum (Dainippon Pharmaceutical Co, Osaka, Japan), 1 mM sodium pyruvate (Invitrogen), 1 μM each of adenosine, guanosine, cytidine, uridine and thymidine (Sigma, MO, USA), 100 U/ml penicillin (Invitrogen) and 0.1 mg/ml streptomycin (Invitrogen). CBCs were cultured in CESM with or without rchLIF in 24-well culture plates at 38.5 °C in 5% CO_2.

Preparation of immunogens. We prepared two different immunogens: rchLIF

(Immunogen I), which was produced in *E. coli* as described previously [20]; and a synthetic peptide (Immunogen II; Fig. 1) composed of 17 residues from chLIF, including Site III, which was synthesized using a solid-phase method in a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan). The synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH; Imject Maleimide Activated mcKLH; Pierce, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from 1, 3, 6 and 9-day CBC cultures with ISOGEN-LS (Nippon Gene Co, Tokyo, Japan). Poly(A)$^+$RNA was purified from total RNA with Oligotex-dT30 (Nippon Roche, Tokyo, Japan). For expression analysis, 0.5 μg Poly(A)$^+$RNA was reverse-transcribed at 50 °C using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) in a 20-μl reaction mixture containing oligo$(dT)_{20}$ primer, according to the manufacturer's protocol. cDNA was extracted with phenol-chloroform, then ethanol-precipitated and resuspended in TE buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA). Semi-quantitative PCR was carried out using the ABI PRISM 7700 sequence-detection system (Applied Biosystems, Tokyo, Japan) with SYBR Green core reagents (Applied Biosystems). The amounts of cDNA were normalized to chicken β-actin SYBR Green PCR according to the manufacturer's instructions and equal quantities were then used as templates in the PCR reactions. PCR amplification was carried out with 0.4 μM primers, 0.2 mM of each dNTP, 1 mM $MgCl_2$, 10 x *Ex Taq* buffer (Mg^{2+} free) and 1 U *Ex Taq* (Takara, Tokyo, Japan) in a final volume of 25 μl using a Gene Amp PCR System 9700 (Applied Biosystems). The PCR reaction consisted of 30-35 cycles (specified below) of 30 s at 94 °C, 30 s at 54-65 °C (specified below) and 1 min at 72 °C. The PCR products were analyzed on 1.5% agarose gels and visualized with EtBr. The upstream and downstream primer pairs,

annealing temperature and cycle numbers used for each gene were as follows: *chLIF* (TCCTCAACGCCTCACTGG and GCCCTGCTGCTTCTTCTT, 65 °C, 35 cycles); *chgp130* (GCAGATGGACAGATTGAGC and TCACTACTGATCCTGTAGCG, 54 °C, 35 cycles); *chLIFRβ* (TTTCTGCTTGGAGTTGTGAC and AGGCTGATACATTGATTGAA, 65 °C, 35 cycles); *β-actin* (CACCTTCCAGCAGATGTGGA and GCAAATGCTTCTAAACCGGACT, 60 °C, 30 cycles). We confirmed that 30 or 35 PCR cycles represented the exponential phase of the amplification. Chicken *β-actin* amplification products were used as an internal control.

Production of rchLIF by Chinese hamster ovary (CHO)-K1 cells. CHO-K1 cells were routinely grown in HAM F-12 (Invitrogen) containing 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2. The coding region of mature chLIF was amplified using the following primers: forward primer (5′-CCAAGCTTGCGGGCGCTGCTGGGGACGAG-3′) and reverse primer (5′-CCCTCGAGGGCACTGAAACTCCTGGTCT-3′). PCR products were digested with *Hind* III and *Xho* I and sub-cloned into the pSecTag2A plasmid (Invitrogen) containing the c-myc epitope and the histidine tag. For expression in eukaryotic cells, the recombinant plasmid was transfected into CHO-K1 cells using Polyfect Transfection Reagent (Qiagen, Tokyo, Japan) and recombinant cells were selected in medium containing 0.25 mg/ml zeocin (Invitrogen). Several stable CHO-K1 cell lines secreting biologically active rchLIF were selected. Culture supernatants were collected and purified on a ProBond resin (Invitrogen) and used as rchLIF.

