ELSEVIER

Journal of Immunological Methods 241 (2000) 43–59

JIM Journal of Immunological Methods

www.elsevier.nl/locate/jim

Monoclonal antibodies against the human interleukin-11 receptor alpha-chain (IL-11Rα) and their use in studies of human mononuclear cells

Chrystel Blanc[a], Patricia Vusio[b], Karin Schleinkofer[c], Olivier Boisteau[b], Stefan Pflanz[c], Stéphane Minvielle[b], Joachim Grötzinger[c], Gerhard Müller-Newen[c], Peter C. Heinrich[c], Yannick Jacques[b], Félix A. Montero-Julian[a,*]

[a]*Immunotech: A Beckman–Coulter Company, 130 av. de Lattre de Tassigny, BP 177, 13276 Marseille Cedex 9, France*
[b]*INSERM U463, Institut de Biologie, 9 Quai Moncousu, 44035 Nantes Cedex 01, France*
[c]*Institut für Biochemie RWTH Aachen, Klinikum Pauwelstr. 30, 52057, Aachen, Germany*

Received 23 December 1999; received in revised form 15 March 2000; accepted 4 April 2000

Abstract

A panel of 14 hybridoma cell lines secreting monoclonal antibodies against the human interleukin-11 receptor alpha chain (hIL-11Rα) was obtained using two different approaches. Two antibodies were raised against peptides of the N- and C-terminal sequences, respectively, of the extracellular part of the hIL-11Rα. Another group of 12 antibodies was generated against a hybrid protein consisting of the extracellular part of the hIL-11Rα fused to mature full-length human IL-2. All these antibodies recognized native hIL-11Rα and most also recognized the denatured receptor on immunoblots after SDS–PAGE. Four different epitopes were identified on the extracellular part of the hIL-11Rα. One epitope, defined by the E27 antibody, is located at the N-terminus and the other three epitopes are clustered in the membrane-proximal, C-terminal region. The antibodies defining epitopes I and II recognized membrane-bound hIL-11Rα expressed in gp130/hIL-11Rα-co-transfected Ba/F3 cells. The E27 antibody cross-reacted with murine IL-11Rα, in agreement with the fact that the N-terminal region is highly conserved between species. The other 13 antibodies all recognized a region between amino acids 319 and 363, which is the membrane-proximal part of the hIL-11Rα. This region, which is less conserved between mouse and human, is shown here to be an immunodominant region. Anti-IL-11Rα monoclonal antibodies, which have not been described previously enabled us to explore the expression and tissue distribution of IL-11Rα on human peripheral blood mononuclear cells and cell lines. The antibodies provide powerful tools for the study of the regulation and function of the receptor. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Interleukin-11, Interleukin-11 receptor; Cytokine receptors; Monoclonal antibody

Abbreviations: CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IL-11Rα, interleukin-11 receptor alpha chain; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl polyacrylamide gel electrophoresis

*Corresponding author: Tel.: +33-4-9117-2752; fax: +33-4-9117-2753.
E-mail address: montero@immunotech.fr (F.A. Montero-Julian)

0022-1759/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0022-1759(00)00194-0

Lassen - Exhibit 1015, p. 1

DOCKET ALARM

Find authenticated court documents without watermarks at docketalarm.com.

1. Introduction

Interleukin-11 (IL-11) was first described as a hematopoietic cytokine produced by bone marrow stromal cells (Paul et al., 1990). A variety of cells produce IL-11. It is involved in the development and proliferation of hematopoietic progenitor stem cells, as well as at different stages of hematopoiesis, such as the enhancement of megakaryocytopoiesis and platelet formation (Neben and Turner, 1993; Du and Williams, 1997). Its role in platelet formation recently led to its therapeutic use in the prevention of thrombocytopenia associated with chemotherapy (Tepler et al., 1996). IL-11 exerts pleiotropic activities in different tissues, including the induction of acute phase proteins (Baumann and Schendel, 1991), the stimulation of the differentiation of B lymphocytes into immunoglobulin-secreting cells (Anderson et al., 1992), the development of osteoclastic cells (Girasole et al., 1994), a role in neuronal development (Mehler et al., 1993), and the induction of protective effects in the intestinal mucosa (Booth and Potten, 1995). Recently, IL-11 was implicated in female reproduction (Robb et al., 1998) and was shown to have potent anti-inflammatory properties (Trepicchio et al., 1996; Hill et al., 1998).

