Abrogation of Viral Interleukin-6 (vIL-6)-Induced Signaling by Intracellular Retention and Neutralization of vIL-6 with an Anti-vIL-6 Single-Chain Antibody Selected by Phage Display

Marina Kovaleva,^{1,†‡} Ingo Bussmeyer,^{1,†} Björn Rabe,¹ Joachim Grötzinger,¹ Enge Sudarman,² Jutta Eichler,² Udo Conrad,³ Stefan Rose-John,^{1*} and Jürgen Scheller¹

Department of Biochemistry, Christian Albrechts Universität, Kiel, Germany¹; Gesellschaft für Biotechnologische Forschung GmbH, Braunschweig, Germany²; and Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK), Gatersleben, Germany³

Received 28 February 2006/Accepted 7 June 2006

Human herpesvirus 8 (HHV-8) encodes several putative oncogenes, which are homologues to cellular host genes known to function in cell cycle regulation, control of apoptosis, and cytokine signaling. Viral interleukin (vIL-6) is believed to play an important role in the pathogenesis of Kaposi's sarcoma as well as primary effusion lymphoma and multicentric Castleman's disease. Therefore, vIL-6 is a promising target for novel therapies directed against HHV-8-associated diseases. By phage display screening of human synthetic antibody libraries, we have selected a specific recombinant antibody, called monoclonal anti-vIL-6 (MAV), binding to vIL-6. The epitope recognized by MAV was localized on the top of the D helix of the vIL-6 protein, which is a part of receptor binding site III. Consequently, MAV specifically inhibits vIL-6-mediated growth of the primary effusion lymphoma-derived cell line BCBL-1 and blocks STAT3 phosphorylation in the human hepatoma cell line HepG2. Since it was previously found that vIL-6 can also induce signals from within the cell, presumably within the endoplasmic reticulum, we fused the recombinant antibody MAV with the endoplasmic retention sequence KDEL (MAV-KDEL). As a result, COS-7 cells expressing MAV-KDEL and synthesizing vIL-6 ceased to secrete the cytokine. Moreover, we observed that vIL-6 that was bound to MAV-KDEL and retained in the endoplasmic reticulum did not induce STAT3 phosphorylation in HepG2 cells. We conclude that the activity of the intracellularly retained vIL-6 protein is neutralized by MAV-KDEL. Our results might represent a novel therapeutic strategy to neutralize virally encoded growth factors or oncogenes.

All members of the hematopoietic interleukin-6 (IL-6) cytokine family exert their biological effects via binding to a receptor homodimer of gp130 or a heterodimer of gp130/leukemia inhibitory factor receptor (LIFR), gp130/oncostatin M receptor (OSMR), and gp130/IL-27 receptor (WSX-1). gp130 as well as LIFR, OSMR, or WSX-1 are the signal transduction receptors (β receptors) activated by binding of the cytokine. Some cytokines (IL-6, IL-11, IL-27, and ciliary neurotrophic factor [CNTF]) cannot bind to β receptors unless they first form a complex with their specific ligand-binding α receptor (IL-6-R, IL-11-R, Epstein-Barr virus-induced protein 3 [subunit of IL-27], and CNTF-R) (62, 72, 76).

Human IL-6 first binds to a cell surface receptor (IL-6-R) and thereupon associates with the signal-transducing protein gp130. Dimerization of gp130 leads to the activation of kinases of the JAK family and of the STAT1 and STAT3 transcriptional activators (19, 76).

A naturally occurring soluble form of the IL-6-R (sIL-6-R) was found in various body fluids (27, 50). sIL-6-R together with IL-6 can stimulate cells that express only gp130 (38, 76), which was named transsignaling (60, 68). Furthermore, it was shown

that the sIL-6-R strongly sensitizes IL-6-responsive target cells (59). transsignaling cell types that are exclusively responsive to IL-6/IL-6-R but not to IL-6 alone include hematopoietic progenitor cells (61), endothelial cells (67), osteoclasts (77), smooth muscle cells (32), and neuronal cells (39, 40).

Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus, was first identified by Chang et al. (12) in Kaposi's sarcoma tissues of AIDS-infected persons and appears to be the first member of the human gammaherpesviruses (rhadinoviruses). HHV-8 is linked to the development of Kaposi's sarcoma as well as primary effusion lymphoma (PEL) (11, 47) and multicentric Castleman's disease (MCD) (53, 74). It has also been associated with multiple myeloma (63, 65). Similar to other human herpesviruses, HHV-8 has a particular cell tropism, infecting predominantly endothelial and other mesenchymal cells as well as B lymphocytes.

Viral IL-6 (vIL-6) (48) encoded by HHV-8 is a structural homologue of human IL-6 (huIL-6) and murine IL-6 and affects the cells via binding to the signal transducer gp130 (26). Although cellular and viral IL-6 proteins show only ~25% sequence homology, the crystal structure of a receptor complex demonstrated a strong relationship of the binding epitopes to their receptors (15, 44). vIL-6 protein folds, as most cytokines do, into so-called four-helical bundles (7, 23).

Viral IL-6 mimics a number of huIL-6 activities such as stimulation of IL-6-dependent B-cell-line growth (9) and activation of the JAK/STAT signal transduction pathway in HepG2 cells (44). There is still debate about the involvement of the IL-6-R in cellular responses to vIL-6. Whereas vIL-6

* Corresponding author. Mailing address: Department of Biochemistry, Christian Albrechts Universität zu Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany. Phone: 49-431-880 3336. Fax: 49-431-880 5007. E-mail: rosejohn@biochem.uni-kiel.de.

† M.K. and I.B. contributed equally to this work.

‡ Present address: North-West Cancer Research Fund Institute, University of Wales, Bangor, United Kingdom.

responses were shown to be blocked by neutralizing IL-6-R monoclonal antibodies (9), other groups have questioned the requirement of IL-6-R for vIL-6-induced activation of cells (33, 44, 46, 78).

Viral IL-6 is constitutively expressed in lytically infected Kaposi spindle cells and AIDS-related B-cell lymphoma cell lines (BCBL and PEL) (45) and in Castleman's disease (56) and induces proliferation, angiogenesis, and hematopoiesis in IL-6-dependent cell lineages (26, 45). Furthermore, viral IL-6 can serve as an autocrine growth factor for PEL cell lines (30). Expression of vIL-6 accelerates hematopoiesis and induces vascular endothelial growth factor, a factor implicated in PEL and Kaposi's sarcoma pathogenesis (3).