Production of anti-chLIF mAbs. Six-week-old female BALB/c mice were

immunized intraperitoneally with 50 μg rchLIF or synthetic peptide-KLH conjugate, mixed with an equal volume of complete Freund's adjuvant. After three biweekly boosts with the same dose of immunogen in 0.1 ml of phosphate-buffered saline (PBS), mice with high serum titers of anti-rchLIF antibodies were identified by ELISA and Western blot analysis. These mice were injected intravenously with 50 μg rchLIF or synthetic peptide in PBS and 3 days later their splenocytes were fused with SP2/0 Ag14 myeloma cells [26] using established methods [27]. Hybridomas secreting anti-chLIF antibodies were screened by ELISA and Western blot analysis, and cloned by limiting dilution. The immunoglobulin isotypes for chLIF reactive mAbs were determined using a mouse isotyping kit (Amersham Biosciences, NJ, USA).

ELISAs. ELISAs were carried out in NUNC-immunomodule plates (Nalge Nunc International, NY, USA), coated overnight at 4 °C at 50 μl/well with 5 μg/ml rchLIF produced in *E. coli* or CHO cells, mLIF (Chemicon, CA, USA) or BSA in PBS. The plates were blocked with 200 μl/well 25% Block Ace (Dainippon Pharmaceutical Co.) in PBS for 2 h at 37 °C. A total of 50 μl supernatant from fusion wells or established hybridoma cultures was added to the wells and incubated for 1 h at 37 °C. After 4 washes in 0.05% Tween-20 in PBS (Tween-PBS), 50 μl/well ALP-labeled goat anti-mouse-κ (Southern Biotech, AL, USA), diluted 1:1000 in 10% Block Ace-PBS, was added and incubated for 1 h at 37 °C. After eight washes with Tween-PBS, ALP activity was developed with 100 μl/well 1 mg/ml *p*-nitrophenyl phosphate in 1 mM $ZnCl_2$, 1 mM $MgCl_2$ and 100 mM glycine (pH 10.4) for 15 min at room temperature (rt). Absorbance was read at 405 nm in a microplate reader (model 680; Bio-Rad, CA, USA).

Western blot analysis with anti-rchLIF mAbs. The rchLIF produced in *E. coli* or CHO cells or mLIF were run on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were incubated in blocking buffer (20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 25 mM EDTA, 0.2% Tween 20 and 4% nonfat milk) overnight at 4 °C. After three washes in Tween-PBS, membranes were incubated with hybridoma culture supernatants or mouse anti-myc mAb (Invitrogen) diluted 1:3000 in blocking buffer containing 1% nonfat milk (dilution buffer) for 1 h at rt. After three washes (as before), blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig; KPL, MD, USA), diluted 1:3000 in dilution buffer for 1 h at rt, washed three times and incubated with ECL Plus Western blotting detection reagent (Amersham Biosciences). Results were recorded using an LAS-3000 image reader (Fujifilm, Tokyo, Japan).

Western blot analysis to detect activated STAT3. CBCs were incubated in cytokine- and serum-free CESM with 20 ng/ml rchLIF, which had been previously incubated with anti-chLIF mAbs, or with mouse IgG2b (Chemicon) or mouse IgA (Southern Biotech) as controls, for 1 h at 37 °C. After 15 min at 38.5 °C, the cells were lysed with ice-cold extraction buffer containing 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM Na_3VO_4, 0.1% SDS, 0.1% sodium deoxycholate and 1% Nonidet P-40. One tablet of a complete protease-inhibitor cocktail (Roche, Mannheim, Germany) was added per 25 ml extraction buffer. Lysates were centrifuged at 14,000 g for 10 min at 4 °C, and the protein concentration of the cleared lysates was determined using the BCA protein assay kit (Pierce). Samples (12 μg) of the lysates were separated on 7.5%

