

# Reconstitution of two isoforms of the human interleukin-11 receptor and comparison of their functional properties

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**Abstract** Long-term stable Ba/F3 transfectants (B13R $\alpha$ 1 and B13R $\alpha$ 2) expressing two isoforms of the human IL-11R $\alpha$  receptor ( $\alpha$ 1 full length or  $\alpha$ 2 lacking the cytoplasmic domain) in combination with human gp130 were established. IL-11R $\alpha$ 1 and IL-11R $\alpha$ 2 were each expressed and detected as three bands upon Western blot analysis, with apparent molecular masses in agreement with those of the polypeptide backbone (47 and 44 kDa, respectively) with no, one or two N-linked sugars. B13R $\alpha$ 1 and B13R $\alpha$ 2 bound IL-11–thioredoxin with similar efficiencies and proliferated with superimposable dose–response curves to IL-11, demonstrating that the intracellular domain of IL-11R $\alpha$  has no significant contribution on ligand binding and signaling. Analysis of a set of anti-human gp130 mAbs confirmed the similar responsiveness of B13R $\alpha$ 1 and B13R $\alpha$ 2 transfectants.

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**Key words:** Human interleukin-11 receptor; Isoform; Transfection (Ba/F3 cells); Proliferation; Monoclonal antibody

## 1. Introduction

Interleukin-11 (IL-11) is a cytokine produced by a variety of cells from mesenchymal origin such as fibroblasts, keratinocytes, chondrocytes, synoviocytes, bone marrow stromal cells and osteoblasts. It also displays multiple biological activities, many of which are similar to those exerted by interleukin-6 (IL-6) and, to a lesser extent, by other pleiotropic cytokines such as LIF (leukemia inhibitory factor), OSM (oncostatin M) and CNTF (ciliary neurotrophic factor) [1–4]. For example, IL-11 and IL-6 act in synergy with IL-3 to support the growth of pluripotent bone marrow progenitors and to stimulate megakaryogenesis [5]. They both regulate the synthesis and secretion of acute-phase proteins by hepatocytes [6], promote the differentiation of B lymphocytes into immunoglobulin-secreting cells [7], function as adipogenesis inhibitory factors [8] and participate in the development of osteoclastic cells [9]. The molecular identification of the components of the functional high-affinity receptors for these pleiotropic cytokines has provided some explanation for this redundancy of action. All cytokines of this family require the

common gp130 transmembrane glycoprotein to transduce their biological activities. The gp190 glycoprotein (low-affinity LIF receptor) is also required for signal transduction through LIF, OSM (type I), CNTF and CT-1 receptors [10,11].

In the case of the CNTF, IL-6 and IL-11 receptors, additional receptor components ( $\alpha$  chains) have been described which confer cytokine specificity. The CNTF-R $\alpha$  chain is a 72 kDa glycoprotein (gp72) which is attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor [12]. The IL-6R $\alpha$  chain is a 80 kDa membrane anchored glycoprotein with a 82 amino acids intracellular domain [13]. More recently IL-11R $\alpha$  chains have been described in mouse [14] and human [15,16]. We have shown the existence of two human IL-11R $\alpha$  cDNAs resulting from alternative exon splicing [15,17]. The first cDNA encodes a transmembrane glycoprotein containing, like the IL-6R $\alpha$  chain, a cytoplasmic domain (32 amino acids). The second cDNA isoform encodes a protein lacking, like the CNTF-R $\alpha$  chain, a cytoplasmic domain. In this study, we describe the establishment and characterization of stable Ba/F3 cell lines transfected with the two human IL-11R $\alpha$  isoforms and use these cells to evaluate the contribution of the intracytoplasmic domain of IL-11R $\alpha$  in ligand binding and signal transduction.

## 2. Experimental procedures

### 2.1. Expression constructs

Human IL-11 cDNA was subcloned into pTrx-Fus (Invitrogen BV, Leek, The Netherlands) to construct a thioredoxin–IL-11 fusion cDNA. Expression was performed in *E. coli* and the fusion protein (IL-11–Trx) was prepared as a crude extract from *E. coli* osmotic shock treatment [18]. *Eco*R1 fragment of BQM15.C cDNA clone containing the human IL-11R $\alpha$ 1 coding sequence was inserted into the pLXSPuro (Transgen) eukaryotic expression vector to obtain pLXSP/IL-11R $\alpha$ 1 and selected via the puromycin-resistance gene. Human IL-11R $\alpha$ 2 complete coding sequence was obtained by PCR amplification with the oligonucleotides, 5'-GCGGAATTCATGAGG-GACCAATGGCAGT and 5'-GGACTTTCTAGATGCTGGCAC, using BQP32.21 cDNA clone as template and ligated to the *Eco*R1–*Xba*I fragment of BQM15.C cDNA. The sequence of the PCR fragment was confirmed by double-strand DNA sequencing. This insert was subcloned in pLXSPuro to obtain pLXSP/IL-11R $\alpha$ 2.

