RESEARCH ARTICLE

Characterization of new anti-IL-6 antibodies revealed high potency candidates for intracellular cytokine detection and specific targeting of IL-6 receptor binding sites

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Accepted for publication June 04, 2018

To cite this article: Chouman K, Korioth-Schmitz B, Sack M, Schmitz JE, Pham AT, Fischer R, Barth S, Klockenbring T, Fendel R. Characterization of new anti-IL-6 antibodies revealed high potency candidates for intracellular cytokine detection and specific targeting of IL-6 receptor binding sites. *Eur. Cytokine Netw.* 2018; 29(2): 59-72 doi:10.1684/ecn.2018.0409

ABSTRACT. Interleukin-6 (IL-6) expression and secretion, induced by inflammatory processes, stimulate the acute phase response cascade. The overexpression of IL-6 contributes to a variety of inflammatory diseases, *e.g.* rheumatoid arthritis, Castleman's disease, multiple myeloma, and prostate cancer. Screening for high amounts of IL-6 in the patients' blood serum can be crucial for an adequate treatment. In this study, five novel murine monoclonal antibodies (mAbs) reactive to human IL-6 were generated. The mAbs were characterized for potential diagnostic purposes and recombinant antibodies were derived thereof. Initial epitope mapping using a combination of blocking experiments and Hyper-IL-6, a fusion protein consisting of IL-6 and the soluble IL-6 receptor revealed distinct but overlapping binding sites. At least one of the mAbs was found to interact with the region of IL-6/ IL-R complex formation. Three mAbs were applied successfully in intracellular staining by flow cytometry, whereas one of the mAbs showed comparable binding as a reference reagent. Furthermore, the mAbs were tested for applications in various immunological assays such as ELISA, Western blot and surface plasmon resonance spectroscopy (SPR), using IL-6 from commercial sources as well as in-house produced protein (IL-6_IME). The limit of detection was determined by sandwich ELISA (0.5 ng/mL, SD ±0.005). Our results also demonstrated that the recombinant IL-6 produced was functional and correctly folded. These findings support the use of the generated mAb clones as promising candidates for application in various immunological assays for diagnostic and scientific purposes.

Key words: interleukin-6, antibody characterization, intracellular detection

Interleukin 6 (IL-6) is an important inflammatory member of the cytokine superfamily. Originally, IL-6 was named B-stimulating factor (BSF-2) because the molecule, released by T lymphocytes, was shown to induce the final maturation of B cells into immunoglobulin-secreting cells [1]. Contrary to the initial assumption that BSF-2 was a humoral factor originating solely from T lymphocytes, IL-6 is actually produced by a wide variety of cell types such as lymphocytes, monocytes, macrophages, fibroblasts, and endothelial cells [2]. IL-6 molecules occur in multiple forms with different molecular masses varying from 20 to 30 kDa. The molecular heterogeneity is based on the glycosylation sites present or absent in the molecule, such as O-, N-, or non-glycosylation [3, 4]. However, these changes have no effect on the biological activity [5].

Particularly worth mentioning aspects are the pleiotropic activity of IL-6 and its key roles in modulating immune responses and regulating metabolic and regenerative processes under physiological as well as pathological conditions. While IL-6 is present at low concentrations in the serum of healthy individuals [6-8], inflammatory processes cause increased levels, as can be measured, *e.g.*, in the serum and synovial fluid of patients with rheumatoid arthritis [9, 10]. The overproduction of IL-6 in the long term leads to chronic inflammation, causing a wide array of chronic inflammatory diseases, like fibrosis, cardiovascular events, rheumatoid arthritis, inflammatory bowel disease, and others [11-13].

IL-6 also is an inflammatory serum marker that is well recognized as a biomarker for cardiovascular events so that it can be used, *e.g.*, as a predictor of ischemic stroke. It

doi: 10.1684/ecn.2018.0409

has been proposed as a tool for risk assessment in patients with atherosclerotic lesions of the carotid artery [14]. The ability to measure the activity of biological processes in peripheral blood during vascular or cardiovascular disease, including the progression or destabilization of atherosclerotic plaques, provides the advantage that patients can be closely monitored with a simple assay using peripheral blood, without the need for repeated invasive intravascular diagnostic techniques that carry higher risks for the patient's health [15].

Immunological assays play a key role in IL-6 diagnostics and therapeutic approaches. Within this study, several mAbs have been developed and recombinant formats have been derived thereof. In order to provide functional binding characteristics of the antibody, a recombinant human IL-6 protein has been produced in eukaryotic cells and used in various assays. Here, we are reporting on the basic characteristics of the mAb clones as well as quantitative and functional qualities in various immunological methods.

MATERIALS AND METHODS

Generation of monoclonal anti-IL-6 antibodies

Monoclonal antibodies against IL-6 were generated by hybridoma technology, as previously described by Puettmann *et al*. [16] with modifications. All animal experiments were performed in accordance with the requirements of the German Animal Welfare Laws (Tierschutzgesetz) and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (official file number 8.87-51.05.30.10.077). Briefly, six 6-8-week-old female BALB/c mice (Janvier, Saint-Berthevin, France) were immunized by subcutaneous route with 60 μg of recombinant human IL-6 produced in bacteria (IL-6) (GenScript, Piscataway, NJ, US) formulated with 40 μLGerbu MM adjuvant (GerbuBiotechnik, Heidelberg, Germany). For boost immunizations, the dose of IL-6 (GeneScript) was reduced to 30 μg, mixed in 20 μL adjuvant on days 14 and 21 or in phosphate-buffered saline (PBS) on days 28, 29, 30, and 32. Antibody titers in mouse serum collected from the immunized mice by tail vein bleeding were measured by ELISA. Briefly, 96-well high binding plates (Greiner, Frickenhausen, Germany) were coated with the antigen (50 ng/well at 4°C overnight and blocked with 1% (w/V) bovine serum albumin (BSA; Thermo Fisher Scientific, Waltham, MA, U.S.A.) in PBS (Thermo Fisher Scientific) for 1h at room temperature (RT). Cell culture supernatant was applied for 1h at RT. Detection of bound antibodies was carried out by peroxidase-coupled goat anti-mouse IgG (Fc specific) (GaM PO) (Jackson Immuno Research, West Grove, PA, U.S.A.) diluted 1:5000 and visualized by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany) using an ELISA Reader at 405 nm. When the serum IgG titer reached 1:100,000 the murine spleens were harvested.

For the generation of stable hybridoma cell lines, spleen cells were fused with the myeloma cell line Sp2/0Ag14 (ATCC CRL-1581) at a ratio of 1:1 on day-33. Cell fusion was induced by polyethylene glycol 1500 (PEG; Roche, Mannheim, Germany). Fused cells were cultured in RPMI 1640 (Thermo Fisher Scientific) containing hypoxanthine/aminopterin/thymidine (HAT) selection medium supplemented with 20% (v/V) bovine calf serum (Biochrom AG, Berlin, Germany), 100 U/mL streptomycin (Thermo Fisher Scientific), 100 μM hypoxanthine, 16 μM thymidine, and 0.4 μM aminopterin (Sigma-Aldrich, Taufkirchen, Germany), 80 U/mL murine IL-6 (Calbiochem, San Diego, USA) and 50 μM ß-mercaptoethanol (Thermo Fisher Scientific) in 96-well plates. Supernatants were analyzed with IL-6 (GeneScript) for anti-IL-6 antibody production 12 days after fusion by ELISA. Hybridomas in high responding wells (>1.0 O.D. 405 nm) were selected for limiting dilution and cultivated in HT medium (RPMI 1640 supplemented with 20% (v/V) bovine calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μM hypoxanthine, 16 μM thymidine, 80 U/mL IL-6 and 50 μM ß-mercaptoethanol). Monoclonal lines were expanded in standard growth medium (RPMI 1640 supplemented with 10% (v/V) bovine calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin) for 7 days. Stable cell lines were adapted to serum free hybridoma culture medium ISF-1 (Biochrom AG) and expanded in bioreactor flasks (CELLine Classic bioreactor flask, Sigma Aldrich, Germany) for bulk production of antibodies.