IL-11 has been grouped into the interleukin-6 (IL-6) family (for reviews, see, Kishimoto et al., 1995; Heinrich et al., 1998). This family is composed of IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1). All members of this cytokine family share the transmembrane glycoprotein gp130 as a common signal transducing subunit. An IL-11 receptor alpha-chain (IL-11Rα) binds IL-11 with low affinity (K_d ~10 nmol/l) and is responsible for ligand-binding specificity (Hilton et al., 1994). The IL-11/IL-11Rα complex triggers the association of two gp130 molecules (Yin et al., 1993), leading to the formation of a high-affinity receptor (K_d ~400–800 pmol/l) (Hilton et al., 1994) able to transduce signals via the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signaling pathway (Dahmen et al., 1998). The IL-11Rα gene has been cloned in the mouse (Hilton et al., 1994) and human (Chérel et al., 1995; Nandurkar et al., 1996). The mouse genome contains two distinct IL-11Rα loci (Bilinski et al., 1996); one of these genes is expressed strongly in the testis and is restricted to some mouse strains. In the human, only one locus has been identified (Chérel et al., 1996; Van Leuven et al., 1996), but two different cDNAs were isolated, both coding for a membrane-anchored receptor with one lacking the cytoplasmic portion (Chérel et al., 1995, 1996). The two isoforms are both active (Lebeau et al., 1997). The sequence homology between murine and human IL-11Rα is 84% at the amino acid level. At present, there is little information about the expression of IL-11Rα receptor protein, and the existence of a soluble form of the receptor remains to be demonstrated. A functional role for IL-11 and for its α-receptor moiety and possible involvement in human disease has not been established. In order to address these questions, highly specific reagents directed at the IL-11Rα have to be developed, and we report here the generation and characterization of a panel of monoclonal antibodies against human IL-11Rα. We took advantage of these antibodies to analyze IL-11Rα receptor surface expression on peripheral blood mononuclear cell subsets and numerous human cell lines.

2. Materials and methods

2.1. Reagents and antibodies

Fine chemicals, unless otherwise stated, were from Merck (Darmstadt, Germany) and cell culture reagents from Biowhittaker (Gagny, France). Human recombinant IL-2 and IL-11, biotinylated rabbit polyclonal anti-IL-11 and recombinant murine IL-11RαFc chimera were from R&D Systems (Abingdon, UK). Restriction enzymes were purchased from Eurogentec (Brussels, Belgium). HAT reagent was purchased from Sigma (St. Louis, MO). Rabbit polyclonal anti-murine IL-11R (N20) was purchased from Santa Cruz Biotechnology (California, USA). This antibody was generated using a peptide coding for the first 20 amino acids of the extracellular domain of the murine IL-11Rα and displayed 95% homology with the corresponding hIL-11Rα peptide. Peroxidase-, biotin- or phycoerythrin-conjugated goat anti-mouse and goat anti-rabbit antibodies, as well as streptavidin conjugated to phycoerythrin or peroxidase, were obtained from Immunotech (Marse-

Lassen - Exhibit 1015, p. 2

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

ille, France). Anti-human Fc-domain antibody was from Jackson Laboratories (New Jersey, USA).