polyacrylamide slab gels and electroblotted onto PVDF membranes. Membranes were blocked for 2 h at 37 °C and then probed sequentially with anti-STAT3 (Calbiochem, CA, USA), anti-MEK1/2 (Cell Signaling Technology, MA, USA), anti-phospho-STAT3 (Tyr705, Cell Signaling Technology) and anti-phospho-MEK1/2 (Ser217/221, Cell Signaling Technology) antibodies. The blots were incubated with HRP-labeled anti-rabbit IgG (KPL) and developed with ECL Plus. Bands were quantified using Image Gauge software (Fujifilm), and the inhibition of STAT3 and MEK1/2 phosphorylation was determined using the following equation: % Inhibition = $(1-X/Y) \times 100$. Here, X was the luminescence intensity of phosphorylation in the presence of anti-chLIF mAb and Y was the luminescence intensity of phosphorylation in the absence of anti-chLIF mAb.

Inhibition assay. CBCs were prepared from stage X chicken embryos, as described above, and were cultured in CESM, with or without HUL-1 or HUL-2, in 24-well culture plates at 38.5 °C in 5% CO_2. Normal mouse IgG2b or IgA was added to the cultures as a control. The cultured cells were observed for 5 days using an Olympus IX71 (Olympus, Tokyo, Japan).

Results

Production and characterization of mAbs to rchLIF

We prepared two types of immunogen in order to produce anti-chLIF mAbs (Fig. 1): Immunogen I was full length mature chLIF constructed as previously reported [20], and Immunogen II was a synthetic peptide encoding the region including Site III

residues, Phe^{156} and Lys^{159}. The fusions produced two hybridomas making anti-rchLIF antibodies, as shown by indirect ELISAs and Western blot analysis, which were cloned by limiting dilution. The resulting two mAbs were named HUL-1 and HUL-2, and were IgG2b-κ and IgA-κ immunoglobulins, respectively. HUL-1 and HUL-2 gave rise to Immunogen I and Immunogen II, respectively.

In indirect ELISAs using rchLIF produced in *E. coli* or CHO cells, and mLIF, HUL-2 recognized rchLIF but not mLIF, and HUL-1 did not bind to either rchLIF or mLIF (Fig. 2). As shown in Fig. 3A, both mAbs detected a band with a molecular mass of 19 kDa on Western blots using rchLIF, but not using mLIF. Both mAbs were also tested for recognition of rchLIF produced in eukaryotic cells, in supernatant from transfected CHO-K1 cells, by Western blot analysis. As shown in Fig. 3B, HUL-1, HUL-2 and anti-c-myc antibody bound to a band of 28-37 kDa, which was consistent with the molecular mass of the rchLIF-c-myc fusion protein.

Effects of mAbs on the LIF-signaling pathway in CBCs

The effects of the two mAbs on the LIF-signaling pathway were investigated by examining their ability to inhibit the phosphorylation of STAT3 and MEK1/2, induced by rchLIF in CBCs. HUL-2 inhibited the chLIF-induced phosphorylation of STAT3 by 3-32% in a dose-dependent manner, whereas HUL-1, normal IgA and normal IgG2b had no effect on STAT3 phosphorylation (although HUL-1 had a minor inhibitory effect of 3-8%; Fig. 4 A, BI). By contrast, MEK1/2 phosphorylation was not inhibited by HUL-2 (Fig. 4 A, BII). Thus, HUL-2 has an influence on STAT3 but not on the MEK1/2 signal pathway.

Effect of mAbs on CBCs in culture

The effects of adding the mAbs to CBCs in culture were observed over 5 days, both with and without rchLIF in the medium. Control cultures contained mouse IgG2b or IgA (data not shown). As shown in Fig. 5A and B, cell growth was greater with added rchLIF than with no exogenous LIF, and CBCs started to form a few cyst-like embryoid bodies (EBs). The addition of HUL-1 had no effect on the CBC cultures (Fig. 5C, D). However, in the presence of HUL-2, many small EBs were observed when rchLIF was added to the cultures (Fig. 5E) and large EBs were observed in the absence of rchLIF (Fig. 5F). The formation of EBs after only 3 or 4 days in culture was especially striking in the cultures to which HUL-2 had been added without rchLIF (data not shown). These results suggested that CBCs produced some chLIF in culture, which acted as an autocrine signal.