Generation of recombinant antibodies

Isolation of murine antibody sequences was performed as previously described by Kapelski *et al*. with minor modifications [17]. The V-gene regions were amplified with the forward primers for variable heavy (V_H) and variable light (V_L) chains [18] and with the reverse primers for variable heavy (V_H) and variable light (V_L) chains [19]. The amplified variable regions were cloned into pTT5 vectors expressing antibodies with the human IgG1 and kappa light chain constant domains as described before [20]. Antibody production was performed by transient transfection of HEK293-6E cells (obtained from the National Research Council Canada; HEK 293 expression platform L-10894/11266/11565) [21]. The transfection efficiency was monitored by simultaneously expression of enhanced green fluorescent protein (GFP) by co-transfection with the GFP containing vector pTT-o-GFPq. The cell culture supernatants were purified by Protein A chromatography.

IL-6 genetic sequence amplification and cloning

RNA was extracted from peripheral blood mononuclear cells (PBMC) using the RNeasy® Micro or Mini Kit (Qiagen). Total RNA was purified according to the manufacturer's instructions. Using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen), first strand cDNA was synthesized from the total RNA primed with oligo (dT) provided by the kit. The primers were designed based on the sequence in the database of the National Center of Biotechnology Information (NCBI) for human IL-6, transcript variant X2, mRNA (reference sequence XM_011515390.2). The sequence was amplified using the primers 5'-AGA TAC GAG CTA GCA TGA ACT CCT TCT CCA CAA GCG C-3' and 3'-ATT TAT CTC AGC GGC CGC CAT TTG CCG AAG AGC CCT CAG-5'.

The IL-6 gene fragment containing a His-tag was cloned into the pMS expression vector [22], using NotI and NheI as restriction enzymes. The recombinant construct was confirmed by Sanger sequencing carried out at the Fraunhofer IME sequencing facility using an ABI Prism 3700 Capillary-Sequencer (Applied Biosystems, Foster City, CA, USA) with BigDye^{TM} cycle sequencing terminator chemistry and the Applied Biosystems Sequencing Analysis Program. The sequences were analyzed with CLC Genomics Workbench software (Qiagen Informatics).

Protein expression using HEK-293T cells

HEK-293T cells were transfected using 1 μg of pMS plasmid DNA and 3 μL of RotiFect (Carl Roth GmbH; Karlsruhe, Germany), according to the manufacturer's recommendations. The transfected cultures were grown in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 50 μg/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 100 μg/mL Zeocin (components obtained from Invitrogen, Carlsbad, CA). To produce larger amounts of protein, triple flasks were inoculated and 500-1000 mL of supernatant was collected for purification.

Protein purification

Recombinant IL-6_IME was enriched by immobilized metal ion affinity chromatography (IMAC) on an Äkta Purifier System (GE Healthcare, Uppsala, Sweden) using IMAC Sepharose 6 Fast Flow as resin (GE Healthcare). After loading, the column was washed with wash buffer containing 10 mM imidazole until the baseline was reached. Elution was carried out at a flow rate of 1 mL/min using elution buffer (250 mM imidazole). If needed, the elution was concentrated using a Vivaspin Ultrafiltration Device 30 K (Sartorius, Göttingen, Germany). Finally, the elution fraction was dialyzed against PBS at 4°C overnight.

The mAbs were purified from hybridoma supernatants by protein A chromatography (ÄKTA FPLC systems, Amersham Biosciences, Freiburg, Germany) according to the manufacturer's recommendations, with 0.2 M Tris–HCl (pH 9.0) as binding and washing buffer, and 0.2 M sodium citrate (pH 2.7) as the elution buffer. Eluted fractions were immediately neutralized with 1 M Tris–HCl (pH 9.0). The mAbs were dialyzed against PBS overnight and adjusted to a concentration of 1 mg/mL and stored in 0.5 mL aliquots at -20°C.

Protein quantification

Densitometry

Samples of the respective protein as well as the reference protein BSA (Sigma Aldrich) were prepared for SDS-PAGE as described below. The gel was loaded with pre-stained broad range protein marker (kDa) (New England BioLabs, Frankfurt am Main, Germany) and 1, 2 and 5 μL of the analyte as well as with 0.1, 1.0, 2.0, and 2.5 μg BSA reference on a 12% SDS-PAGE. After electrophoretic separation, the protein bands were stained with Coomassie brilliant blue. Densitometric analysis was performed with Aida5 software (Advanced Image Data Analyzer, Ray test, Straubenhardt, Germany).

Bradford assay

Purified proteins were quantified using the Roti-Nanoquant (Roth, Karlsruhe, Germany) Bradford Assay according to the manufacturer's instructions. Serially diluted BSA (Sigma-Aldrich) at known concentrations was used to generate a standard curve. The final determination of protein concentration was performed with Microsoft Excel for Windows.

Mass spectrometry of IL-6_IME

The excised SDS-PAGE protein bands were digested with trypsin. Mass spectrometric analyses were performed with a Micromass Electrospray Q-Tof-2 mass spectrometer and Masslynx 5.3 software. From the obtained mass spectra, peptides were automatically selected for further fragmentation. The resulting MS/MS data were transformed into PKL files and transferred to the MASCOT search engine (Perkins *et al*., 1999, accessible at www.matrixscience.com). The search was carried out within the NCBI database.

Antibody conjugation

Selected IL-6 mAb clones (#1, #4, #6 and #8) were labeled with horseradish peroxidase (HRP; Roche, Basel, Switzerland cat. No. 11428861001) according to the manufacturer's procedure.

To reach a desired concentration of 4 mg/mL, the purified mAbs were spun using concentrating centrifugal devices (Vivaspin 30 kDa MWCO, Hydrosart membrane, Sartorius, Göttingen, Germany). For conjugation IgG and HRP were mixed at a molar ratio of 1:5 and incubated overnight at 4°C. The reaction was stopped with a mixture of 40 μL of 2 M triethanolamine solution pH 8.0 and 50 μL of 200 mM of sodium borohydride solution, and incubated for 30 min at 4°C. Then 25 μL of 2 M triethanolamine were added, followed by an incubation for 2 h at 4°C. Subsequently, 10 μL of 1 M glycine solution at pH 7.0 were mixed in for the stabilization of the conjugate. The coupling was transferred to a storage buffer and stored at 4°C.

FITC (fluorescein isothiocyanate) labeling

Antibody FITC conjugation was performed with the Pierce FITC Antibody Labeling Kit (cat. No. 53027, Thermo Fisher SCIENTIFIC) according to the manufacturer's instruction.

All steps were carried out at room temperature and protected from light. Briefly, antibody concentration was adjusted to 2 mg/mL as described above. 40 μL borate buffer (0.67 M) was added to 0.5 mL antibody solution. The antibody solution was added to the vial of FITC reagent and gently mixed until the dye was completely dissolved. The sample was spun down to the bottom of the tube and incubated for 1 h. Protein purification was carried out with the provided spin columns and collection tubes. After removal of the storage solution and equilibration of the resins with the provided suspension buffer by a first centrifugation step the labeling reaction was applied (250 μL per column each) and spun for 45 sat 1,000 × g. The purified protein samples were stored in 20 μL aliquots at -20°C.

Detection of IL-6 by direct enzyme-linked immunosorbent assay (ELISA)

For ELISA testing, 96-well high binding microplates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4°C with 50 ng/well of antigen (IL-6_IME) in 50 μL of 100 mM carbonate buffer (pH 9.5). Plates were washed three times with PBS containing 0.02% (v/V) Tween (PBS-T) between all steps. Subsequently, the plates were blocked with 100 μL/well of 2% (v/V) BSA IgG free (Invitrogen) for 1 h at RT. Anti-IL-6 mAbs (1:1000) or hybridoma supernatants were added for 1 h at RT. The binding of anti-IL-6 mAbs was detected by goat anti-mouse IgG antibody (peroxidase conjugated, H+L; Sigma-Aldrich). Tetramethylbenzidine (TMB; Invitrogen) was used for the colorimetric reaction. The reaction was stopped with 1 M HCl immediately after the reaction turned blue. Absorbance was measured at 450 nm on an ELISA reader (Biotek Instruments, Bad Friedrichshall, Germany). Measurements were performed in triplicates.

Sandwich ELISA

The 96-well high binding ELISA plates were coated with the respective IL-6 capture antibody at 100 ng/well in 100 mM carbonate buffer (pH 9.5) overnight at 4°C. Washing and blocking steps were performed as described above. The antigen incubation was carried out for 1h at RT. Anti-IL-6 antibody HRP conjugates (1:1000) were added and incubated for 1h at RT. Tetramethylbenzidine (TMB; Invitrogen) was used for the colorimetric reaction. The reaction was stopped with 1 M HCl immediately after the reaction turned blue. Absorbance was measured at 450 nm. The thresholds were set at two standard deviations (SD) above the average optical density (OD) value of the negative control (NC).