2.2. Cells and cell culture

The IL-3-dependent mouse pro-B-cell line Ba/F3 and the stable transfectant Ba/F3/130/IL-11Rα expressing both the human signal transduction subunit gp130 and the human IL-11 receptor α were generated as described (Lebeau et al., 1997). Ba/F3 cells were maintained in RPMI-1640 containing 10% (v/v) heat-inactivated fetal calf serum (FCS), supplemented with additives (2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 500 U/ml penicillin/streptomycin) and 2% (v/v) WEHI-3-conditioned medium as a source of murine IL-3. Transfected cells were cultured in the same medium supplemented with 1 ng/ml of human recombinant IL-11 instead of IL-3. Mouse myeloma cell line X63.Ag.653 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and additives. The hybridoma cell lines were grown in DMEM supplemented with 20% FCS, additives and HAT (hypoxanthine–aminopterin–thymidine). Chinese hamster ovary (CHO) cells deficient in dehydroxyfolate reductase (DHFR −/−) were grown in DMEM/F12 medium with 10% FCS, 2 mM glutamine, 500 U/ml penicillin/streptomycin. Transfected CHO cells were grown in RPMI-1640 supplemented with 10% FCS, additives and 750 μg/ml of G418. Meg01 and MO7E megakaryoblastic leukemia, HEL and TF1 erythroleukemia, KG1a bone marrow acute myelogenous leukemia, K562 chronic myelogenous leukemia, HL60 promyelocytic leukemia, THP-1 monocytic leukemia, NK3.3 NK cells, RPMI8866 B-cell lymphoma, Daudi Burkitt lymphoma, Peer and Molt13 leukemic T-cell lymphoma, MG63 and Saos2 osteosarcoma, SW620 colon adenocarcinoma, INT407 embryonic intestine, A375 melanoma, MDA-MD-157 and MCF-7 breast carcinoma, JAR choriocarcinoma, SVK14 transformed keratinocyte, HELA epithelial carcinoma, SRJH30 rhabdomyosarcoma, SK-N-SH, SK-N-MC and IMR32 neuroblastoma, SNB-19, A172 and U373MG glioblastoma cell lines were obtained from the ATCC (Rockville, MD). The WM35 Melanoma cell line was provided by Dr M. Herlyn (Wistar Institute, Philadelphia, PA). All cell lines were maintained in RPMI-1640 containing 10% (v/v) FCS supplemented with additives.

2.3. Preparation of soluble IL-11R–IL-2 fusion protein, transfection of CHO cells and purification of sIL-11R–IL-2

A cDNA encoding a soluble form of the human IL-11Rα (amino acids 1–363) was generated by polymerase chain reaction (PCR) amplification from the complete cDNA of human IL-11Rα (Chérel et al., 1995) as template, with 5′-GGAATTCGAAATGAGCAGCAGCTGCTCAG-3′ as sense primer and 5′-TGCATGCATCACAGAGTCCCTGTGATCA-3′ as anti-sense primer. The fragment was cloned into a Bluescript plasmid coding for human Interleukin-2 (hIL-2) opened with *Eco*RI/*Pst*I. The resulting fused sIL-11R–IL-2 gene contains two additional codons as linker between the sIL-11Rα and IL-2 genes, coding for the dipeptide Met–Gly. The sequence of the hybrid sIL-11R–IL-2 gene was confirmed by sequencing. The sIL-11R–IL-2 was sub-cloned into the mammalian expression vector pKCR6 between the *Xho*I/*Not*I sites. The Bluescript plasmid containing hIL-2 and the vector pKCR6 were provided by Dr M. Bonneville (INSERM, Nantes).

DHFR −/− CHO cells were co-transfected with both the pKCR6 sIL-11R–IL-2 plasmid and with the pCDNA3 plasmid (Invitrogen, Netherlands) which carries the neomycin resistance gene, thus providing an additional means of selection. The transfection was performed using LipofectAMINE PLUS Reagent (Life Technologies) according to the manufacturer's protocol. Clones producing sIL-11R–IL-2 protein were detected using a commercial enzyme immunoassay for the detection of human IL-2 (Immunotech). One clone, 1.22, was selected for its maximal production of sIL-11R–IL-2 of about 300 ng/ml, based on the measurement of the immunoreactivity of human IL-2 in the supernatant. The supernatant of clone 1.22 was collected after 7 days, when the cell monolayer was confluent and was concentrated by precipitation with ammonium sulfate at 60% saturation. An IL-2-immunoaffinity column was prepared by grafting an anti-IL-2 mAb (IL-2.66, Immunotech) onto CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) following the protocol of the manufacturer. The concentrated supernatant was

Lassen - Exhibit 1015, p. 3

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

dialyzed and loaded onto the column. The column was washed exhaustively with 50 mM Tris, 0.15 M NaCl pH 7.5 and eluted with 50 mM sodium acetate pH 4.2. The concentration of sIL-11R–IL-2 was determined in the fractions collected with the IL-2 immunoassay. Active fractions were pooled, dialyzed against PBS and stored at −20°C. Purity of the product was assessed by SDS–PAGE (Miniprotean Ready Gels, BioRad, Ivry-sur-Seine, France). Proteins were stained with Coomassie Blue or transferred onto a nitrocellulose membrane (Hybond ECL, Amersham, Les Ullis, France) using a transblot cell (BioRad). After incubation with blocking reagent (Roche Molecular Biochemicals, Germany), the membrane was probed with either the anti-IL-2 mAb described above or anti-murine IL-11Rα rabbit polyclonal (N20), followed by incubation with appropriate peroxidase-conjugated secondary antibodies. Development was performed using an ECL (Enhanced ChemoLuminescence) kit (Amersham).