However, the precise role of vIL-6 in HHV-8 pathogenesis is not well understood. Viral IL-6 may act as an antiapoptotic factor for the survival of HHV-8-infected cells and/or promote the proliferation of HHV-8-infected cells or potential host cells. In lymphoma cells infected with HHV-8, the viral cytokine protects cells against innate immune defenses triggered by viral infection via blocking of the interferon signaling pathway (13).

Recombinant soluble gp130Fc (sgp130Fc), consisting of the extracellular domain of human gp130 fused to the constant region of a human immunoglobulin G1 (IgG1) heavy chain (31), was shown to specifically inhibit cellular responses via the IL-6/sIL-6R complex, whereas cellular responses triggered by IL-6 alone remained unaffected. The sgp130Fc protein is expected to be a valuable therapeutic tool to specifically block disease states in which soluble IL-6-R *trans* signaling responses exist (5, 8, 29, 51) and might also be used to block vIL-6-induced signals (46).

Here, we report the isolation and characterization of a human recombinant antibody recognizing viral IL-6, which might be used as a molecular tool for the specific inhibition of signaling induced by vIL-6 but not by huIL-6 or IL-6/sIL-6R. Specific recombinant antibodies binding to vIL-6 were selected from an unbiased human synthetic V_H -plus- V_L single-chain variable fragment (scFv) library by phage display screening. One of the single-chain Fv fragments isolated (called monoclonal anti-vIL-6 [MAV]) bound to vIL-6 but not to huIL-6. Moreover, MAV was found to specifically inhibit vIL-6-mediated growth of the PEL-derived cell line BCBL-1. It was reported previously that secretion of vIL-6 is not necessarily needed for signal induction. Moreover, vIL-6 seems to have the ability to induce signals by an autocrine mechanism that relies on intracellular binding to its receptor gp130 within the endoplasmic reticulum (ER) (43). Therefore, we decided to use MAV as an intrabody (antibodies for intracellular applications) to influence the intracellular localization of vIL-6. Intrabodies have been successfully used to intracellularly modulate the function of proteins in various applications (32–34). The ER retention signal KDEL (58, 69) was fused to the C terminus of MAV at the cDNA level. The cDNA coding for MAV-KDEL was transiently transfected into COS-7 cells. The antibody localized in the ER could then bind viral IL-6 and prevent its further transport via the secretory pathway to the cell membrane, leading to an abrogation of vIL-6 signaling.

MATERIALS AND METHODS

Cells and reagents. COS-7 and HepG2 cells were bought from the ATCC (Rockville, MD) and were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The vIL-6 DNA sequence was originally obtained from F. Neipel and B. Fleckenstein (University of Erlangen, Germany) (48) and has been used in previous studies (26, 46). The PEL-derived BCBL-1 cells were a kind gift from M. Gramatzki's group at the University of Kiel (Germany) and were maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 2 mmol/liter L-glutamine (Life Technologies, Grand Island, NY), 10% fetal calf serum (BioWhittaker), and 5 μ g/ml gentamicin (Life Technologies). The Ba/F3 cell lines (Ba/F3-gp130, IL-6-R, and Ba/F3-gp130) used in this study were described previously (22, 55). EBNA-293 cells were used as described elsewhere previously (71). The human single-framework scFv libraries Tomlinson A and Tomlinson B (T. Tomlinson, MRC, Centre for Protein Engineering, Cambridge, United Kingdom) were provided by the MRC (see below). The anti-mouse alkaline phosphatase conjugate was bought from Sigma-Aldrich (Munich, Germany), the polyclonal rabbit antibody anti-phospho-STAT3 (Tyr705) and mouse anti-STAT3 were obtained from Cell Signaling Technology (Beverly, Calif.), and polyclonal horseradish peroxidase coupled to goat anti-rabbit and anti-mouse antibodies was obtained from Pierce (Erlangen, Germany). Anti-mouse Alexa Fluor 594 antibody was obtained from Molecular Probes (Invitrogen). The anti-vIL-6 rabbit serum was described previously (26). [3 H]thymidine was obtained from Amersham-Pharmacia (Freiburg, Germany). Recombinant cytokines used in this study were produced as described previously (21). Soluble gp130Fc is a fusion protein of the extracellular portion of gp130 and the Fc portion of IgG1 and was expressed and purified as previously described (31).

Cloning of MAV-KDEL, vIL-6-green fluorescent protein (GFP), vIL-6-KDEL, and vIL-6. Standard cloning procedures were performed as described previously by Sambrook et al. (70). The ER retention signal KDEL was added to the C terminus of MAV by PCR (MK404 [5'-GTGGTTTAAACCTAGAGTTCGTC TTTCCGCGGATTACAGATCTCTTCTGA-3'] and MK304 [5'-ATG GCC GAG GTG CAG CTG-3']). The signal peptide needed for eukaryotic expression of MAV-KDEL was amplified from human IL-6 (MK104 [5'-CA CGT TTA AAC GCC ACC ATG AAC TCC TTC TCC ACA-3'] and MK704 [5'-CAA CAG CTG CAC CTC GGC CAT CAC GTG TAC TGG GGC AGG GAA GGC-3']), fused to MAV-KDEL by slicing by overlapping extension PCR (25, 28), and cloned into vector p409B. The resulting plasmid was named p409B-MAV-KDEL. vIL-6 was amplified by PCR (MK504 [5'-CA CTA GCT AGC GCC ACC ATG TGC TGG TTC AAG TTG-3'] and MK604 [5'-CCG CTC GAG CTT ATC GTG GAC GTC AGG-3'], template p409-vIL-6) and inserted into plasmid pcDNA3.1-GFP via *NheI* and *XhoI* digestion, leading to pcDNA3.1-vIL-6. To generate an ER-retained vIL-6 expression construct, vIL-6 was amplified by PCR (5'-TAC TAC TCG AGC CCA CCA TGT GCT GGT TCA AGT TG-3' and 5'-TAT TGC GGC CGC CTT ATC GTG GAC GTC AGG-3') and inserted into pDCC-MAV-KDEL opened with *XhoI* and *NotI*, leading to pDCC-vIL-6-KDEL. As a control, vIL-6 was also amplified by PCR (5'-TAC TAC TCG AGG CCA CCA TGT GCT GGT TCA AGT TG-3' and 5'-CGCGGATCCCTACAGATCCTCTCTGAGAT-3') and cloned into pDCC-vIL-6-KDEL opened with *XhoI* and *BamHI*, leading to pDCC-vIL-6.