Surface plasmon resonance (SPR) analysis

The interactions between IL-6 variants and the mAbs were analyzed by SPR spectroscopy using a Biacore T200 biosensor (GE Healthcare). Polyclonal rabbit anti-mouse (RaM) Fc was immobilized to the surface of a CM5 sensor chip (GE Healthcare) using an antibody capture kit (mouse; GE Healthcare) according to the manufacturer's recommendations. The mAbclones #3, #4, #6 and #8 were diluted to 150 nM in HBS-EP buffer (GE Healthcare) and injected into the flow cell for 180 s at a flow rate of 30 μL/min using HBS-EP as the running buffer. Subsequently, the following antigens were added: IL-6_IME protein (produced as described above) at 40 nM, IL-6_reference also at 40 nM, Hyper-IL-6 fusion protein at 20 nM, or a buffer control. The biochip surface was regenerated with a 60 s pulse of 30 mMHCl after the measurements.

Mouse monoclonal anti-human Fc was immobilized to the surface of a CM5 sensor chip (GE Healthcare) using a human antibody capture kit (GE Healthcare) according to the manufacturer's recommendations. Next, 150 nM of the chimeric antibody clone #3 diluted in HBS-EP buffer (GE Healthcare) was injected into the flow cell for 180 s at a flow rate of 30 μL/min using HBS-EP as the running buffer. Subsequently, 40 nM of the hIL-6_IME was added. Then mAbs #4, #6 and #8 were injected in different orders at 150 nM for 180 s at a flow rate of 30 μL/min using HBS-EP as the running buffer. The surface was regenerated with a 60 s pulse of 3 M $MgCL_2$.

Human PBMC preparation and stimulation of monocytes with LPS

Blood samples for the isolation of PBMCs were kindly provided by the Centre of Transfusion Medicine/ Blood Donation Service of the University Clinic RWTH Aachen from volunteers giving blood donations. At the time of blood sampling all donors were healthy.

PBMC were isolated by density gradient centrifugation on Ficoll-Paque solution (GE Healthcare). Isolated PBMC were incubated at 37°C overnight on a microtiter plate at concentrations of 10^6 cells per well in RPMI 1640 medium (Sigma-Aldrich R-8005) with 10% (v/V) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 5×10^{-5} M 2-mercaptoethanol and antibiotics. To induce the expression of IL-6 in monocytes, lipopolysaccharides (LPS) from *Escherichia coli* (K 235, Sigma-Aldrich) were added to all wells at a final concentration of 5 μg/mL. The IL-6 content of the supernatant was quantified with the Human IL-6 Quantikine ELISA kit (R&D Systems Europe, LTD., Abingdon, UK) according to the manufacturer's instruction.

Intracellular IL-6 cytokine detection by flow cytometric analysis

For intracellular cytokine staining, PBMCs were isolated. Non-stimulated PBMCs and stimulated PBMCs with LPS were used as negative and positive controls respectively. Protein secretion was blocked using 10 μg/mL of brefeldin-A (Sigma-Aldrich) and incubated at 37 °C, 5 % CO_2 and 70 % humidity for 4 hours.

The cells were transferred to FACS tubes and fixed using BD cell fix (BD Bioscience, Heidelberg, Germany) for 40 min. Cells were permeabilized using PBS containing 3% (w/V) saponin (Merck Millipore, Darmstadt, Germany) for 15 min. For detection/differentiation of monocytes, cells were stained with anti-CD14-phycoerythrin (PE; Sigma-Aldrich; P5435-50TST) at an antibody dilution of 1:200. For subsequent intracellular IL-6 detection, the cells were incubated for 15 minutes with the commercial fluorescein isothiocyanate (FITC)-coupled anti-IL-6 antibody (eBioscience; MP5-20F3) or the murine antibody clone described here at an antibody dilution of 1:100. Between all steps, the cells were washed with PBS containing 0.5% (w/V) BSA and 2 mM EDTA and centrifuged at 300 *g* for 5 min. The staining was performed on ice in the dark. The cells were immediately analyzed by flow cytometry on a BD FACSVerse™, collecting 10,000 events per sample.

The staining index SI was calculated by the formula:

$$SI_{non-parametric} = median_{positive} - median_{negative} \times rDS^{-1}$$

$median_{positive}$ refers to the gated monocytesand $median_{negative}$ to the unstained lymphocyte population. *rSD* indicates the robust standard deviation.

RESULTS

Recombinant human IL-6 (subsequently called IL-6_IME) was produced in HEK239-T cells. The expression cassette of the used vector system (pMS) is shown in *figure 1A*. The cytokine was secreted into the cellular supernatant based on the Igκ-leader peptide. The cells express GFP

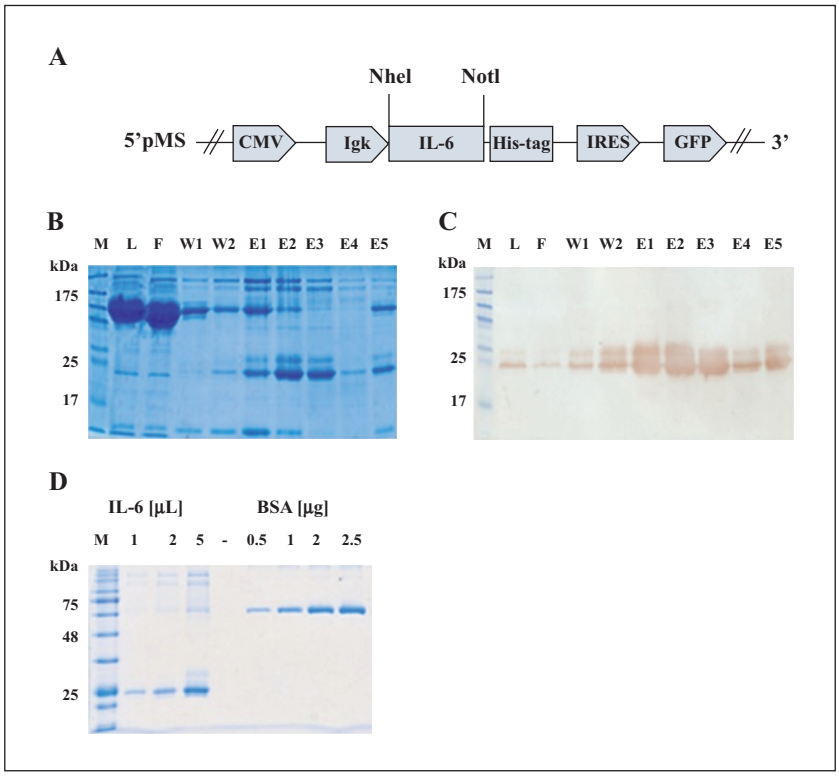

Figure 1

IL-6 cloning and expression in HEK 293 6E. (A) The pMS vector includes the recombinant human IL-6_IME (insert) fragment with an Ig kappa leader peptide, a c-terminal his-tag for purification, an internal ribosome entry site (IRES), GFP, and the selection markers zeocin and ampicillin. (B) The recombinant expressed IL-6_IME was purified by IMAC. The purity was assessed by SDS PAGE. (C) Recombinant protein was detected by Western blot using a mouse-anti-penta-His antibody (1:5,000; Qiagen, Germany) and a goat anti mouse IgG FC alkaline phosphate conjugate (Sigma-Aldrich, Germany). Pre-stained broad range protein marker (New England, BioLabs). Abbreviations: kDa: kiloDalton; M: molecular weight marker; L: load; F: flow; W: washing steps; E: elution steps. (D) Densitometrical protein quantification by SDS PAGE. Recombinant expressed IL-6_IME (1 µl, 2 µl, and 5 µl) was stained with Coomassie brilliant blue. Quantitative analysis was performed by Advanced Image Data Analyzer (Raytest) against the internal standard BSA (0.5 µg, 1 µg, 2 µg, and 2.5 µg).