Expression analysis with semi-quantitative PCR

To determine whether the CBCs produced chLIF, we analyzed the expression of chLIF, gp130 and LIFRβ mRNAs in culture. The pattern of expression of these mRNAs by CBCs, as reflected by the cDNAs from semi-quantitative PCR, is shown in Fig. 6. ChLIF was highly expressed on day 1 in culture. Its expression had decreased by day 3, but subsequently increased again by days 6 and 9. mRNAs for gp130 and LIFRβ were not expressed on day 1, but their expression increased transiently by day 6 and decreased again by day 9 in culture.

Discussion

The two novel anti-chLIF mAbs described here, HUL-1 and HUL-2, were both shown to be specific for rchLIF produced in *E. coli* and CHO-K1 cells, according to Western blot analysis (Fig. 3). However, HUL-1 did not recognize rchLIF in ELISAs (Fig. 2), suggesting that the epitope that it recognizes is hidden within the tertiary structure of rchLIF. As HUL-2 recognized rchLIF in ELISAs as well as Western blots (Figs. 2 and 3A), the mAb must bind an epitope that is exposed in more native forms of chLIFs. The molecular mass of eukaryotic chLIF was 28-37 kDa, which is bigger than that of prokaryotic chLIF (Fig. 3B). This might be due to glycosylation, because the chLIF protein contains six asparagine-linked glycosylation sites [20]. When it was demonstrated by the deglycosylation of eukaryotic chLIF, the chLIF was detected as a single band of approximately 20 kDa (our unpublished data). No cross-reactivity was detected for mLIF by either mAbs in ELISAs or Western blot analysis (Figs. 2 and 3B). For HUL-1, this might not be surprising, as chLIF and mLIF have only 39.3% identity at the amino-acid level [20]. However, the lack of cross-reactivity with mLIF was more surprising for HUL-2. Hudson and colleagues [22] have shown that the major residues contributing to the high-affinity binding of human LIF to LIFRβ are Phe^{156} and Lys^{159} in Site III, and both of these amino acids are conserved between human, mouse and chicken LIFs [20]. However, the whole amino-acid sequence represented in immunogen II is only 35.2% homologous between chLIF and mLIF, suggesting that HUL-2 recognizes amino acids within this sequence that differ between the two species.

We used these mAbs to investigate LIF binding to its receptor and the two major signaling pathways downstream of its gp130 subunit, the Jak-STAT and Shp2-ERK

pathways [12]. In ES cells, STAT1 and STAT3 are activated, and the activation of the latter by LIF is sufficient to maintain the undifferentiated state of these cells [13, 14]. In the current study, HUL-2 binding to rchLIF led to the inhibition of STAT3 phosphorylation in CBCs, but had no effect on the phosphorylation of MEK1/2 in the Shp2-ERK pathway (Fig. 4). This indicated that HUL-2 did not simply inhibit the physical binding of chLIF to LIFRβ. Interestingly, a similar phenomenon was observed when CBCs were treated with mLIF [20]. Our results, together with other evidence, suggest that the binding between chLIF and its receptor must be precise for STAT3 and MEK1/2 to be phosphorylated, and that the epitope within immunogen II, which is recognized by HUL-2, has a crucial role in inducing STAT3 phosphorylation.

As HUL-2 inhibited the phosphorylation of STAT3 in CBCs, we examined the effects of the mAbs in CBC cultures. The addition of HUL-2 to the CBC culture medium, with or without rchLIF, promoted the appearance of cyst-like EBs (Fig. 5). This suggests that the phosphorylation of STAT3 by chLIF is sufficient to maintain the undifferentiated state of chicken ES-like cells, as is the case for mouse ES cells. A further novel observation made in this study was that EBs appeared relatively early in CBCs cultured with HUL-2 but without rchLIF. Our previous studies showed that CBCs began to form EBs in the absence of rchLIF after 5 or 6 days in culture [20]. However, as the addition of HUL-2 resulted in the formation of EBs in the absence of rchLIF after only 3 or 4 days, it seemed likely that the CBCs themselves produced chLIF in culture, which acted as an autocrine signal. Although we demonstrated the expression of mRNAs for chLIF and its receptor subunits by cultured CBCs (Fig. 6), we were unable to detect chLIF in the CBC culture supernatant by either ELISA or Western blot analysis. We therefore assume that chLIF is present in the CBC culture supernatants at extremely low concentrations.