2.4. Preparation of the soluble IL-11R/FP

The soluble IL-11R/FP was prepared as described (Pflanz et al., 1999). Briefly, this protein contains domains II and III of the hIL-11Rα (amino acids 109–318) followed by a 21 amino acid spacer linked to mature human IL-11. This protein does not contain the Ig-like domain of the hIL-11Rα. The gene encoding this protein was cloned into pPICZαA plasmid (Invitrogen). Competent *P. pastoris* GS115 were transfected with pPICZαAIL-11R/FP applying the LiCl method according to the manufacturer's instructions. Transfected cells were selected and conditions for the expression of IL-11R/FP were optimized.

2.5. Preparation of a soluble domain III protein of hIL-11Rα (IL-11RD3), expression in E. coli, folding and purification.

The domain III-encoding region (amino acids 212–337) of the hIL-11Rα, IL-11RD3, was amplified by PCR using hIL-11Rα-cDNA as a template (Chérel et al., 1995). *Nco*I and *Bam*HI sites were introduced into the 5′- and 3′-primers, respectively to make possible cloning of the amplified DNA in the corresponding sites of the *E. coli* expression vector pET8c/3d (Stratagene, La Jolla, CA, USA) (sense primer, 5′-GGT GGT GCC ATG GAG AGC ATC TTG CGC CCT GAC-3′; anti-sense primer 5′-CCG GAA GCT TAC TCC ACC TCT GGC TGC GT-3′). The IL-11RD3 cDNA construct was confirmed by restriction analysis and DNA sequencing. *E. coli* BL21 (DE3) (Studier et al., 1990) was transformed with the IL-11RD3 expression vector. Expression of the recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (final concentration: 0.5 mM) for 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100, 1 mM dithiothreitol (DTT)). Complete lysis of bacteria was achieved by three freeze–thaw cycles followed by four steps of sonication. The inclusion body pellet containing the recombinant protein was purified through ten cycles of washing and centrifugation. Inclusion bodies were solubilized in 6 M guanidine–HCl, 50 mM Tris–HCl, pH 8.0, 100 mM DTT. The final concentration of the recombinant protein was approximately 5 mg/ml. Solubilized inclusion bodies were submitted to an in vitro folding process by rapid 1/100 dilution in 20 mM Tris–HCl pH 8.5, 1 mM EDTA, 200 mM NaCl, 1 mM DTT. The folded recombinant protein was purified on a Superdex 75 (26/60) column (Amersham Pharmacia Biotech). Fractions were analyzed by SDS–PAGE, pooled and concentrated.

2.6. IL-11/sIL-11R–IL-2 binding assay

Interaction of IL-11 with sIL-11R–IL-2 was measured by an ELISA-based binding assay using the purified protein sIL-11R–IL-2 as described previously (Tacken et al., 1999).

2.7. Generation of hybridoma-producing monoclonal anti-hIL-11Rα antibodies

2.7.1. Immunization of mice with peptides and screening

Peptides (Genosys, Cambridge, UK) corresponding to amino acids 23–43 (SSPCPQAWGPPGVQYG-QPGRS) of the N-terminal region (peptide 1) and to amino acids 345–363 (PPRPSLQPHRRLLDH-RDSV) of the C-terminal region of the extracellular domain of the hIL-11Rα (peptide 2) were conjugated

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

to carrier proteins, ovalbumin or KLH (Keyhole Limpet hemocyanin) (Pierce, USA). BALB/c mice (Iffa Credo, Les Oncines, France) were immunized three times at 3-weekly intervals with 100 μg of either peptide conjugated to KLH, intraperitoneally in complete Freund's adjuvant (CFA) (Sigma). Three weeks after the final injection, one of the mice received 50 μg of peptide conjugated to ovalbumin intravenously and was sacrificed 3 days later. Spleen cells were fused with mouse myeloma X63.Ag8.653 using polyethylene glycol 1500 (Sigma) and selected in standard HAT medium. Hybridoma supernatants were screened on 96-well plates coated with peptides or with sIL-11R–IL-2 protein. Positive hybridomas were cloned under limiting dilution and two hybridomas were established: E27 directed against the N-terminal peptide and A39R against the C-terminal peptide. Both antibodies recognized not only the immunizing peptide but also the recombinant hybrid protein sIL-11R–IL-2. The isotypes were determined using a mouse isotyping kit (Amersham). Ascitic fluids were generated from both hybridomas and purified by affinity chromatography using protein L (Interchim, Montlucon, France) for E27 mAb which is of the IgA isotype or protein A (Pharmacia) for A39R mAb which is of the IgG1 isotype.