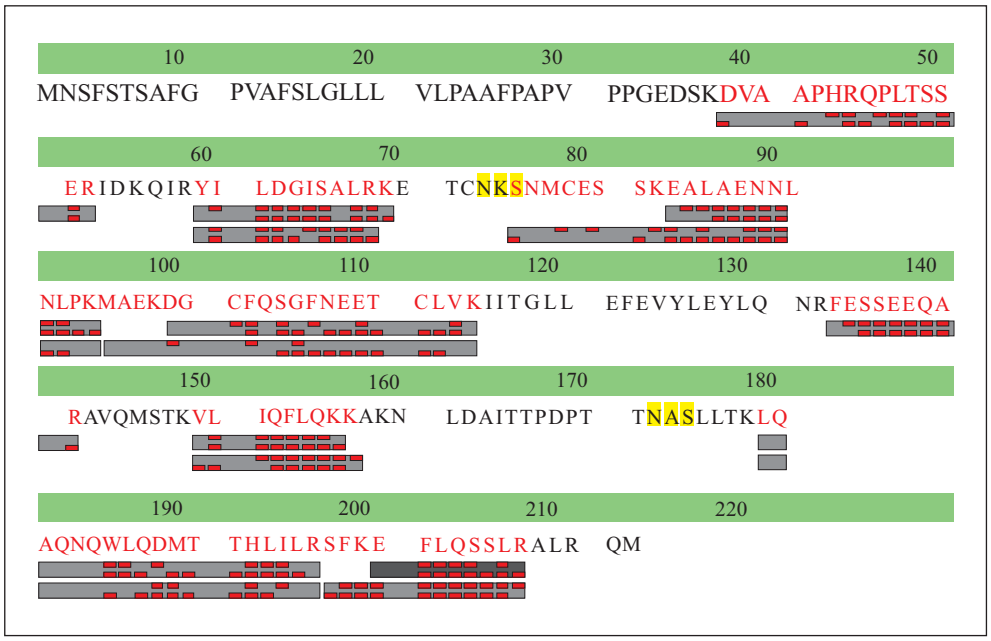

Figure 2

Mass spectroscopy of the recombinant human IL-6_IME. MS-identified amino acids compared with the sequences from NCBI are shown in red. Yellow marked positions indicate putative glycosylation sites.

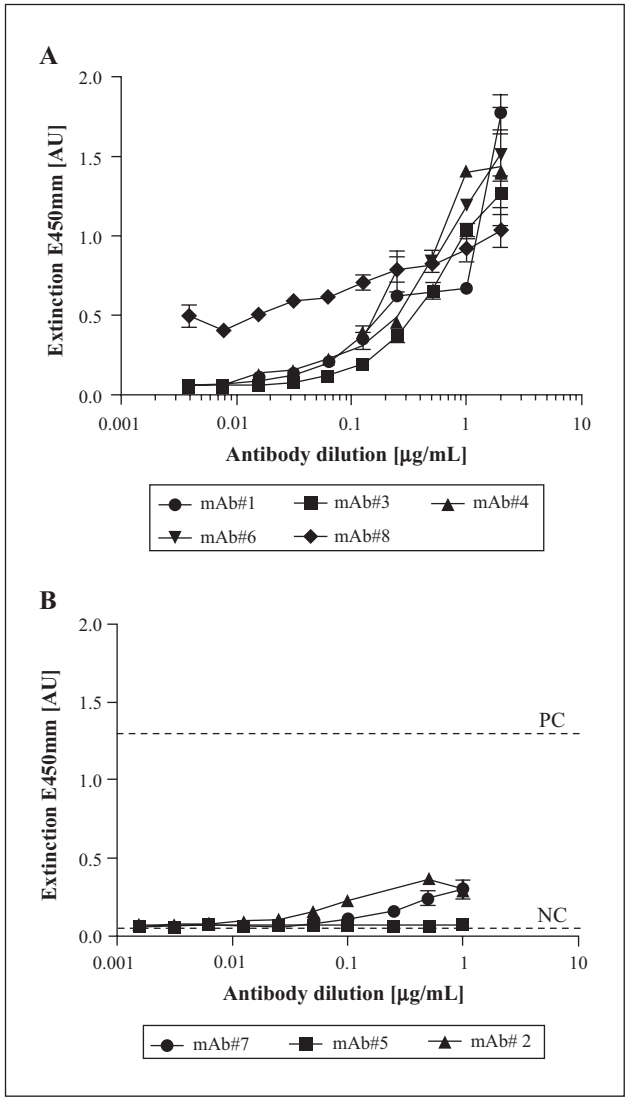

Figure 3
Antibody binding assays for IL-6 detection. (A) Direct ELISA with anti-IL-6 mAbs clones #1, #3, #4, #6, and #8. (B) Direct ELISA with anti-IL-6 mAbs clones #2, #5 and #7. In both graphs the serial dilutions of the hybridoma supernatants were plotted against the absorbance. PBS was used as negative control (NC) instead of the primary antibody. The anti-IL-6 mAb (Invivogen) was used as positive control (PC). Maximal OD of NC and PC are indicated by dashed lines. (n = 3 ±SD).

intracellularly in order to control for transfection efficiency and to allow the selection of positively transfected cells by flow cytometry.

High levels of GFP expressing HEK293-T cells were observed after 2 weeks of cultivation (>50%). One litre of HEK293-T cell supernatant was harvested and the his-tagged IL-6_IME was purified by immobilized metal affinity chromatography (IMAC). Elution fractions were analyzed by SDS-PAGE and Western blotting using the anti-his antibody (*figure 1B* and *C* respectively). The purification yielded approximately 5 mg/L of IL-6_IME after purification and buffer exchange. The recombinantly expressed IL-6_IME showed a size of 25 kDa. A second band at 30 kDa most likely represents the glycosylated isoform.

The concentration of IL-6_IME was determined bySDS PAGE using the program Aida5 (Advanced Image Data Analyzer) against the internal standard BSA (0.5 µg, 1 µg, 2 µg, and 2.5 µg). The concentration was 340 ng/ µL (*figure 1D*).

To further verify the protein identity, mass spectrometry analysis of the purified IL-6_IME protein was carried out. The result showed an amino acid sequence coverage of 53.3% compared to the human IL-6 sequence from the GenBank (*figure 2*). 14 peptides were matched and reached a MOWES score of 824.9.

Murine mAbs

mAbs against the recombinant human IL-6 (GenScript) were generated by hybridoma technology. By limiting dilution, eight monoclonal cell lines (clones #1-#8) were isolated. The cell lines were expanded, and supernatants were tested for confirmation of specific binding to IL-6 by direct ELISA. The antibody clones #1, #3, #4, #6 and #8 showed efficient binding and were selected for further investigations (*figure 3A*). In contrast, binding to IL-6 could not be confirmed for the mAbs #2, #5 and #7 and thus these were excluded from further investigations (*figure 3B*).

The supernatants from the monoclonal cell lines were purified by Protein A chromatography, and mAbs were analyzed by SDS-PAGE (*figure 4A*). Binding to denatured IL-6_IME was tested by using Western blot (*figure 4B*). Interestingly, all mAbs similarly recognized IL-6_IME in Western blot.

An aliquot of each antibody was conjugated to horseradish peroxidase, as a prerequisite for the setup of a sandwich assay. Different antibody combinations were tested by sandwich ELISA to find out the best working antibody pairings and to determine the limit of detection (LOD) for IL-6. Among the mAbs tested, the combination of the mAb clone #3 for capturing the antigen and the mAbs #1, #4 and #6 coupled with HRP for detection had the lowest LOD with mean values ± SD of 0.8 ±0.005 ng/mL, 0.8 ±0.015 ng/mL, and 0.5 ±0.013 ng/mL, respectively (*figure 5A, B*). The mAb #8 HRP conjugate exhibited a slightly higher LOD of 1.6 ±0.003 ng/mL. When mAbs clone #8 was used as a capture antibody the remaining mAbs failed completely to work as detection antibodies, except for mAb clone#6 which showed a LOD of 6.0 ±0.024 ng/mL.