### 2.2. DNA transfection experiments

Ba/F3/gp130/IL-11R $\alpha$ 1 (B13R $\alpha$ 1) and Ba/F3/gp130/IL-11R $\alpha$ 2 (B13R $\alpha$ 2) were established by electroporating (at 900  $\mu$ F and 300 V) mouse pro-B-cell Ba/F3 with 20  $\mu$ g of pLXSP/IL-11R $\alpha$ 1 or pLXSP/IL-11R $\alpha$ 2 and 20  $\mu$ g of pRCNeo/. Transfected cells were selected in a culture medium containing: RPMI-1640, 10% fetal calf serum, 1% glutamine, 0.8 mg/ml G418 (Sigma-Aldrich, St. Quentin

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**Abbreviations:** IL-11R $\alpha$ , interleukin-11 receptor; Trx, thioredoxin; s-IL-6R $\alpha$ , soluble IL-6 receptor; mAb, monoclonal antibody

Fallavier, France), 5 µg/ml puromycin (Sigma) and 5 ng/ml (0.25 nM) human IL-11. Ba/F3/IL-11Rα1 (BRα1), Ba/F3/IL-11Rα2 (BRα2) and Ba/F3/gp130 (B13) were obtained using the same procedure except that cells were only transfected with one construct and selected in medium containing 10% of WEHI-3-conditioned medium as a source of IL-3 instead of IL-11 and either 5 µg/ml puromycin (BRα1, BRα2) or 0.8 mg/ml G418 (B13).

### 2.3. Cytokines and monoclonal antibodies

Human *E. coli* recombinant IL-11 was from Peprotech Inc. (London, UK). IL-6, sIL-6Rα and the anti-IL-6Rα monoclonal antibody (mAb) PM1 were kindly given by Dr. Yasukawa (Tosoh Corp., Tokyo, Japan). The anti-human gp130 mAbs B-P8, B-S12, B-R3, B-P4 and the anti-human IL-6Rα mAb B-N12 were from Diaclone (Besançon, France). Their initial characterisation and analysis of functional properties have already been described [19–21].

### 2.4. Proliferation assays

Transfected Ba/F3 cells were washed twice in culture medium lacking growth factor and seeded in 96-well microtiter plates at a density of  $15 \times 10^3$  cells in 50 µl per well. In a first type of assay, cells were incubated (in a final volume of 100 µl) with serial dilutions either of IL-11 (5 nM), of IL-6 (20 nM) in the presence or not of sIL-6Rα (fixed concentration of 8 nM), or of a mixture of B-P8 (6.66 nM) plus B-S12 (20 nM). In a second type of assay, cells were stimulated with IL-11 (0.25 nM) or IL-6 (0.6 nM) plus sIL-6Rα (8 nM), and serial dilutions of B-N12 (20 nM), PM-1 (20 nM), B-R3 (20 nM) or B-P4 (20 nM). Assays were performed in triplicate. After incubation at 37°C for 36 h, cellular proliferation was assessed by a (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based assay (Sigma).

### 2.5. Flow cytometric analysis

Cells ( $1 \times 10^5$ ) were preincubated for 3 h in culture medium lacking growth factor and washed 3 times. For gp130 staining, the B-R3 mAb was added at a final concentration of 67 nM and incubated on ice for 1 h. After washing (3 times), cells were stained with a phycoerythrin-

labeled goat anti-mouse antiserum (1:200; Immunotech, Marseille, France). For IL-11Rα staining, cells were incubated with IL-11-Trx (30 nM), washed 3 times, further incubated for 1 h with a mouse anti-Trx antibody (1:5000) (Invitrogen), washed again (3 times), and finally stained with a phycoerythrin-linked goat anti-mouse antiserum (Immunotech). In some experiments, a 42-fold molar excess of human IL-11 (1.25 µM) was added together with IL-11-Trx.