Expression of recombinant vIL-6. EBNA cells expressing vIL-6 were grown in culture medium containing 10% FCS to 80 to 100% confluence. The medium was then changed to FCS-free medium, and expressed vIL-6 protein was collected in the supernatant for 7 days. Fifty milliliters of cell culture supernatant was centrifuged and incubated at room temperature (RT) with 300 μ l Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN, Hilden, Germany) for 2 h. The agarose was washed with phosphate buffer containing 500 mM NaCl at pH 7.4, and recombinant vIL-6 was eluted with the same buffer containing 0.5 M imidazole and dialyzed to 50 mM Tris buffer, pH 7.4, without imidazole.

Phage display. The phage display screening was done as described previously (41). Briefly, panning of two phage libraries (A and B; MRC) was done in 96-well microtiter plates (Nunc Maxisorb) coated at 4°C overnight with 1 μ g/well purified Flag-vIL-6-His. Three rounds of biopanning were carried out against vIL-6 immobilized on microtiter plates. Eluted phages were used to infect TG1 cells and were rescued with M13K07 helper phage. The culture supernatant, containing the polyclonal phage populations from each round of biopanning, was used to assess the binding to vIL-6 by enzyme-linked immunosorbent assay (ELISA). To assess the binding of monoclonal phage scFvs, single colonies from titration plates of the third round were cultured and analyzed by ELISA. Clones that gave the highest signals were selected to infect *Escherichia coli* HB2151 cells to express soluble scFvs. Infected HB2151 cells were induced by IPTG (isopropyl-

β -D-thiogalactopyranoside), and the supernatant containing the soluble scFvs was analyzed by ELISA.

ELISAs. Microtiter plates were coated with 10 μ g/ml vIL-6 (or other antigens for cross-reactivity ELISA) or 1 μ g/mls gp130Fc for the sandwich ELISA and incubated at RT overnight. The wells were blocked with 3% BSA for 2 h at RT. Specific binding of scFvs to vIL-6 or to the vIL-6/sgp130Fc complex (sandwich ELISA) or of Flag-tagged vIL-6 to sgp130Fc was detected by ELISA with an anti-c-myc antibody or with an anti-Flag antibody, respectively, and anti-mouse antibodies conjugated with alkaline phosphatase followed by incubation with substrate (1 mg/ml *p*-nitrophenylphosphate in diethanolamine buffer). The color intensity was measured as extinction at 405 nm using an ELISA reader.

Production and purification of soluble MAV. The MAV coding sequence was cut out from pIT1-MAV (library vector) by NcoI and NotI and inserted into the pET22b vector via NcoI and NotI. pET22b encodes the PelB leader sequence, which is a signal peptide for periplasmic expression of the gene of interest. The antibody sequence together with the PelB leader was then cloned into vector pET23a-SM12 (our unpublished results) by XbaI and NotI, so that MAV obtained C-terminal c-myc and His tags. *E. coli* BL21 cells were transformed with vector pET23a-MAV. Transformed *E. coli* BL21 cells were grown with shaking at 37°C to an optical density at 600 nm of 0.5. Periplasmic expression of MAV was induced by adding 0.4 mM IPTG to cells and shaking them overnight at 30°C. The bacterial culture was centrifuged at 6,000 $\times g$ for 20 min. The supernatant containing soluble scFv was purified with Ni-NTA agarose. One hundred milliliters of the bacterial supernatant was incubated with 0.5 ml Ni-NTA agarose (QIAGEN, Germany) at RT for 2 h. The matrix was then washed with 50 mM phosphate buffer, pH 7.4, containing 500 mM NaCl, and bound MAV was eluted with 0.5 M imidazole in the same buffer and dialyzed to 50 mM Tris buffer, pH 7.4, without imidazole.

CD spectroscopy. Circular dichroism (CD) measurements were carried out using a Jasco 720 CD spectrometer equipped with a temperature control unit and calibrated with a 0.1% aqueous solution of *d*-10-camphorsulfonic acid according to the method described previously by Chen and Yang (14). The spectral bandwidth was 1.5 nm. The time constant was 2 s, and the cell path length was 0.1 mm.

Calculation of protein concentrations. Protein concentrations were calculated from absorption spectra in the range of 240 to 320 nm using the method described previously by Waxman et al. (79).

Western blot analysis. For Western blotting of cell lysates, cells from a 10-cm dish were lysed with 1 ml 2 $\times$ Laemmli buffer (36), 10 μ l of which was applied to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. To analyze the proteins of the cell supernatant, 15 μ l out of 5 ml was used. Proteins separated by SDS-PAGE were transferred for 1 h at 1 mA/cm² to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in 6% bovine serum albumin (BSA)-Tris-buffered saline (TBS). The filters were then incubated with an appropriate antibody before being labeled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed with the Amersham enhanced chemiluminescence kit in accordance with the manufacturer's instructions. Band intensities were estimated from photographic films by densitometry using ImageJ software (National Institutes of Health).

Immunoprecipitation of vIL-6 with MAV. COS-7 cells were transiently transfected with p409-vIL-6 using the DEAE-dextran method (42, 64). The next day, DMEM containing 10% FCS was changed to 5 ml FCS-free DMEM. After 24 h, the supernatant was removed and centrifuged at 10,000 $\times g$ for 2 min to remove cell debris. One milliliter of supernatant containing vIL-6 was incubated with 15 μ g of MAV at 4°C overnight under continuous rolling. Immunocomplexes were collected with 25 μ l Talon beads (Dynal) during incubation at 4°C for 2 h. After washing with phosphate-buffered saline (PBS)-Tween and PBS buffers, the MAV/vIL-6 complexes were eluted with 20 μ l 0.5 M imidazole in PBS. The eluates were separated by SDS-PAGE, and Western blotting was done using anti-vIL-6 serum as described above.