2.7.2. Immunization of mice with sIL-11R–IL-2 and screening

BALB/c mice were immunized subcutaneously twice at 3-weekly intervals with 15 μg of sIL-11R–IL-2 in CFA. Three weeks after the second immunization, one mouse received intravenously 5 μg of sIL-11R–IL-2 and was sacrificed 3 days later. Spleen cells were fused as described above. Hybridoma supernatants were screened both on 96-well plates coated with sIL-11R–IL-2 or hIL-2 and by cytometric analysis on Ba/F3/130/IL-11Rα cells. Twelve clones, E1.8, E12.7, E10.1, I12.3, C4.2, I7.4, D14.7, D16.1, E24.2, C8.7, B24.3, A3.4 were established. All mAbs were of the IgG1 isotype. Ascitic fluids were generated and mAbs purified using protein A Sepharose (Pharmacia).

2.8. Epitope analysis of anti-IL-11Rα mAbs

The definition of the epitopes recognized by the different anti-IL-11Rα mAbs was performed in an ELISA format by cross-pairing each mAbs with all other mAbs. Antibodies were biotinylated with biotin-*N*-hydroxy-succinimide-1 ester (Roche Diagnostic) following the manufacturer's instructions. The 96-well plates were coated with mAbs at 5 μg/ml in PBS for 24 h at 4°C and saturated with 3% BSA in PBS. Then 100 μl/well of CHO-derived sIL-11R–IL-2 supernatant at 30 ng/ml were incubated for 2 h at room temperature, followed by three washes with an automatic washer (SLT, Salzburg, Austria). One hundred μl/well of each biotinylated anti-IL-11Rα mAbs at 1 μg/ml (except A39R at 5 μg/ml) were incubated for 2 h at room temperature. After three additional washes, 100 μl/well of peroxidase-streptavidin (1/50,000) was added and the plates incubated for 1 h. The enzymatic activity was revealed using 100 μl/well of substrate (1% tetramethybenzimide, 0.1% H_2O_2 in 0.1 M sodium acetate, pH 5.5). After development in the dark for 10 min, the reaction was stopped and the absorbance at 450 nm was measured using an ELISA reader (Molecular Device, UK).

2.9. Surface plasmon resonance (SPR) studies

The affinity and dissociation constants for each antibody were calculated by SPR studies with the BIAcore 2000 optical biosensor (BIAcore). Briefly, the chimeric sIL-11R–IL-2 protein was covalently linked to the activated carboxylated dextran matrix of the biosensor chip (CM5, BIAcore) via its primary amine groups, as recommended by the manufacturer. The coupling reaction was carried out for 7 min at a flow-rate of 5 μl/min at a sIL-11R–IL-2 concentration of 20 μg/ml and the chip was blocked with ethanolamine. A control chip was prepared by blocking carboxymethyl groups directly with ethanolamine. Concurrently, peptide 2 was covalently linked to another biosensor chip at a concentration of 2.3 μg/ml. Anti-IL-11R mAbs were allowed to bind sequentially to sIL-11R–IL-2 or peptide 2 in Hepes-buffered saline. Concentration (a) of mAb bound to sIL-11R–IL-2 or peptide 2 and the reaction rates (da/dt) are given by the BIAlogue software. Six different mAb concentrations (1, 2, 5, 10, 20 and 50 μg/ml) were run. Regeneration of the flow cells was achieved with 10 mM glycine HCl pH 1.8. Kinetic rate constants (k_{on} and k_{off}), as well as apparent

Lassen - Exhibit 1015, p. 5

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

WHAT WILL YOU BUILD? | sales@docketalarm.com | 1-866-77-FASTCASE

fastcase® Smarter legal research.