### 2.6. Immunoblot analysis

Cells were washed 3 times in 10 ml of phosphate-buffered saline (PBS) and pelleted by centrifugation. Pellets were frozen (−80°C) and thawed once, homogenized in a Dounce homogenizer, and centrifuged for 20 min at 2500 rpm at 4°C. The supernatant was centrifuged for 30 min at 14000 rpm at 4°C. Pellets were resuspended in 10 mM Tris buffer (pH 7.4), containing leupeptin at 0.1 mg/ml (Sigma). The protein concentration was determined by the Bicinchoninic Acid (BCA) method (Pierce). Membrane proteins (50 µg/lane) were resolved by 10% SDS-PAGE and transferred onto a PVDF membrane (Amersham, Arlington Heights, USA). After blocking, the membrane was incubated with a rabbit polyclonal antibody (1:100) directed against a 20-amino-acid peptide of murine IL-11R (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using chemiluminescence (ECL kit, Amersham).

## 3. Results

### 3.1. Generation of Ba/F3 transfectants with long-term proliferation in response to IL-11

The murine Ba/F3 cell line has been widely used to study the reconstitution of cytokine receptors [22]. It is absolutely dependent on IL-3 for growth and does not proliferate in response to IL-6 or LIF. Human IL-11Rα1 or IL-11Rα2 cDNAs were transfected in Ba/F3 cells either alone or in combination with full-length human gp130 cDNA. Transfected cells were subsequently cultured in selection media con-

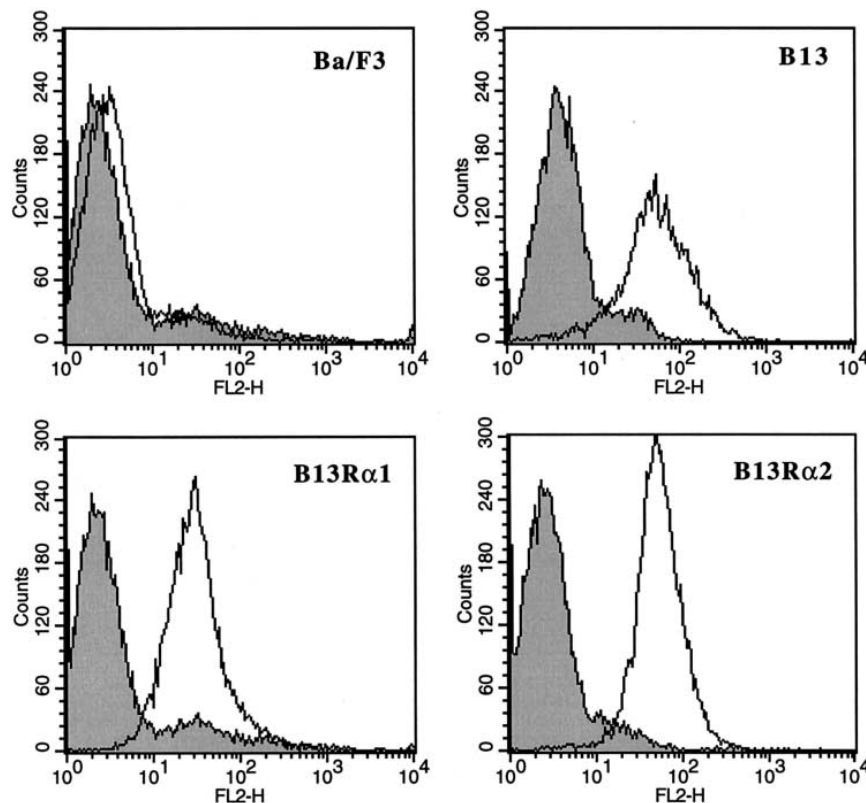


Fig. 1. Flow cytometric analysis of human gp130: B-R3 mAb and phycoerythrin-conjugated second antibody (open area). Control: second antibody alone (closed area).