Surface plasmon resonance. Forty micrograms of recombinant vIL-6 in 10 mM Na-acetate, pH 5.5, was immobilized onto a surface plasmon resonance sensorchip (IAsys). The binding experiment was performed at room temperature with different concentrations of MAV using the IAsys optical biosensor. Affinograms were analyzed by nonlinear regression with FASTfit software, which uses the Marguard-Levenberg algorithm for iterative data fitting (54).

Immunostaining of transfected COS-7 cells. Approximately 40,000 COS-7 cells grown on glass coverslips for 24 h were washed with PBS containing 0.1% acid and fixed with cold methanol at -20°C for 10 min. The cells were then washed two times with TBS and incubated with blocking buffer (1% BSA, 0.2% glycerine, TBS) at room temperature for 2 h. A 1:100 dilution of the anti-c-myc antibody was applied at 4°C overnight. The cells were washed with TBS-Tween and TBS and incubated with the Alexa Fluor 594 anti-mouse antibody at 37°C for

1 h. The washing was repeated as before, and the glass coverslips were mounted onto slides with SlowFade Light (Molecular Probes) and analyzed using fluorescence microscopy. A $\times 543$ magnification was used to photograph the cells.

Proliferation assay. BCBL-1 and Ba/F3-gp130 cells were washed three times with sterile PBS and resuspended in RPMI medium containing 10% FCS at 5×10^3 cells per well of a 96-well plate. The cells were cultured in a final volume of 100 μ l for 72 h and subsequently pulse-labeled with 0.25 μ Ci [³H]thymidine for 4 h. The specific activity of [³H]thymidine was 25 Ci/mmol. Cells were harvested on a glass fiber filter (Filtermat A; Wallac, Turku, Finland), and the filter was baked in a microwave oven for 2 min. Subsequently, the filter was soaked in 4.5 ml liquid scintillation cocktail (Betaplate Scint; Wallac, Turku, Finland). [³H]thymidine incorporated into cellular DNA was determined by scintillation counting using a MicroBeta TriLux counter from Perkin-Elmer (Wellesley, MA). Bioassays were performed, with each value being determined in triplicate.

RESULTS

Isolation of human recombinant antibody binding to viral IL-6. The modular episomal vector pCEP-Pu (34), based on the Epstein-Barr virus genome, was used for the expression of vIL-6. The expression constructs contained the vIL-6 sequence with either an N-terminal His tag or with two affinity tags, an N-terminal Flag tag and a C-terminal His tag. Both variants of vIL-6 were produced by stably transfected EBNA-HEK293 cells, and the vIL-6 variants were purified from the cell culture supernatants with Ni-NTA (Fig. 1A). The correct folding of the recombinant vIL-6 proteins was confirmed by CD spectroscopy. vIL-6 expressed and purified from EBNA-293 cells displayed a typical α -helical spectrum (Fig. 1B). The purified vIL-6 variants were biologically active, as shown by the induction of proliferation of the cytokine-dependent murine pre-B-cell line Ba/F3-gp130 (Fig. 1C). Native Ba/F3 cells proliferate in the presence of the cytokine IL-3. After stable transfection of a cDNA coding for gp130, these cells (Ba/F3-gp130), in the absence of IL-3, gain the ability to proliferate in the presence of IL-6/sIL-6-R, hyper-IL-6 (a fusion protein of IL-6 and sIL-6-R) (21), or viral IL-6. Previous studies have shown that Ba/F3-gp130 cells proliferate in response to hyper-IL-6 at a concentration below 1 ng/ml, whereas 100 ng/ml of viral IL-6 was needed to induce Ba/F3-gp130 cell proliferation (26, 46).

In this study, Flag-vIL-6-His and His-vIL-6 expressed in EBNA-293 cells induced the proliferation of Ba/F3-gp130 cells at similar concentrations. In contrast, bacterially expressed and refolded viral IL-6 protein was not correctly folded, did not show the typical CD spectrum of a four-helical protein (Fig. 1B), and did not induce proliferation of Ba/F3-gp130 cells (Fig. 1C). Biologically active vIL-6 has been produced in *E. coli* previously with a different expression strategy (26). In EBNA-293 cells, the expression level of Flag-vIL-6-His was threefold higher than that of His-vIL-6. Thus, Flag-vIL-6-His was produced in milligram amounts to be used for the screening of a scFv phage library. The correct processing of the signal peptide was verified by N-terminal sequencing of the purified secreted protein (data not shown).

For the production of recombinant antibodies against vIL-6, phage display technology was chosen (for a review, see reference 17), and the Tomlinson A and B libraries (T. Tomlinson, MRC, Center for Protein Engineering, Cambridge, United Kingdom), comprising 10^9 scFv fragments, were used. The libraries are based on a single human framework for the variable regions of heavy (V_H) and light (V_L) human immunoglobulin chains, in which the CDR3 loops have been random-

FIG. 1. Expression and characterization of vIL-6. (A) Purified vIL-6 expressed by EBNA-293 cells. (I) Silver staining of vIL-6-His and Flag-vIL-6-His. (II) Western blot of vIL-6-His and Flag-vIL-6-His (anti-vIL-6 antiserum). (B) Circular dichroism spectra. Shown is the α -helical fold of Flag-vIL-6-His and vIL-6-His expressed by and purified from EBNA-293 cells and that of vIL-6-His expressed in bacterial cells. (C) Induction of proliferation of Ba/F3-gp130 cells by vIL-6 and hyper-IL-6. Ba/F3-gp130 cells were stimulated with increasing amounts of Flag-vIL-6-His or His-vIL-6 or bacterially expressed vIL-6-His or hyper-IL-6. Proliferation was measured by [^3H]thymidine incorporation. Squares, hyper-IL-6; diamonds, Flag-vIL-6-His (EBNA); triangles, His-vIL-6 (EBNA); circles, His-vIL-6 (bacterial expression).

ized in sequence and length (for a detailed protocol, see <http://www.mrc-cpe.cam.ac.uk/g1p.php?page=1808>). After three rounds of panning and selection, clones were tested to bind to vIL-6 as soluble scFvs. Only two clones did not cross-react with other irrelevant proteins (data not shown). Sequencing of these two independently selected clones revealed that they were identical, suggesting that in the course of two different selection experiments, the same antibody fragment had been isolated. This underlines the specificity of the screening process. Thus, we obtained one vIL-6-specific scFv antibody, named MAV.