In conclusion, the mAbs produced in this study recognize different epitopes on chLIF. HUL-2, in particular, has provided insights into the signaling pathways induced by LIF binding to its receptor. STAT3 phosphorylation is dependent upon interaction at Site III on rchLIF and specifically affects the differentiation of CBCs in culture. Further experiments using these mAbs will aid the development of chicken stem cells and provide further insights into the LIF-receptor interaction.

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Figure legends

Fig. 1. Diagrams showing the structures of Immunogens I and II. Immunogen I was a rchLIF expressed in *E. coli.* Immunogen II was a synthetic peptide composed of 17 amino acids from the carboxy-terminal region of chLIF, conjugated to KLH. The conserved residues, Phe^{157} and Lys^{160}, in Site III of chLIF are indicated by asterisks.

Fig. 2. Binding of mAbs, HUL-1 and HUL-2, to chLIF and mLIF in ELISAs. Microplates were coated with equal concentrations of prokaryotic rchLIF (black bars), eukaryotic rchLIF (dark gray bars), mLIF (light gray bars) or BSA (white bars), and incubated sequentially with HUL-1 or HUL-2, ALP-conjugated anti-mouse-κ antibody and *p*-nitrophenyl phosphate substrate. Data represent the mean absorbance at 405 nm for triplicate samples ± the standard deviation (SD).

Fig. 3. Binding of mAbs, HUL-1 and HUL-2, to chLIF and mLIF in Western blot analysis. (A) Western blot analysis with LIF produced in *E. coli;* rchLIF was run in tracks labeled "1" and mLIF was run in tracks labeled "2". The position of rchLIF (19 kDa) is indicated on the right. (B) Western blot analysis with LIF produced in CHO-K1 cells. The position of rchLIF (28-37 kDa) is indicated on the right. MAb binding was detected using HRP-anti-mouse Ig and ECL plus. The relative mobility of protein markers is indicated on the left. "-" indicates tracks incubated with negative control antibodies.

Fig. 4. Inhibition of STAT3 and MEK1/2 phosphorylation in CBCs by mAbs. (A) CBCs in cytokine- and serum-free CESM were incubated with or without 20 ng/ml rchLIF,

pre-incubated with different dilutions of HUL-1 or HUL-2 mAbs. The cells were lysed and analyzed by Western blot analysis. The application of equivalent amounts of proteins was confirmed by determining nonphosphorylated STAT3 and MEK1/2. The control antibodies were mouse IgG2b or IgA. (B) Quantification of the inhibition of phosphorylation of STAT3 (I) and MEK1/2 (II) by mAbs at different concentrations was calculated using the formula described in the Materials and Methods.

Fig. 5. Inhibitory effect of anti-chLIF mAbs on CBCs in culture. CBCs were cultured in CESM with or without 20 ng/ml rchLIF and 150 μg/ml HUL-1 or HUL-2 for 5 days. The cultured CBCs were photographed after 5 days in culture. Arrows indicate cyst-like EBs. Scale bar: 200 μm.

Fig. 6. Expression of mRNAs for chLIF and its receptor subunits during CBC culture. Total RNA was extracted from CBCs after 1, 3, 6 and 9 days in culture, and reverse transcribed to generate cDNA. A 1-μl sample of cDNA was amplified by PCR with primers specific for *chLIF*, *gp130* or *LIFRβ* genes. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Figure 1. Y. Yamashita et al.