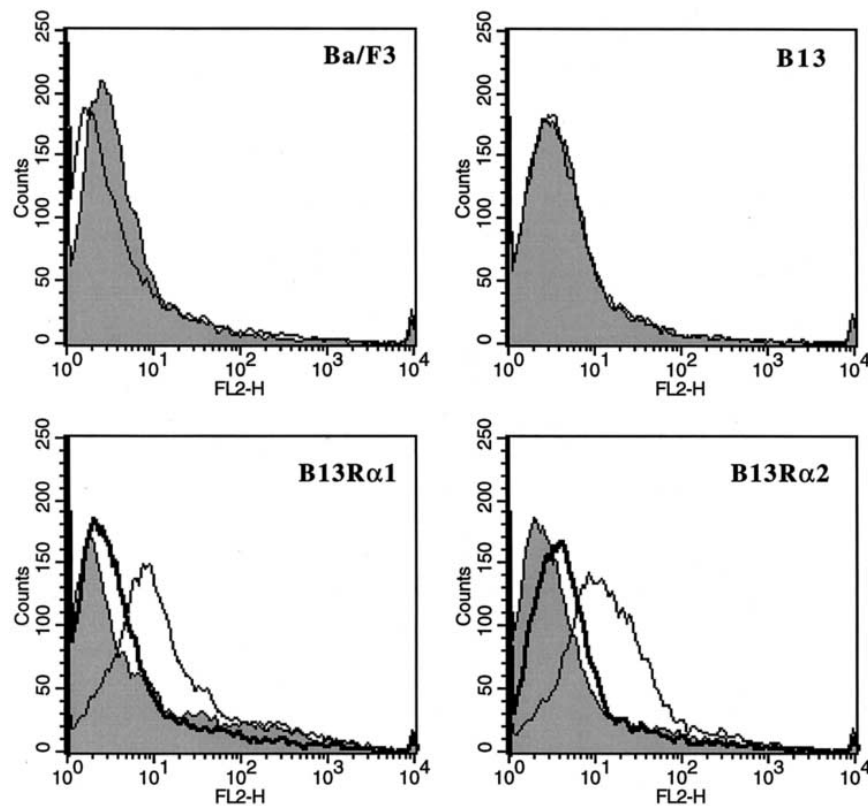


Fig. 2. Flow cytometric analysis of human IL-11R $\alpha$ : IL-11–Trx/anti-Trx antibody and phycoerythrin-conjugated second antibody in the absence (thin lane, open area) or presence (thick lane, open area) of a 42-fold molar excess of human IL-11. Control: anti-Trx plus second antibody (closed area)

taining appropriate antibiotic(s) and IL-3 conditioned medium or 5 ng/ml of human recombinant IL-11. In the presence of IL-3, cellular expansion was observed in all transfected cultures. In IL-11 containing medium, cellular expansion was only observed from Ba/F3 cells co-transfected with IL-11R $\alpha$ 1 and gp130 (B13R $\alpha$ 1) or IL-11R $\alpha$ 2 and gp130 (B13R $\alpha$ 2). Furthermore, when cells selected in IL-3 conditioned medium were shifted to IL-11 containing medium, only cells co-transfected with gp130 and IL-11R $\alpha$ 1 or IL-11R $\alpha$ 2 continued to grow, whereas cells transfected without insert, with IL-11R $\alpha$ 1 alone (BR $\alpha$ 1), IL-11R $\alpha$ 2 alone (BR $\alpha$ 2) or gp130 alone (B13) rapidly died. These results were consistently obtained in several independent transfection experiments. Long-term proliferation of B13R $\alpha$ 1 and B13R $\alpha$ 2 were observed. These cell lines are now routinely grown (for more than 6 months) in IL-11 containing medium and retain their IL-11 growth-dependency (see Fig. 4A).

### 3.2. Analysis of the expression of the transfected receptor chains

Ba/F3 cells expressing different combinations of the transfected cDNAs were established as stable cell lines. The level of expression at the cell surface of the transfection products was evaluated by flow cytometry and Western blots. Fig. 1 shows the flow cytometric histograms obtained with an anti-gp130 mAb (B-R3). In contrast to the parental Ba/F3 cell line which was negative, B13, B13R $\alpha$ 1 and B13R $\alpha$ 2 expressed gp130 with similar intensities. Due to the lack of a suitable anti-IL-11R $\alpha$  for flow cytometric analysis, the expression of IL-

11R $\alpha$  was measured with the combination of an IL-11–thioredoxin fusion protein (IL-11–Trx) and an anti-Trx antibody. As shown in Fig. 2, both B13R $\alpha$ 1 and B13R $\alpha$ 2 transfectants were similarly labeled with IL-11–Trx, whereas Ba/F3 and B13 cells were negative. The specificity of IL-11–Trx binding was assessed by the observation that a 42-fold molar excess of unlabeled recombinant IL-11 almost completely inhibited this binding.