Production of recombinant mAbs

Recombinant mAbs offer advantages over native antibodies regarding modifications and adaptions for further applications in immunoassays and therapy. In this study, recombinant formats were derived from murine hybridomas by cloning the heavy and light chain V genes 5'- of a human IgG1 heavy chain constant domain or a human Ig kappa constant domain, respectively. HEK293-6E cells were co-transfected with the pTT5 vector containing the chimeric murine Vh-human IgG1 heavy chain construct and a corresponding pTT5 vector with the light chain for each antibody clone (#3, #6 and #8), and intracellular production of GFP to control for transfection efficiency. High levels of GFP-expressing HEK cells (>50%) were observed 4 days post-transfection. One litre of supernatant of each of the chimeric mAbs from the HEK293-6E cells was harvested and purified by protein A chromatography. Samples from the supernatant, were washed, and elution steps were analyzed by SDS-PAGE (*figure 6A*) and

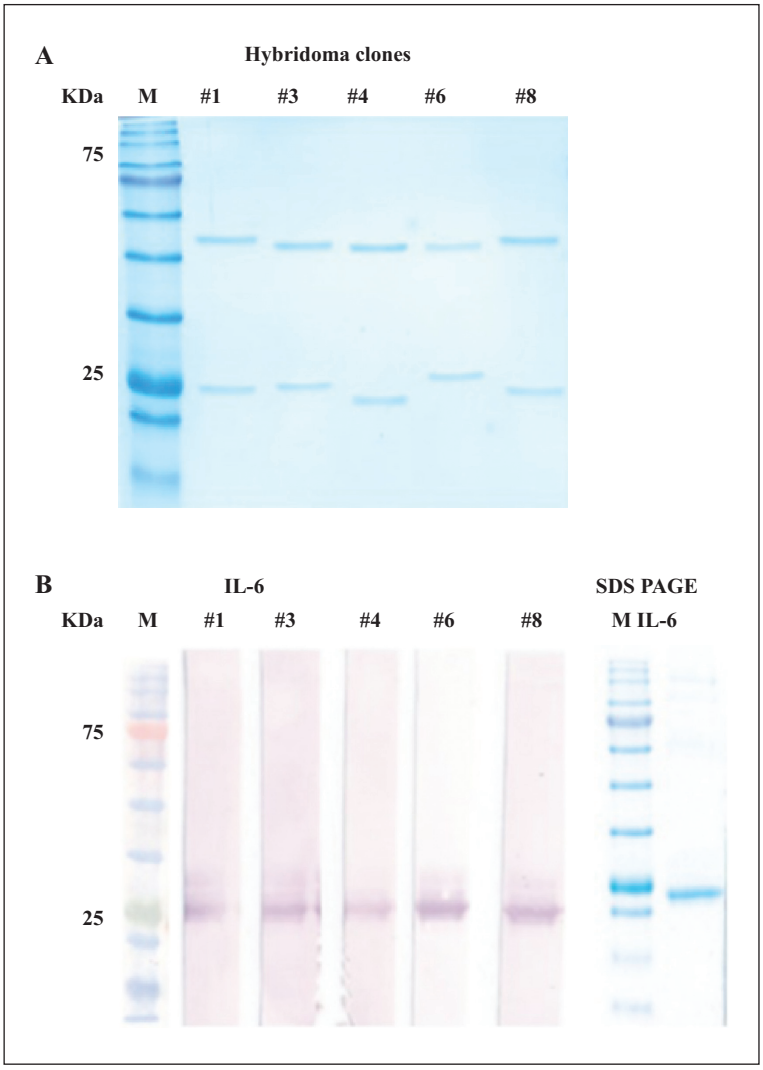

Figure 4

SDS PAGE and Western blot of Protein A purified mAb clones #1, #3, #4, #6 and #8. (A) SDS PAGE of purified mAbs. The heavy chain band appears at 50 kDa and the light chain band at 25 kDa (B) Western blot detection of IL-6_IME by mAbs clones #1, #3, #4, #6 and #8. M: molecular weight marker.

Western blot (*figure 6B*). The yield of each chimeric antibody after purification and buffer exchange amounted to an average of about 0.02 g/L. Binding was tested by ELISA (data not shown) and for chimeric clone #3 also by SPR analysis and intracellular cytokine staining (see below).

SPR analysis

SPR analyses were performed to investigate the binding of various IL-6 variants to the mABs, and for gaining initial information about the antibody epitopes.

Clone #1 failed to bind to soluble IL-6 (data not shown), which is in contrast to the reactivity observed in ELISA and Western blot . A possible explanation might be that this antibody may recognize an epitope only exposed in the coated and denatured IL-6 protein. The other mAbs #3, #4, #6 and #8 bound IL-6_reference and IL6_IME in a similar manner and exhibited distinct binding behaviour. The mAb clone #6 had the fasted association rate but also exhibited a lower activity. Interestingly, mAb #4 exhibited a faster dissociation rate and did not bind to Hyper-IL6, whereas mAbs #3, #6 and #8 all exhibited a slow dissociation rate and binding to Hyper-IL-6. Hyper-IL-6 is a recombinant fusion protein that combines the alpha chain of the IL-6 receptor and IL-6 connected by a flexible peptide linker. This construct mimicks the biological features of the native IL6/sIL6 receptor complex [23]. Thus, we deduce from the binding profile of mAb #4 that its epitope overlaps with the binding site of IL-6 to the alpha chain of IL-6R. Likewise, the epitopes of mAbs #3, #6 and #8 do not overlap with the binding site for IL-6R (*figure 7*).

Additional SPR analysis was performed to determine overlapping regions between the described mAbs by multi-binding assays. For this purpose, the chimeric antibody #3 was used. The experimental sequence of the competition approach starts with the capturing of the chimeric clone #3 by a mouse anti-human-Fc antibody, which had been covalently conjugated on the chip surface. In a second step, IL-6_IME was added, followed by injection of the murine mAbs#4 (Box 1), #6 (Box 2) and #8 (Box3), (*figure 8*). Each of the mAbs bound to the preformed chimeric clone #3-IL-6_IME complex, demonstrating that the epitope of the chimeric clone #3 did not overlap with those of mAb clones #4, #6 and #8. Next, all of these sandwich

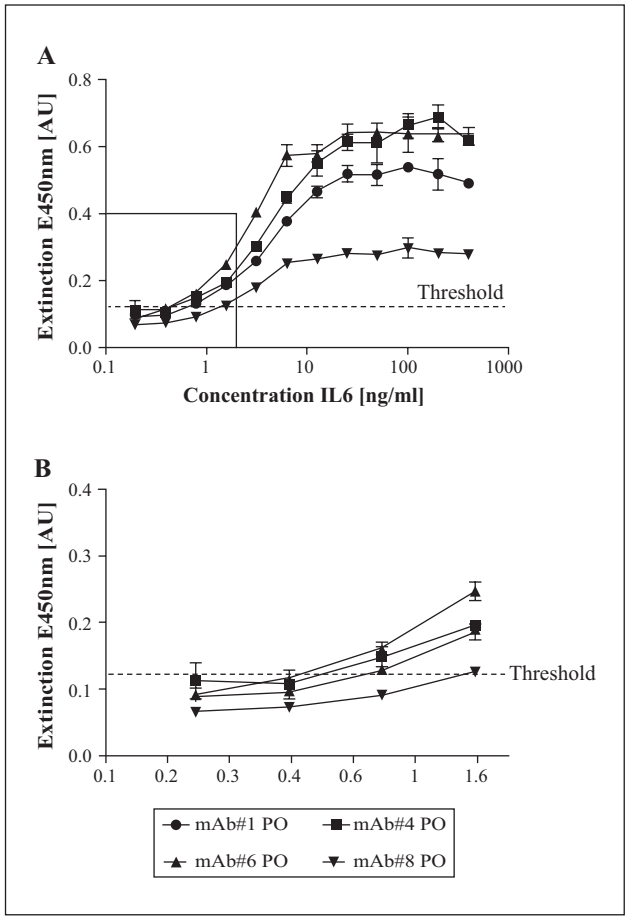

Figure 5
Determination of the limit of detection for IL-6. (A) Serial dilutions of IL-6_IME were assayed by sandwich ELISA to compare the detection limits of various sandwich antibody combinations. Antibody clone # 3 was used as capture antibody. HPR conjugates of mAb clones #1, #4, #6 and #8 were applied as secondary antibody. The dashed line indicates the threshold (NC+ 2 times SD). (B) Enlarged section corresponding to the box in *figure 5A* ($n = 3 \pm$SD).

complexes were probed with the remaining antibody clones. At this stage, the multiple binding assay revealed overlapping binding sites between mAb clones #4 and #6 but not with mAb #8. mAb #6 again showed the fasted association rate. These results demonstrate that all four antibodies recognize unique epitopes on the IL-6 protein (*figures 8 and 9*).

Intracellular IL-6 staining

Intracellular cytokine staining was performed to detect IL-6 prior to secretion, demonstrating the applicability of the mAbs for intracellular staining in flow cytometric assays. The mAbs were tested in a monocyte population of LPS-treated human PBMCs.

The intracellular detection assay was performed with antibody clones #3, #6 and #8-fluorescein isothiocyanate (FITC) conjugates in comparison to a commercially available anti-IL-6-FITC reference antibody from eBioscience. Human PBMCs were subjected to LPS overnight to induce high expression levels of IL-6. The IL-6 concentration of the supernatant was 0.19 ng/mL (data not shown). Untreated PBMCs were used as negative control.