Expression, purification, and characterization of MAV by Western blot, immunoprecipitation, and ELISA and in a vIL-6-phospho-STAT3 assay. Next, MAV was produced in milligram amounts by periplasmic expression of the recombinant protein in *E. coli* and purified using Ni-NTA agarose (Fig. 2A). The purified recombinant antibody fragment MAV was analyzed by ELISA for specific binding to vIL-6. Different antigens were coated at a concentration of 10 $\mu\text{g/ml}$ and 0.5 μg MAV per well. MAV bound to Flag-vIL-6-His and His-vIL-6 but did not exhibit any cross-reactivity with other antigens such as huIL-6, His-tagged OSM, BSA, lysozyme, ABI3 (66), and sgp130Fc (Fig. 2B). Because Flag-vIL-6-His was used as a coating antigen for the panning, it was important to test the MAV reactivity with the Flag and His tags. Specific binding of MAV to His-vIL-6 suggested that MAV did not recognize the Flag tag. Only Flag-vIL-6-His and His-vIL-6 were recognized by the scFv antibody MAV. Also, the lack of binding to the His-tagged OSM suggested that there was no cross-reactivity with the His tag.

MAV binding to vIL-6 was also shown by Western blotting. MAV was used as the primary antibody recognizing vIL-6. One hundred nanograms of vIL-6 protein could be detected by MAV (Fig. 2C). Next, we investigated whether MAV bound to vIL-6 in solution. Therefore, COS-7 cells were transiently transfected with an expression plasmid encoding vIL-6, and the supernatants of transfected as well as untransfected cells were incubated with MAV. vIL-6 bound to MAV was coprecipitated by the use of Talon beads binding the His-tagged proteins. The precipitates were analyzed by Western blotting (Fig. 2D) with a vIL-6-specific antiserum (26). The fact that vIL-6 was precipitated with MAV suggested that MAV recognized vIL-6 in solution.

The kinetics of MAV binding to vIL-6 immobilized on a solid phase were analyzed by surface plasmon resonance. Hence, a plot of multiple K_{on} values, derived from a full interaction experiment, allowed the determination of k_{ass} (gradient) and k_{diss} (intercept), which were 14,500.5 $\text{M}^{-1} \text{s}^{-1}$ and 0.005012 s^{-1} , respectively. In this way, the affinity of MAV for vIL-6 was defined as follows: dissociation equilibrium constant = $k_{diss}/k_{ass} = 340 \text{ nM}$ (data not shown).

To identify the epitope of vIL-6 that is recognized by MAV, several chimeric proteins of human and viral IL-6 were used (B. Rabe and J. Scheller, unpublished results). huIL-6 as well as vIL-6 have a four-helical structure (24). gp130 receptor binding sites of these cytokines are defined as site II, composed of helix A and helix C, and site III, consisting of the initial part of the AB loop and helix D. The chimeric proteins represent the huIL-6 sequence with the exchange of sites II and III of vIL-6 (Fig. 3). Because MAV specifically binds to vIL-6 but not

FIG. 2. Expression and characterization of the recombinant human vIL-6 antibody MAV. (A) Expression and purification of MAV. Periplasmic expression of MAV was induced by IPTG stimulation in *E. coli* strain BL21(plysS). (I) Western blot (WB) of MAV expression in *E. coli* BL21(plysS) using an anti-*c-myc* antibody. MAV was expressed in the periplasmic space of the bacteria and purified by Ni-NTA agarose. (II) Purified protein was analyzed by 12.5% SDS-PAGE and visualized by silver staining. (B) Specific recognition of vIL-6 by MAV in an ELISA. MAV-specific binding to Flag-vIL-6-His and His-vIL-6 was demonstrated, but MAV-specific binding to human IL-6 or other antigens was not found. (C) MAV recognition of vIL-6 by Western blot (WB). One microgram, 100 ng, or 10 ng of recombinant vIL-6 was applied to a 12.5% SDS-PAGE gel, blotted, and detected by MAV as the primary antibody. As a secondary antibody, anti *c-myc*-antibody 9E10 (1:50) was used. (D) Binding of MAV to recombinant vIL-6 in solution. Supernatants of COS-7 cells transiently transfected with vIL-6 or left untransfected were directly applied to SDS-PAGE gels or

FIG. 3. Mapping of the vIL-6 epitopes recognized by MAV. (A) Schematic illustration of human (white)/viral (black) IL-6 chimeras. Chimera A has the A helix of vIL-6, chimera C has the C helix of vIL-6, chimera A+C has both helices A and C of vIL-6, AB has the AB loop of vIL-6, and chimera II+III has complete sites II and III of vIL-6. (B) One microgram of vIL-6, huIL-6, or vIL-6/huIL-6 chimeras was separated by SDS-PAGE, immunoblotted with MAV, and detected with the anti-*c-myc* antibody 9E10.

to huIL-6, these chimeras can be used to define which region of vIL-6 is recognized by MAV (1, 2). As shown by Western blotting, MAV recognized vIL-6 and the chimera II+III, which has the complete receptor binding sites II and III of vIL-6 (Fig. 3). Because MAV did not bind to the chimeras A, C, and A+C, one can conclude that the binding epitope of vIL-6 to MAV is not on site II. MAV binding to chimera II+III suggests that site III of vIL-6 can be the recognition site for MAV. However, the AB loop of site III does not seem to be involved in the binding of MAV, because MAV does not bind to the AB chimera. Therefore, we assume that the D helix of site III of vIL-6 is necessary for recognition by MAV. The lower signal intensity of MAV recognizing the chimera II+III compared to that of wild-type vIL-6 indicates that MAV might bind to a conformational epitope rather than to a simple linear epitope. This was further supported by ELISA data, in which folded

used for immunoprecipitation (IP) with MAV. His-tagged MAV and vIL-6 bound to MAV were isolated by using Talon beads and then separated on a 12.5% polyacrylamide gel. Western blotting was done using an anti-vIL-6 serum (26). Detection of unglycosylated as well as glycosylated vIL-6 protein in the supernatant of transfected COS-7 cells was previously described (20, 35).