Western blots were performed on membrane preparations from these different cell lines, using a polyclonal antibody raised against a N-terminal peptide of murine IL-11R $\alpha$  (Fig. 3). This antibody was negative on parental Ba/F3 cell lines and B13 cells and very clearly identified specific bands on IL-11R $\alpha$ /gp130 transfected cells. Three bands with molecular masses of 47, 50 and 54 kDa were detected on cells transfected with the IL-11R $\alpha$ 1 isoform, whereas on IL-11R $\alpha$ 2 transfectants, these three bands had lower masses of 44, 47 and 51 kDa, respectively.

### 3.3. Proliferative responses

We next examined the proliferative response of the various transfectants to different cytokines. All cell lines proliferated in response to IL-3-conditioned medium with comparable dose responses (data not shown). Only IL-11R $\alpha$ /gp130 double-transfected cells did proliferate in response to IL-11 (Fig. 4A), showing that both IL-11R $\alpha$  and gp130 are required and sufficient for IL-11 signaling in Ba/F3 cells. The dose-response curves relative to the effects of IL-11 on the B13R $\alpha$ 1 and B13R $\alpha$ 2 proliferative responses were almost superimposable,

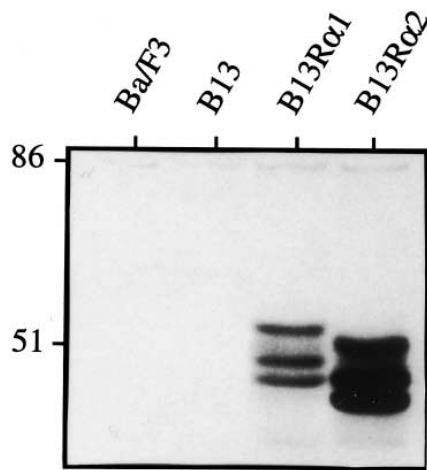


Fig. 3. Western blot analysis of human IL-11R $\alpha$ . Molecular weight markers (left) are in kDa.

thereby indicating that the two isoforms have comparable efficiencies in complementing gp130 for signaling. The concentration of IL-11 inducing half maximal proliferative responses ( $IC_{50}$ ) were 97 and 118 pM, respectively, values compatible with a high-affinity interaction.

The responses of these two double transfectants to the combination of IL-6 plus sIL-6R $\alpha$  were also comparable (Fig. 4B), a result further indicating that the two cell lines express equivalent amounts of functional human gp130. The  $IC_{50}$ s for IL-6 in the presence of a fixed concentration of sIL-6R $\alpha$  (8 nM) were 66 and 75 pM, respectively, values in agreement with its high-affinity equilibrium binding constant [23]. We also tested two anti-human gp130 mAbs (B-P8 and B-S12) which have been described to display synergistic and agonistic functions [19]. In agreement with these findings, B-P8 plus B-S12 induced the proliferation of B13R $\alpha$ 1 and B13R $\alpha$ 2 transfectants (Fig. 4C) with efficiencies comparable to those induced by IL-11 or IL-6/sIL-6R $\alpha$ . Again, the responses of the two transfectants were superimposable, with  $IC_{50}$ s of 0.38 and 0.41 nM.

IL-11 versus IL-6/sIL-6R $\alpha$  proliferative responses of the two transfectants were further assessed by the use of additional monoclonal antibodies (Fig. 5). B-R3 is an anti-human gp130 mAb which interferes with the biological effects of all known cytokines using gp130 as transducing element, whereas B-P4 is an anti-gp130 mAb which has been proposed to interfere specifically with the IL-11 response [19–21]. PM1 and B-N12 are anti-human IL-6R $\alpha$  mAbs. PM1 is a blocking antibody [24], whereas B-N12 is not (J. Wijdenes, unpublished result). For each mAb tested, the responses of B13R $\alpha$ 1 and B13R $\alpha$ 2 were always almost superimposable. The B-N12 antibody had no effect in either system (Fig. 5A,B). The PM1 mAb inhibited the IL-6/sIL-6R $\alpha$  responses ( $IC_{50}$ s: 6.3 and 7.5 nM) (Fig. 5D) while only slightly diminishing the IL-11 driven proliferation (Fig. 5C). The proliferation induced by either IL-11 or IL-6/sIL-6R $\alpha$  was totally abrogated by mAb B-R3 (Fig. 5E,F). The  $IC_{50}$ s describing the inhibitory effects on IL-11 were 0.30 and 0.24 nM, and those on the IL-6 response were 0.44 and 0.41 nM. These values are in full agreement with the binding affinity of B-R3 on these cells as measured by Scatchard analysis ( $K_d$ =0.43 and 0.49 nM on B13R $\alpha$ 1 and B13R $\alpha$ 2 cells respectively; data not shown). The