After blocking the cytokine secretion by brefeldin A, PBMCs were fixed and permeabilized. The cells were stained with anti-CD14-PE and anti-IL-6-FITC antibody conjugates. $CD14^+$ cells were displayed in the forward/side scatter graph by back gating to reinsure co-localization of the putative monocyte region. The $CD14^+$ fraction was referred as monocyte population and used for analysis of IL-6 detection. IL-6 unrelated background fluorescence was determined by FITC labeled isotype antibodies. This population was demarcated by the upper left sector (*figure 10B*). Anti-IL-6 antibody induced shifts into the upper right quadrant that indicates the detection signals of intracellular IL-6. Positive control was established by an anti-IL-6 reference antibody (eBioscienceThermo Fisher Scientific).

The dot plots show the percentage of monocytes stained with the anti-IL-6-FITC mAbs. The plots D, E, and F (*figure 10*) show the percentage of monocytes stained with the mAb clones #3 (84.75%), #6 (50.85%), and #8 (31.19%), respectively. Staining indices (SI) were calculated for each the mAbs which were 11.97 for the anti-IL-6-FITC reference, 11.65 for mAb clone #3, 9.75 for mAb clone #6,6.81 for mAb clone #8 and 5.30 for the isotype control, respectively. mAb clone #3 performed nearly as good as the commercial reference antibody, followed by mAb clone #6 whereas the binding of mAB clone #8 was much weaker.

Table 1 summarizes the binding analysis for the chimeric antibodies and mAbs as well as the overlapping epitope and intracellular detection experiments using the monoclonal cell lines.

DISCUSSION

In the present study, eight murine mAbs raised against human IL-6 were evaluated and corresponding recombinant chimeric antibodies were generated. Besides the determination of basic binding properties, the applicability for different immunological assays was investigated. Furthermore, recombinant human IL-6 protein was produced and checked for functionality.

The anti-IL-6 mAbs were tested by ELISA and Western Blot. The mAbs were successfully applied in sandwich assays such as ELISA and SPR analyses, showing accessibility to different epitopes of IL-6. The mAbs also reacted with native IL-6 when tested with supernatants of LPS-treated human PBMCs by intracellular cytokine staining. These results in combination with the comparative SPR analysis of other IL-6 reference proteins (IL-6_reference and Hyper-IL-6) confirm the identity and proper folding of the in-house produced recombinant IL-6 protein as well as the binding specificity of the mAbs. This is an important verification before progressing *e.g.* to the generation of mutants for epitope fine mapping.

The binding properties to IL-6_IME protein did not significantly vary from IL-6_reference. Minor variations can be explained by conformational and posttranslational variations [3, 24], *e.g.*, by differences in the glycosylation of the expression systems used (IL-6_reference *E. coli versus* in-house produced IL-6 in HEK 293-T cells). Because of the human origin of HEK cells, production of recombinant proteins feature glycosylation patterns close to native

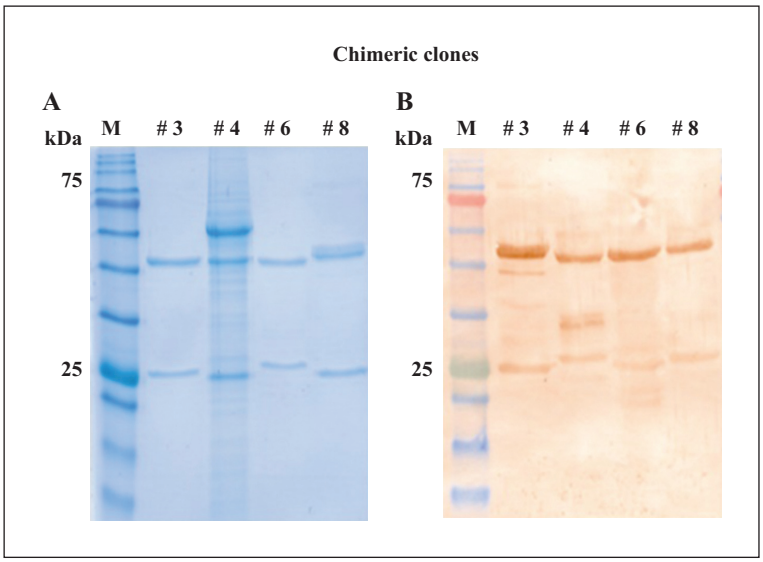

Figure 6
Expression of chimeric anti-IL-6 mAbs. (A) SDS-PAGE of purified chimeric anti-IL-6 antibody clones #3, #6 and #8. SN: supernatant; W: wash; Elu: elution; M: molecular weight marker (B) Corresponding Western blot to (A). Detection was performed using a rabbit anti-human IgG Fc conjugated to peroxidase. (A) SDS-PAGE of the purified chimeric antibody clones #3, #6 and #8 and the supernatant with chimeric clone #4 (B) Corresponding Western blot to (A). Detection was performed using a rabbit anti-human IgG Fc conjugated to peroxidase.

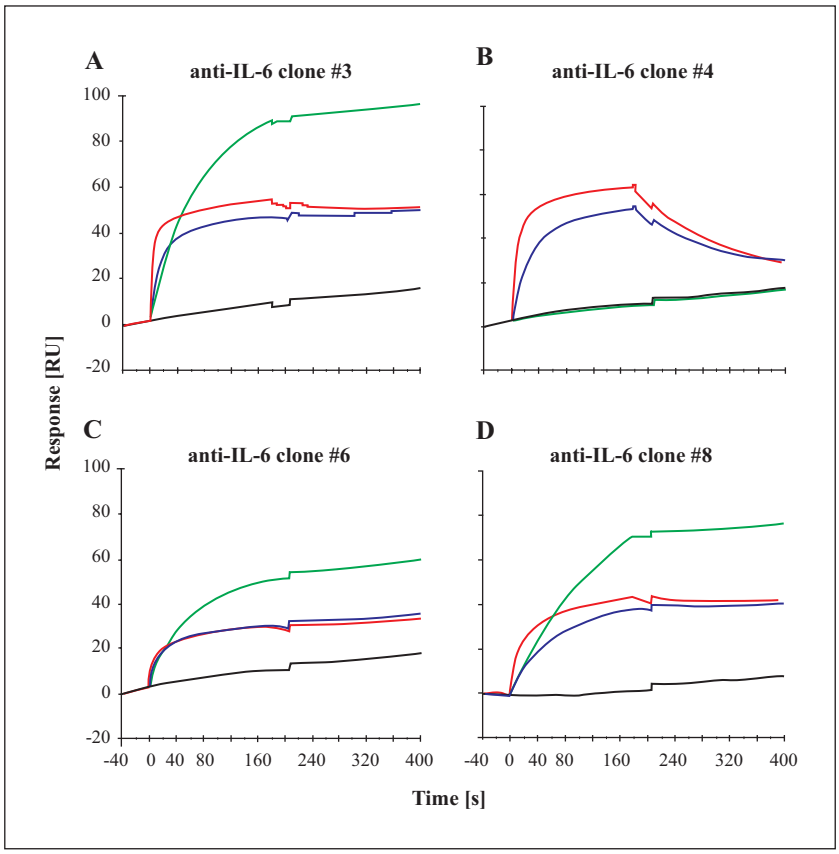

Figure 7
SPR binding of various IL-6 isoforms. Anti-IL-6 mAbs were captured onto a rabbit anti mouse antibody surface followed by injection of IL-6_IME (red line), IL-6_reference (blue line), and hyperIL-6 (green line) and HRP buffer (grey line, negative control). (A) mAb clone #3. (B) mAb clone #4. (C) mAb clone #6. (D) mAb clone #8.