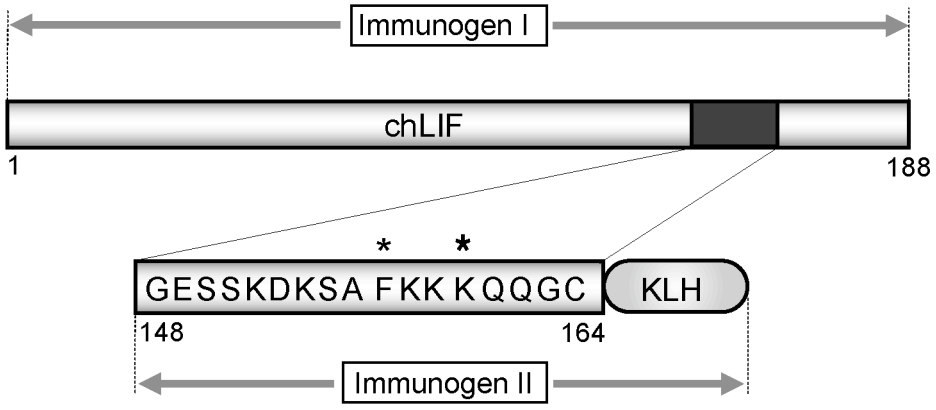

Figure 2. Y. Yamashita et al.

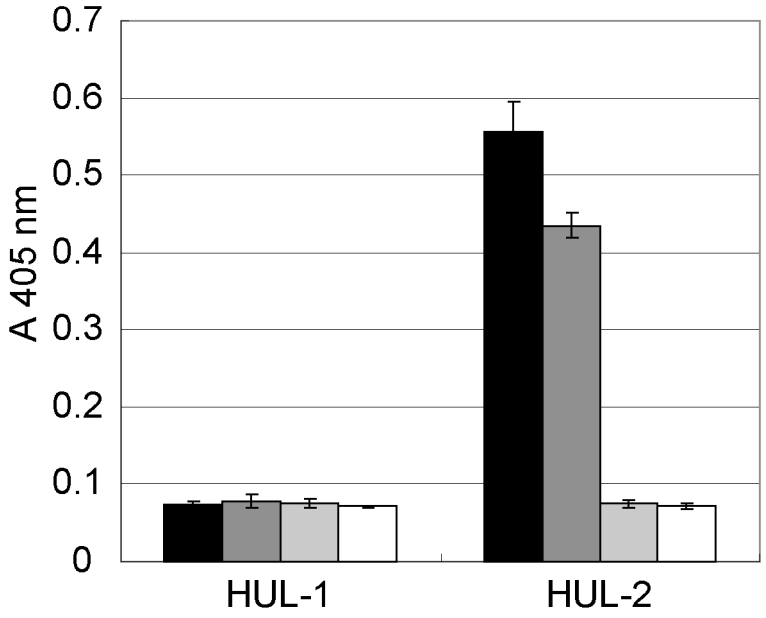

Figure 3. Y. Yamashita et al.