FIG. 4. Specificity and neutralization properties of MAV. (A) Sandwich ELISA. MAV bound to vIL-6 (1 μg/ml) could be detected by an anti-c-myc antibody (second lane). MAV (1 μg/ml) binding to plate-coupled vIL-6 (1 μg/ml) could be prevented by the addition of free vIL-6 (10 μg/ml) (third lane). vIL-6 bound to sgp130Fc (1 μg/ml) could not be detected by MAV anymore (fourth lane). Flag-tagged vIL-6 bound to sgp130Fc could be detected by an anti-Flag antibody (eighth lane). sgp130Fc (gp) or vIL-6 (vIL) was coated. (B) Inhibition of vIL-6-induced STAT3 activation in the human hepatoma cell line HepG2 by recombinant anti-vIL-6-scFv MAV. HepG2 cells were stimulated with 150 ng/ml vIL-6 in the absence or presence of MAV for 10 min. Cells were lysed, and proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Phosphorylated STAT3 (P-STAT3) protein was detected by Western blotting using a phospho-STAT3 antibody.

vIL-6 and the folded chimera II+III were detected with comparable efficiencies (data not shown).

To ask whether MAV binds to a vIL-6-sgp130Fc complex, a sandwich ELISA was performed. First, binding of Flag-tagged vIL-6 to sgp130Fc was shown by anti-Flag antibodies. After coating with sgp130Fc protein, vIL-6 and then MAV were added. Interestingly, vIL-6 bound to sgp130Fc was not detected by MAV, whereas vIL-6 bound directly to the surface of the well was readily detected (Fig. 4A). We conclude from these experiments that the binding sites of MAV and of sgp130Fc overlap and that MAV might therefore have neutralizing properties.

Therefore, we tested the effect of MAV on vIL-6-induced STAT3 phosphorylation of HepG2 cells. vIL-6 binds to the gp130 receptor, leading to JAK/STAT activation (46). Activated STAT3 was detected with an anti-phospho-STAT3 antibody. HepG2 cells were stimulated with 150 ng/ml vIL-6 in the presence or absence of increasing concentrations of MAV. In fact, quantification by densitometry revealed that MAV re-

FIG. 5. MAV inhibits the proliferation of the vIL-6-dependent PEL-derived BCBL-1 cell line. About 5,000 BCBL-1 cells were cultured for 3 days in the presence or absence of MAV or sgp130Fc. Proliferation was measured by pulse-labeling the cells after 72 h with [³H]thymidine for 4 h. Cells were harvested, and incorporated radioactivity was measured by scintillation counting. Bioassays were performed, with each value being determined in triplicate.

duced STAT3 phosphorylation from 100% to 18% in a dose-dependent manner, indicating the neutralizing potential of the scFv MAV protein (Fig. 4B). It should, however, be noted that such a quantification is only an approximation and not an absolutely accurate method to measure the amount of phospho-STAT3 protein.

The proliferation of the vIL-6-dependent PEL-derived cell line BCBL-1 can be inhibited by recombinant MAV. PEL is a distinct type of lymphoma associated with Kaposi's sarcoma-associated herpesvirus infection. Among others, vIL-6 has been described to be a factor responsible for the unrestrained proliferation of the PEL-derived cell line BCBL-1. BCBL-1 cells were found to release huIL-6 and vIL-6 in culture supernatant (30). Here, we have tested whether recombinant MAV can inhibit the proliferation of BCBL-1 cells. For a control, we have used recombinant sgp130Fc, which has previously been shown to specifically inhibit vIL-6-mediated signaling and transsignaling mediated by huIL-6 in complex with soluble IL-6-R (46). sgp130Fc consists of the complete extracellular part of gp130 (domains 1 to 6) fused to a dimerization domain (Fc part of a human IgG antibody) (31). As depicted in Fig. 5, the proliferation of BCBL-1 cells could be blocked by MAV and sgp130Fc by more than 50%. We conclude from this experiment that MAV specifically inhibits vIL-6-mediated growth of the PEL-derived BCBL-1 cell line.

ER retention of viral IL-6 by scFv MAV-KDEL abrogates vIL-6 signaling. Since it was previously reported that vIL-6 can act from within the cell, presumably by binding to gp130 in the

FIG. 6. Intracellular retention of vIL-6 by MAV-KDEL. (A) MAV-KDEL expression in COS-7 cells. Supernatants (SN) of the COS-7 cells transiently transfected with p409B-MAV-KDEL and cell lysates were prepared on the second day after transfection. MAV-KDEL was detected by Western blotting using an anti-*c-myc* antibody. (B) Cotransfection of MAV-KDEL and vIL-6-GFP cDNAs into COS-7 cells resulting in the intracellular retention of vIL-6. COS-7 cells were transiently transfected with pcDNA3.1-vIL-6-GFP alone or were cotransfected together with p409B-MAV-KDEL. Supernatants and lysates of the cells were prepared on the second day after transfection. vIL-6-GFP was detected by Western blotting using an anti-vIL-6 serum (26). (C) Fluorescence microscopy of untransfected COS-7 cells (I) and COS-7 cells transfected with pcDNA3.1-vIL-6-GFP (II). Immunofluorescence of COS-7 cells transfected with p409B-MAV-KDEL is shown. (VI) Cells on the third day after transient transfection were stained with an anti-*c-myc* antibody (1:100) and Alexa Fluor 594 anti-mouse antibody (1:1,500). (III) Control staining was prepared without an anti-*c-myc* antibody. (V) Fluorescence microscopy of vIL-6-GFP of COS-7 cells cotransfected with pcDNA3.1-vIL-6-GFP and p409B-MAV-KDEL. (VI) Immunofluorescence of MAV-KDEL of COS-7 cells cotransfected with pcDNA3.1-vIL-6-GFP and p409B-MAV-KDEL. (VII) Merged image of V and VI. (D) Abrogation of vIL-6-induced STAT3 phosphorylation by intracellular retention and neutralization of vIL-6 with MAV-KDEL. COS-7 cells were transiently transfected with pcDNA3.1-vIL-6-GFP and cotransfected together with p409B-MAV-KDEL or p409B-EYFP. Lysates of the cells were prepared on the second day after transfection. vIL-6-GFP was detected by Western blotting using an anti-vIL-6 serum (26).