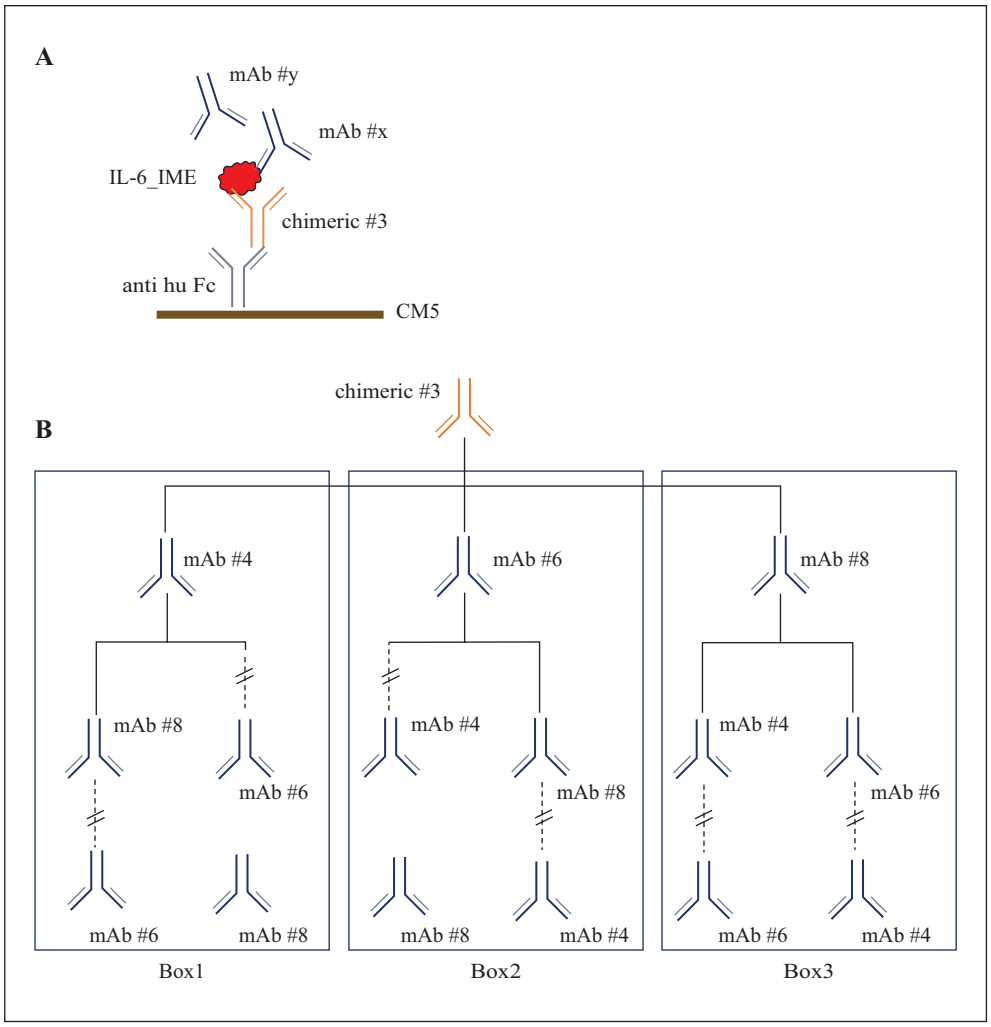

Figure 8
SPR competition experiment. (A) Scheme of the experimental setup. (B) Combinatorial scheme of the antibody competition for IL-6 binding and overview of the outcome of the experiment. Chimeric capture antibody #3 is bound by hu-Fc on the CM5 chip surface. Then IL-6 is added (not indicated in B), followed by three different sequences for mAbs #4, #6 and #8 (Box 1, 2, 3, left and right branch).

human glycosylation characteristics, in contrast to bacterial expression.

To assess the applicability and performance of the mAbs for detection assays, the detection limit of IL-6 was determined by sandwich ELISA. The combination of mAb clone #3 as capture and clone #6 as detection mAbs was the best performing sandwich pair, reaching a limit of detection (LOD) of 0.5 ng/mL. As the levels of IL-6 in the serum of healthy individuals are very low, around 7 to 10 pg/mL [7], further optimization or migration to other assay platforms, such as the Single Molecule Array (SiMOA) or Luminex technology, are required. In this respect mAb #6 seems to have favorable properties. However, in the early phase of inflammatory process IL-6 serum levels can increase by factor 10^5 [25]. Although the conditions did not reach the single-digit pg/mL sensitivity of the commercial kits, this range allows the detection of high IL-6 serum amounts in patients with chronic inflammation or sepsis. Besides blood serum, other fluids containing IL-6 can be used to detect early phases of inflammatory diseases to accelerate the start of treatment. Wimmer *et al.* [26] measured the concentration of IL-6 in synovial fluids using point-of-care testing (POCT) with a mean of 0.4 ng/mL for the aseptic cases. To evaluate acute inflammation in amniotic fluid, Chaemsaithong and coworkers tested another POCT device, assuming 0.7 ng/mL of IL-6 in the fluid as cut off for positive acute inflammation [27]. Feng *et al.* [28] emphasized the relevance in the use of IL-6 as biomarker for sepsis detection. The detection of high levels of IL-6 cytokine can be decisive for the treatment of the patient.

Epitope related features were analyzed by SPR and competitive SPR analysis. Interestingly, all antibodies but anti-IL-6 mAb clone #4 were shown to bind to Hyper-IL-6. This led to the assumption that the site of the clone #4 epitope interferes with the binding site of the IL-6 receptor moiety. Similar findings on different epitopes by binding analysis were made by Liautard [29]; they identified two different epitopes involved in IL-6 binding in a group of several mAbs. The chimeric variant of mAb anti-IL-6 clone #3 was used as capture antibody, and all other antibodies were able to bind to the captured IL-6. In subsequent multiple binding events only mAbs #4 and #6 exhibited crosswise blocking. These experiments clearly showed that

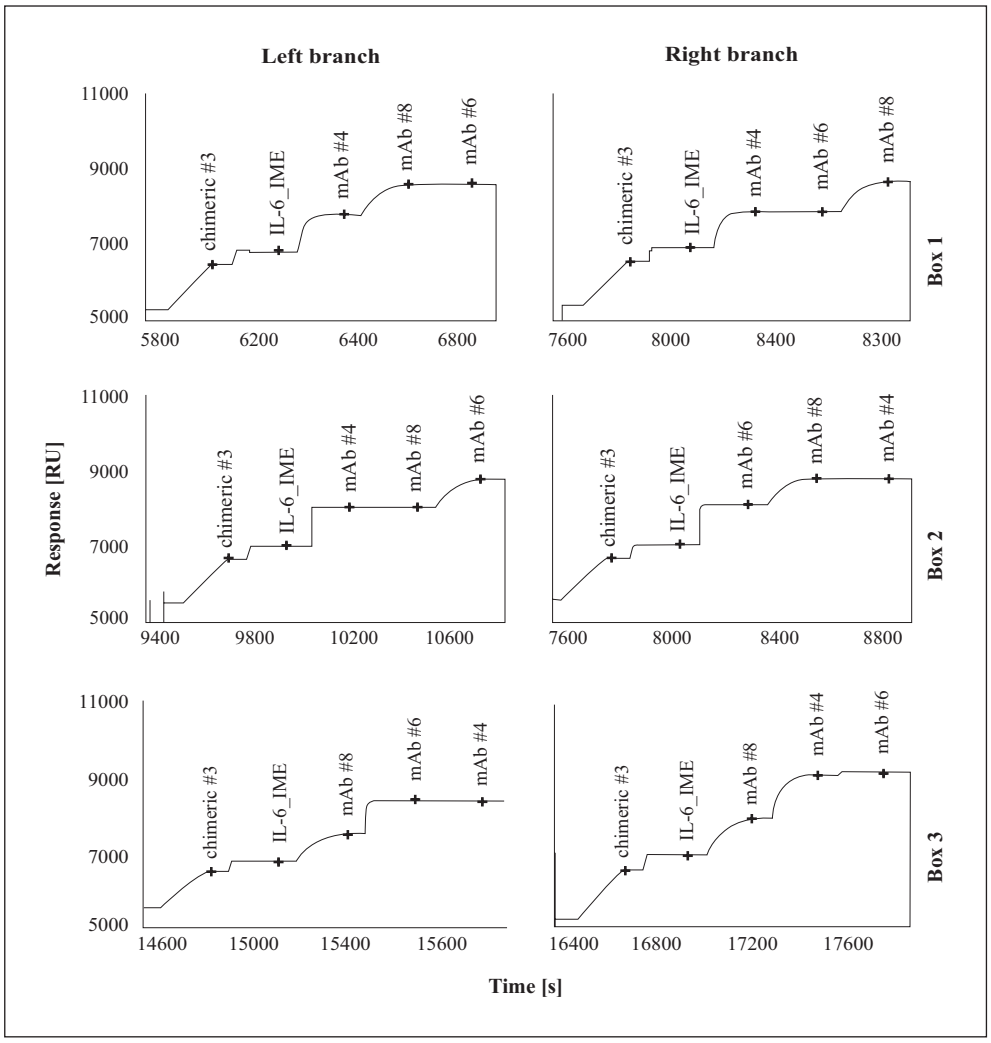

Figure 9

SPR sensograms of the antibody IL-6 competition experiment. The sensograms show binding (shift) or non-binding (no shift). Box 1, 2, 3, left branch and right branch refer to *figure 9*. Black dots indicate the time points of steady state level.

all mAbs recognized distinct epitopes of which only those for mAb #4 and #6 were overlapping.