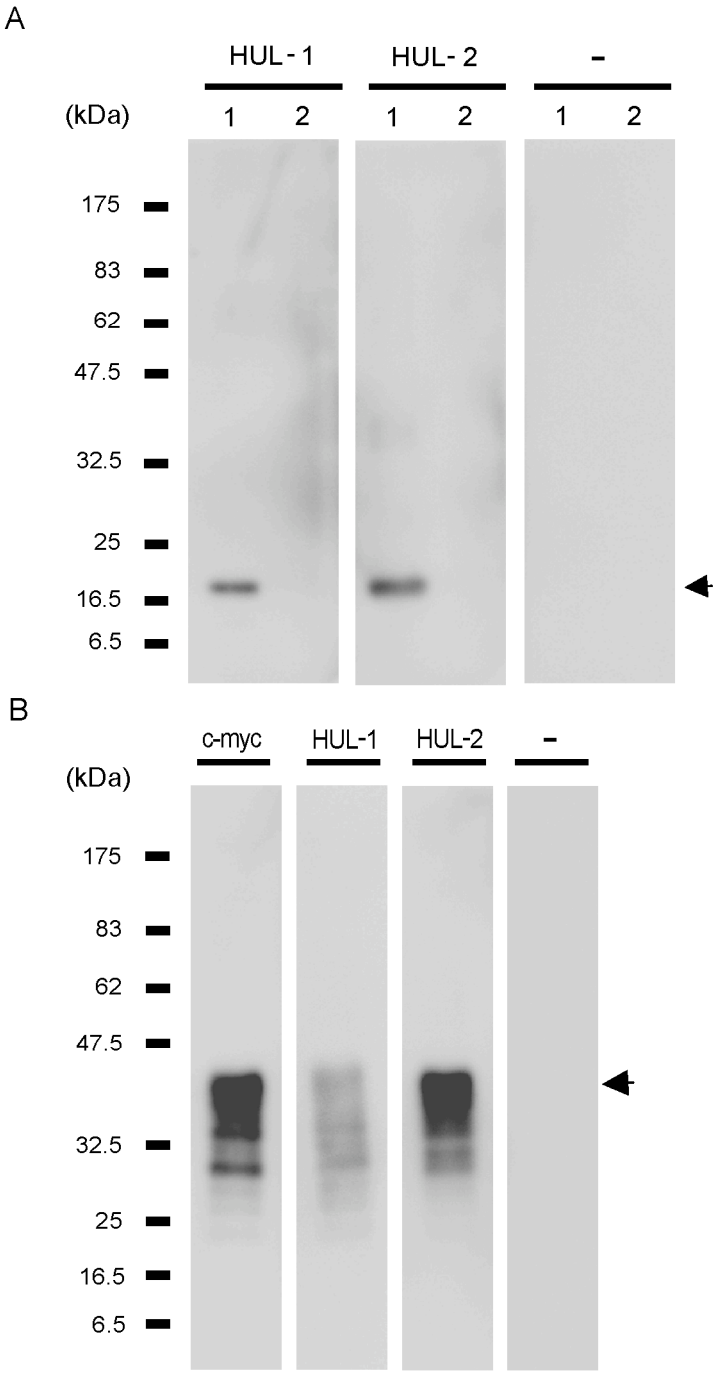

Figure 4. Y. Yamashita et al.