B-P4 antibody abrogated the IL-11 response in B13R $\alpha$ 1 and B13R $\alpha$ 2 cell lines with a similar efficacy as B-R3 ( $IC_{50}$ s=0.24 and 0.18 nM) (Fig. 5G). Unexpectedly, the B-P4 antibody also abrogated the IL-6/sIL-6R $\alpha$  proliferative effect on the two cell lines ( $IC_{50}$ s=0.43 and 0.47 nM) (Fig. 5H).

#### 4. Discussion

This study shows that co-expression in the Ba/F3 cell line of the human IL-11R $\alpha$  with human gp130 is necessary and sufficient for the acquisition of human IL-11 mediated proliferative response and for the establishment of Ba/F3 cell transfectants which can be grown on a long-term basis under the growth promoting effect of recombinant human IL-11. These transfectants are now grown in our laboratory for more than 6 months without any significant changes of their IL-11 growth response sensitivities. These results extend recent ob-

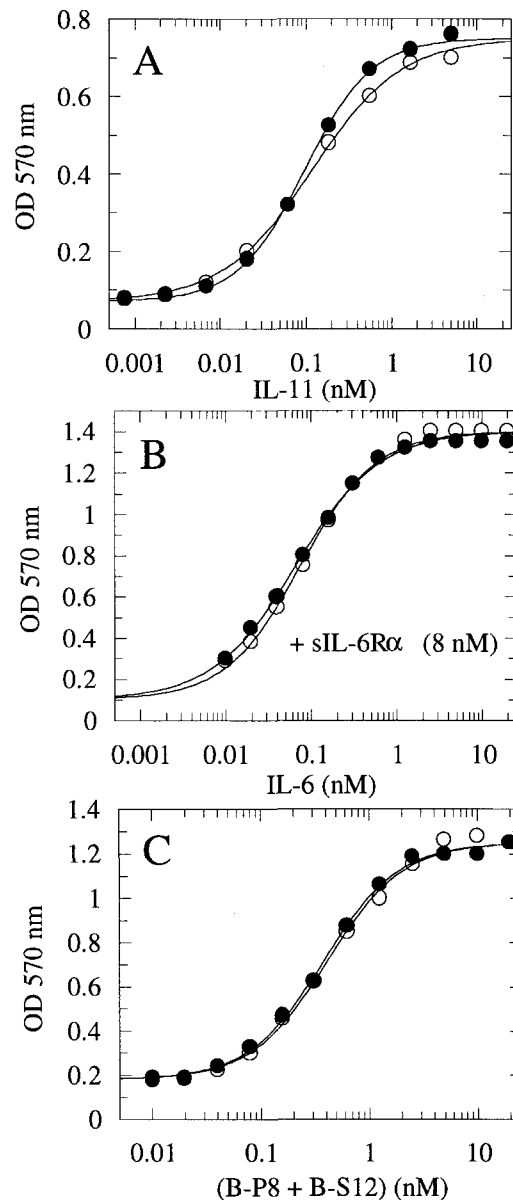


Fig. 4. Proliferative responses of B13R $\alpha$ 1 (●) and B13R $\alpha$ 2 (○) cells.