The use of SPR kinetics to select promising mAbs according to the binding was used in a similar approach by Karlsson and coworkers [30].

Further investigation is needed to determine the epitopes precisely, *e.g.* by epitope fine mapping using point mutants of IL-6, competition experiments using other IL-6 antibodies, namely, Siltuximab, Tocilizumab, and Rituximab, etc. [31], and to investigate antibody effects on IL-6 receptor related interactions including Hyper-IL-6 and gp130 [32]. Moreover, the ability of mAbs to interfere with the IL-6 signal transduction is the most important requirement for antibody based therapeutic approaches. Kalai *et al.* [33] identified several epitopes within the binding sites of the IL-6R α, the gp130/ß1 and the gp130/ß2. From the data presented here, it can be assumed that the mAb #4 and #6 could inhibit the formation of the Il-6/ IL-6-receptor complex. Further an epitope analysis should also address interferences with gp130 binding sites. To identify candidates for clinical applications blocking and/ or neutralizing activities should be investigated for the whole set of mAbs by appropriate cell culture assays. The recombinant antibody formats that we have at hand now will help to modulate any promising candidate for clinical approaches.

The detection of intracellular IL-6 is an important tool to analyze the cytokine levels in activated monocytes, prior to the secretion. Proinflammatory monocytes can be recruited from reservoirs in the spleen, migrating to the inflammation sites far apart [34].

The applicability of the mAbs for intracellular detection of IL-6 was tested by flow cytometric analysis. The anti-IL-6 mAb clone #3 FITC performed very well, also in comparison to the reference antibody that was included as positive control. Therefore, we consider this IL-6 mAb clone as a suitable candidate for the development of diagnostic assays and research applications using intracellular staining of IL-6.

Additional investigations on the interactions of the generated anti-IL-6 mAbs on IL-6 receptor-mediated signaling processes by cell culture based *in vitro* assays may shed further light on the functionality of the mAbs.

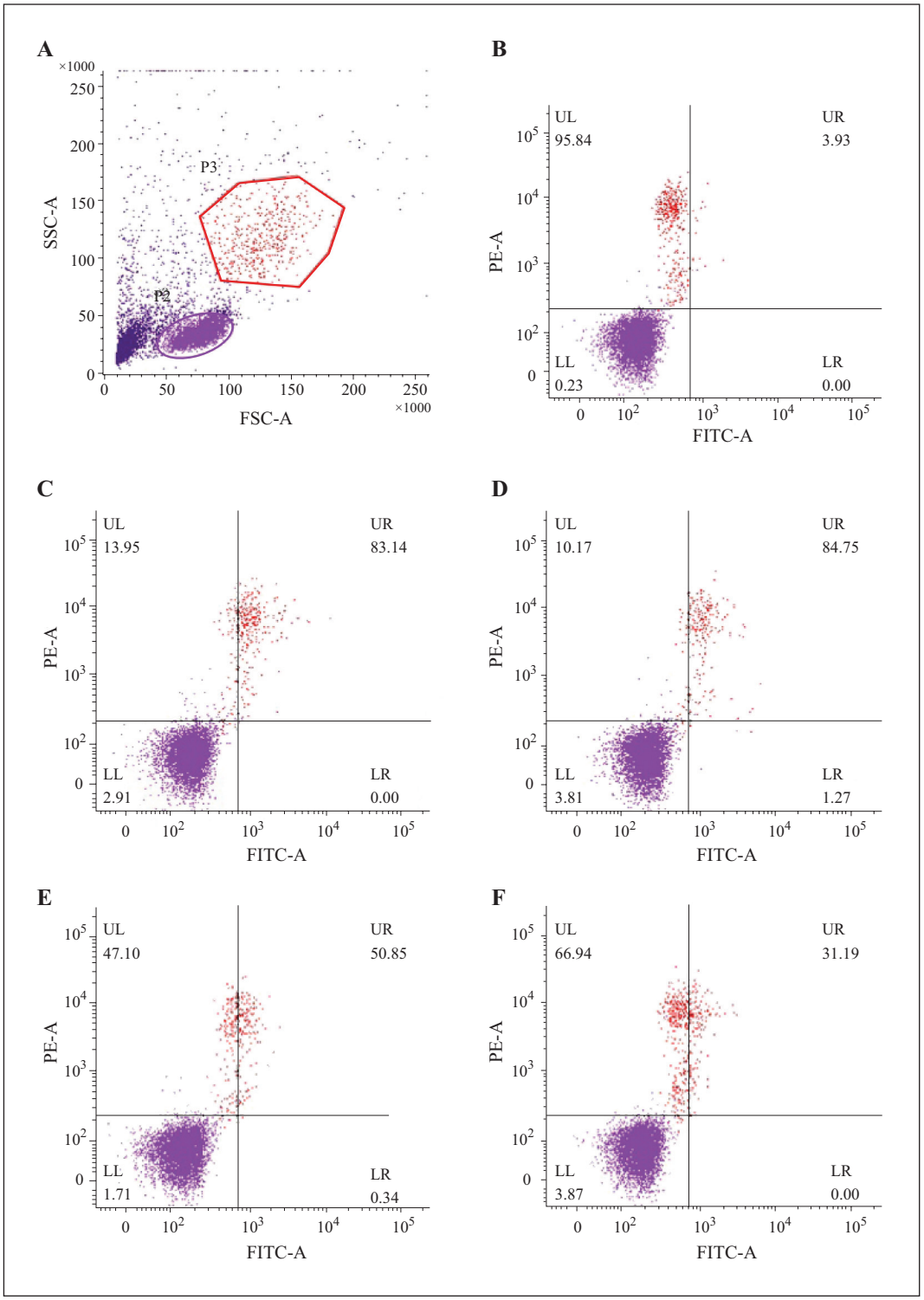

Figure 10

Flow cytometric analysis of intracellular IL-6 detection. PBMCs were treated with LPS overnight. Cells were prepared for intracellular staining by Brefeldin A fixation and Saponinpermeabilization and subsequently subjected to anti CD14-PE and various anti IL-6 antibody-FITC conjugates (A) Gating of lymphocytes (purple) and CD14+ monocytes population (red). (B) CD14+ cells and IgG FITC isotype control. (C) CD14-PE and anti-IL-6 FITC reference antibody (eBioscience). (D) CD14-PE and anti-IL-6 clone #3 FITC. (E) CD14-PE and anti-IL-6 clone #6 FITC. (F) CD14-PE and anti-IL-6 clone #8 FITC.

AUTHOR CONTRIBUTIONS

Chouman K, Korioth-Schmitz B, Sack M, Klockenbring T, Fendel R, Schmitz JE and Anh-Tuan Pham performed all the experiments, analyzed the data, and wrote the manuscript. Fischer R, Barth S; Klockenbring T, Fendel R organized the entire project, analyzed the data, and edited the manuscript. All authors reviewed the manuscript.

Acknowledgments. *This work was supported by the CNPq foundation (National Council for Scientific and Technological Development, Brazil; scholarship program Science without*

Table 1
Summary of the characterization of anti-IL-6 mAbs.

Clone	Hybridoma clones	Chimeric formats	Direct ELISA	Western blot	SPR IL-6	SPR Hyper-IL6	Epitope overlapping	Intracellular IL-6 detection (FACS)
#1	+	ND.	+	+	-	-	/	ND
#3	+	+	+	+	+	+	/	++
#4	+	ND.	+	+	+	-	#6, Hyper-IL6	ND
#6	+	+	+	+	+	+	#4	+
#8	+	+	+	+	+	+	/	+/-

+: binding; -: no binding; /: no overlapping; ND: not done.

borders to K.C.) and by funds of the Fraunhofer Society (Department project). We would like to thank Ivana Chudobova for the mass spectroscopy analysis and Nicole Kündgen-Redding, Gudrun Breuer, Severin Schmies, Reinhard Rosinkeand Kai Fuhrmann for technical assistance. Recombinant IL-6_reference and Hyper-IL-6 were obtained from Prof. Jürgen Scheller (University of Düsseldorf, Germany).

Disclosure. *Financial support: none. Conflict of interest: none.*

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