ER (43), we aimed to use MAV to retain and neutralize vIL-6 within the ER. To clone a construct for eukaryotic expression of MAV-KDEL, the signal peptide (SP) of human IL-6 was added to the N terminus and a DNA sequence coding for the ER retention signal KDEL was added to the C terminus of MAV. SP-MAV-KDEL was transfected into COS-7 cells, and MAV-KDEL expression was analyzed by Western blotting of the cell lysate and the cell supernatant. As expected, MAV-KDEL was detected only in the cell lysate and was not detectable in the cell supernatant (Fig. 6A).

We further constructed a vIL-6-GFP fusion protein in which GFP was fused at the C terminus of vIL-6. Using an anti-vIL-6 serum for Western blot analysis, vIL-6-GFP was detected in the supernatant as well as in the cell lysate of transfected COS-7 cells (Fig. 6B). The double band corresponding to vIL-

6-GFP in the supernatant most likely reflects differentially glycosylated forms of vIL-6-GFP (compare Fig. 6B and 2D).

Cotransfection of vIL-6-GFP and MAV-KDEL into COS-7 cells should result in the intracellular binding of vIL-6-GFP to MAV-KDEL and in the retention of MAV-KDEL/vIL-6-GFP in the endoplasmic reticulum. This was demonstrated by Western blotting of the lysates and supernatants of the cotransfected COS-7 cells (Fig. 6B). Only a very faint band corresponding to vIL-6-GFP was detected in the cell supernatant, suggesting that a very small amount was secreted in the presence of MAV-KDEL.

The expression of MAV and vIL-6-GFP and their localization within the cell were monitored by immunostaining of transfected COS-7 cells. As expected, MAV-KDEL was seen in the region of the endoplasmic reticulum, and no plasma

FIG. 7. Intracellular signaling by vIL-6. (A) vIL-6-KDEL-induced STAT3 phosphorylation. HepG2 cells were transiently transfected with pDCC-vIL6 or pDCC-vIL-6-KDEL. Lysates of the cells were prepared on the second day after transfection. vIL-6 was detected using anti-c-myc-antibodies. (B) STAT3 phosphorylation of HepG2 cells transiently transfected with pDCC-vIL6 or pDCC-vIL-6-KDEL. (C) Cells were washed with PBS, and conditioned supernatants of transfected HepG2-cells were collected after 4 h on the second day after transfection. The supernatant was used to stimulate untransfected cells for 5 min. STAT3 phosphorylation was detected by anti-phospho-STAT3 (P-STAT3)-specific antibodies.

membrane staining was detected (Fig. 6C, panel IV). Strong fluorescence of the ER suggested a high expression level of vIL-6-GFP (Fig. 6C, panel II). Secretion of vIL-6-GFP was seen in vesicles as green dots (Fig. 6C, panel II, white arrows) and observed as green fluorescence in the region of the plasma membrane.

In double-transfected cells, intracellular colocalization of vIL-6 and MAV-KDEL could be observed as yellow staining caused by the overlay of green (vIL-6-GFP) and red (MAV-KDEL-Alexa Fluor 594) fluorescence (Fig. 6C, compare panels V, VI, and VII).

Finally, we asked whether ER retention of vIL-6 in MAV-KDEL cotransfected cells would lead to reduced STAT3 phosphorylation (Fig. 6D). Therefore, we transfected COS-7 cells with 0.5 μ g and 0.25 μ g of the expression plasmids coding for vIL-6 and 3 μ g of expression plasmids coding for MAV-KDEL or GFP. We found a clear reduction of vIL-6-induced STAT3 phosphorylation in the presence of MAV-KDEL, indicating that the retention of vIL-6 in the ER led to the inhibition of STAT3 phosphorylation. Quantification of the phospho-STAT3 Western blots by densitometry showed that we achieved a reduction of 30% when 0.5 μ g vIL-6 and MAV-KDEL plasmids were transfected and a reduction of 69% when 0.25 μ g vIL-6 and MAV-KDEL plasmids were transfected. It should, however, be noted that such a quantification is only an approximation and not an absolutely accurate method to measure the amount of phospho-STAT3 protein.

We conclude from these experiments that the neutralization of vIL-6 is feasible from within the cell and that such a strategy can lead to the neutralization of vIL-6 activity.

Recently, it was reported that vIL-6 has the ability to transduce signals from within the cell. It was speculated that only a small proportion of synthesized vIL-6 is secreted and that intracellular signal activation via a vIL-6-gp130 interaction in the ER is predominant (43). To evaluate this model and to

demonstrate the relevance of our scFv-vIL-6-ER retention strategy, we generated a vIL-6 cDNA C-terminally fused to the ER retention signal KDEL. Interestingly, vIL-6 protein could be detected in the lysate of HepG2 cells transiently transfected with a cDNA coding for vIL-6 or vIL-6-KDEL (Fig. 7A). Due to the ER retention signal, vIL-6-KDEL could be detected at higher amounts in cell lysates than vIL-6, which was also secreted into the supernatant (Fig. 7C and data not shown). Interestingly, cells transfected with a cDNA coding for vIL-6 or vIL-6-KDEL were found to have identical STAT3 phosphorylation intensities (Fig. 7B), indicating that both intracellular and secreted vIL-6 can induce gp130 signal transduction. We stimulated untransfected HepG2 cells with 4-h conditioned medium of vIL-6- or vIL-6-KDEL-transfected HepG2 cells and analyzed STAT3 phosphorylation to see whether any biologically active vIL-6-KDEL was secreted. As expected, untransfected HepG2 cells treated with conditioned medium of vIL-6-transfected HepG2 cells showed phosphorylated STAT3, whereas untransfected HepG2 cells treated with conditioned medium of vIL-6-KDEL-transfected HepG2 cells showed only unphosphorylated STAT3 (Fig. 7C), indicating that the ER retention of vIL-6-KDEL was complete. The concentration of vIL-6 in the conditioned medium was under the detection limit by Western blotting (data not shown). These data support the model that an active vIL-6-gp130 signal transduction complex can be formed within the cell.