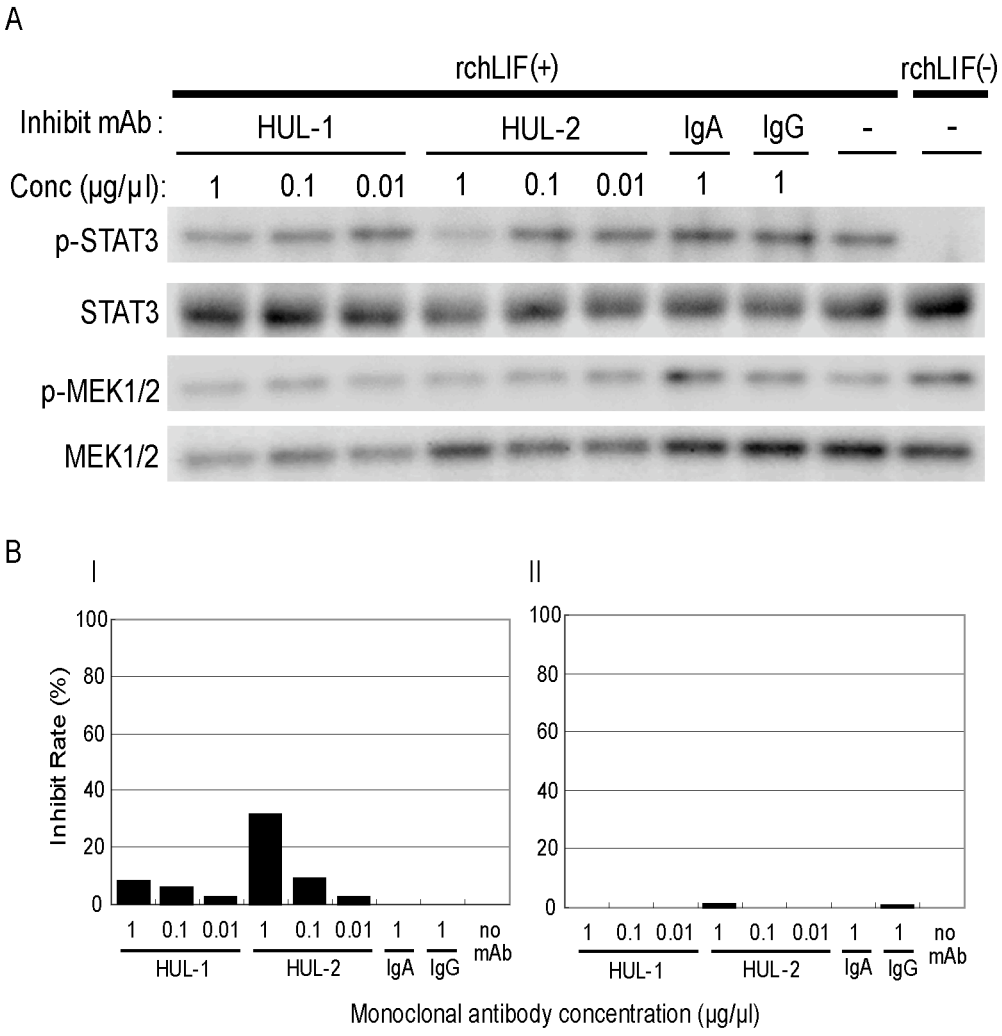

Figure 5. Y. Yamashita et al.

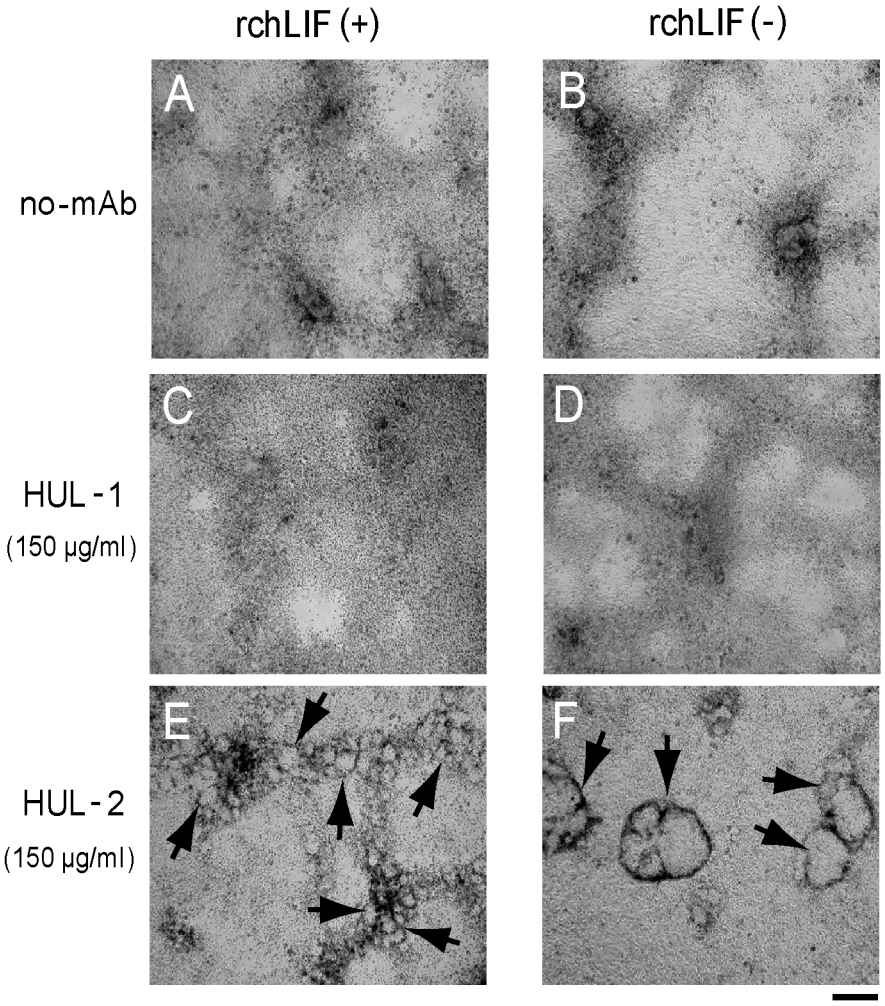

Figure 6. Y. Yamashita et al.