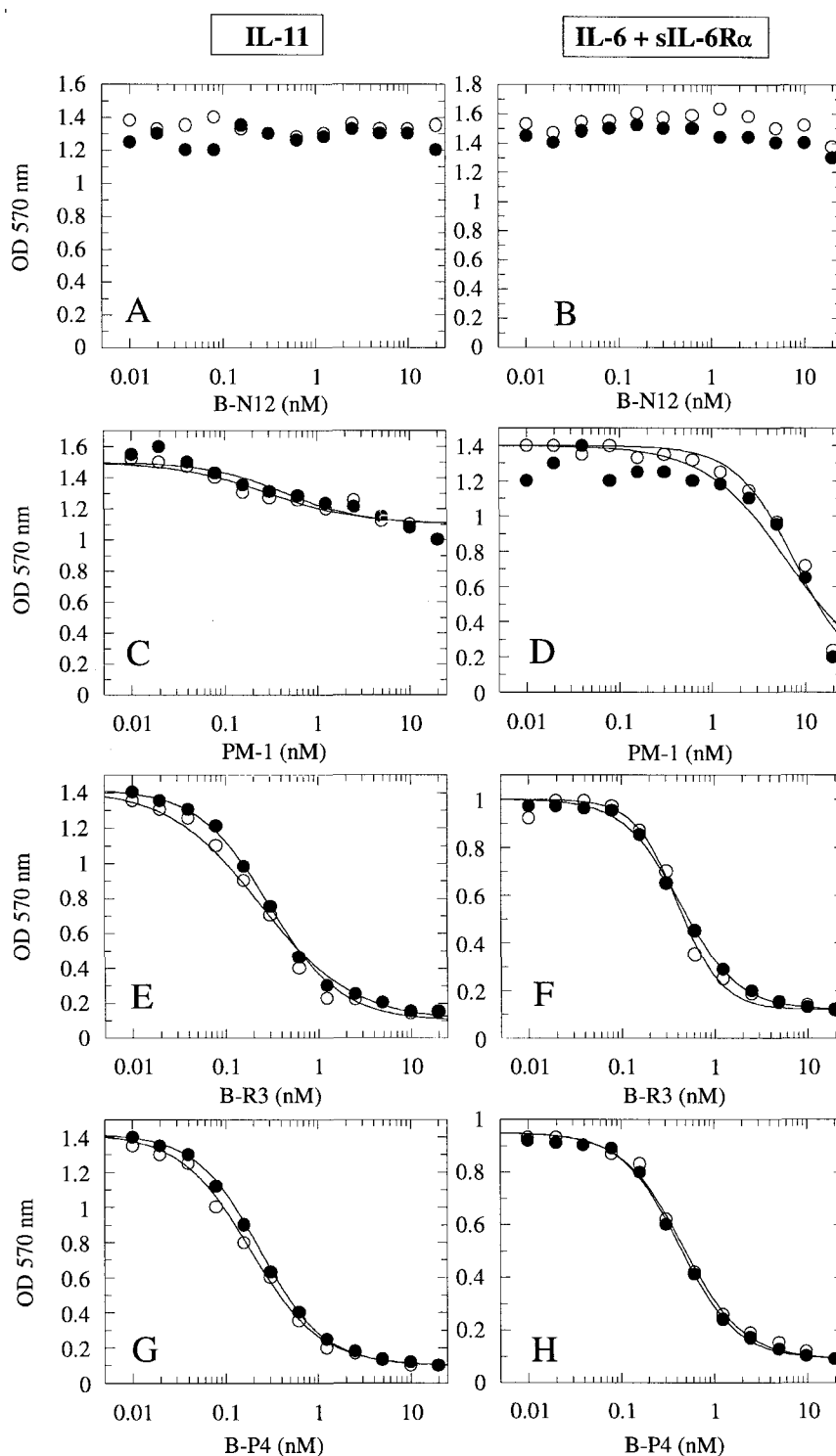


Fig. 5. Effects of anti-gp130 and anti-IL-6R mAbs on B13R $\alpha$ 1 (●) and B13R $\alpha$ 2 (○) proliferative responses induced either by IL-11 (5 nM) (left) or by IL-6 (0.6 nM) plus sIL-6R $\alpha$  (8 nM) (right).

servations on similarly co-transfected Ba/F3 cells showing that IL-11 was able to maintain cell viability on a short (48 h) time period [14,16] and provide for the first time the description of stable human IL-11R $\alpha$ /gp130 Ba/F3 cell transfectants proliferating in response to human IL-11.

We have previously shown that the human IL-11R $\alpha$  chain exists in two different isoforms resulting from alternative

splicing of a single gene. These two naturally occurring IL-11R $\alpha$  isoforms differ only by the presence of the 32-amino-acids cytoplasmic domain [15]. We took advantage of this situation to analyze the functional implication of this cytoplasmic domain in the Ba/F3 system.

A polyclonal antibody raised against the N-terminal peptide (residues 25–44) of mouse IL-11R $\alpha$  was shown to react with

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