DISCUSSION

HHV-8, also called Kaposi's sarcoma-associated herpesvirus, interferes with the body's immune response and causes Kaposi's sarcoma. Kaposi's sarcoma is a cancer of blood vessel cells that often occurs in subepidermal tissues or the mucous membrane. Kaposi's sarcoma is one of the most common malignancies occurring among human immunodeficiency virus (HIV)-infected individuals (45, 52). HHV-8 encodes the cytokine vIL-6, which not only inhibits immune function but also causes cancerous cells to grow. vIL-6 protects virus-infected cells from undergoing growth arrest and apoptosis or cell death, which is one strategy that the immune system applies to limit viral infection. vIL-6 inhibits signaling of the antiviral factor alpha interferon, which prevents virus-infected cells from growing (13). In addition, in some cells, vIL-6 not only stops the suppression of tumors but also causes healthy cells that are not infected with HHV-8 to proliferate abnormally (49). Therefore, vIL-6 is a promising target for novel therapies directed against HHV-8-associated tumors and malignancies. Although vIL-6 was initially isolated from Kaposi's sarcoma lesions, it was also found to be associated with lymphoproliferative diseases (37) such as PEL and MCD. Castleman's disease is a nonmalignant atypical lymphoproliferative disorder characterized by lymphadenopathy with angiofollicular hyperplasia and plasma cell infiltration. Virtually all HIV-infected individuals and nearly 50% of HIV-negative individuals with MCD are infected with HHV-8 (52, 57). Furthermore, some HIV-infected individuals have coexistent Kaposi's sarcomas (18 to 20%) (52). Primary effusion lymphoma is a malignant effusion in the absence of a contiguous solid tumor mass that develops in the pleural, pericardial, or peritoneal cavity. vIL-6 is believed to play an

important role in the pathogenesis of PEL and MCD, acting as an autocrine or paracrine factor in the lymphoproliferative processes common to both diseases (75).

In this study, we aimed to produce recombinant human antibodies against viral interleukin-6 for the neutralization of the biological effect of vIL-6. Encoded by HHV-8, vIL-6 is believed to play an important role in the pathogenesis of Kaposi's sarcoma-associated diseases.

Importantly, vIL-6 stimulates cells expressing gp130 on their surface without the need of the membrane IL-6-R. Recombinant soluble gp130 blocks the antiapoptotic effect of vIL-6 mediated via membrane-bound gp130 (46). On the other hand, soluble gp130 is an inhibitor of human IL-6 responses that are dependent upon sIL-6-R and is therefore a therapeutic tool to specifically block disease states in which IL-6 transsignaling responses exist (31). A highly specific inhibitor of vIL-6 can be a specific antibody against the viral cytokine. Therefore, an antibody screened from a phage display human library is a promising candidate for the therapy of these diseases.

Bacterially produced vIL-6 showed only low biologic activity. Therefore, we produced vIL-6 in a mammalian expression system using EBNA-293 cells stably transfected with vIL-6 cDNA, which led to vIL-6 with high biological activity and correct folding as determined by CD spectroscopy. Moreover, bacterial proteins are usually unglycosylated, and vIL-6 N-glycosylation at position N89 was previously reported to be important for the biological activity of the cytokine and for stimulating vIL-6-dependent cell proliferation (20).

Flag-vIL-6-His was purified in the milligram range from EBNA-293 cell culture supernatants and used for the production and characterization of recombinant antibodies. Phage display technology provides a means by which high-affinity, fully human monoclonal antibodies can be rapidly isolated and also provides a starting point from which a selected antibody can, if necessary, be affinity matured for improved neutralization potency or binding kinetics (6, 41, 73).

One scFv antibody fragment called MAV that was specific for vIL-6 was selected from Tomlinson library A (T. Tomlinson, MRC, University of Cambridge, United Kingdom). A high specificity of MAV for vIL-6 binding was observed by ELISA. Importantly, MAV was not cross-reactive with other antigens and did not bind to human IL-6. The fact that MAV recognized vIL-6 by both ELISA and Western blotting makes the antibody a suitable immunological reagent to detect vIL-6. Furthermore, MAV is capable of recognizing vIL-6 in solution, as was demonstrated when vIL-6 secreted into the supernatant of transfected COS-7 cells was precipitated by MAV. The fact that MAV precipitated unglycosylated as well as glycosylated vIL-6 demonstrated that there was no reactivity of MAV with carbohydrate moieties.

The MAV binding epitope of vIL-6 was identified on the D helix of site III. As previously described, site III of vIL-6 is the binding site for the gp130 receptor (4). If MAV and gp130 interact with the same vIL-6 epitope, the binding of MAV to vIL-6 in vivo should abrogate the signaling that occurs upon vIL-6 binding to gp130 (4). This was shown to be true, since MAV did not recognize vIL-6 when it was bound to gp130, whereas vIL-6 was readily detected when vIL-6 was adsorbed to the ELISA well directly (Fig. 6C). Stimulation of HepG2 cells with vIL-6 resulted in the phosphorylation of STAT3 (44).

Stimulation of vIL-6 in the presence of MAV led to decreased STAT3 phosphorylation (from 100% to 18%) in a dose-dependent manner. Furthermore, we demonstrated the inhibition of the growth of the PEL-derived BCBL-1 cell line by recombinant MAV. MAV was able to inhibit the proliferation of BCBL-1 cells by more than 50%. A complete inhibition of BCBL-1 cell proliferation could not be observed, most likely because BCBL-1 cells are dependent not only on vIL-6 as an autocrine or paracrine stimulatory factor but also on other factors such as IL-10 (30).

An elegant study by Corte-Real et al. recently demonstrated the general feasibility of the intrabody technology to specifically eliminate HHV-8-infected cells. Those authors used a cytoplasmic intrabody directed against the HHV-8 gene product LANA. LANA is expressed in every BCBL-1 cell and is necessary for the maintenance and segregation of the viral episome. Blockade of LANA in BCBL-1 cells led to a loss of the viral episome during transient transfection of the anti-LANA intrabody (18). As we and others have demonstrated, BCBL-1 cells are only partly dependent upon the presence of vIL-6. Moreover, vIL-6 expression was detected in less than 2% of the cells (10). Due to the low transfection efficacy of BCBL-1 cells and the small number of BCBL cells that express vIL-6, we first chose to demonstrate the universal application of MAV as a vIL-6 neutralizing intrabody in COS-7 cells. Studies of the stable transfection of MAV-KDEL into BCBL-1 cells will have to be undertaken in the future to demonstrate the biologic consequences of a stable blockade of vIL-6 in these cells.

Secreted proteins like vIL-6 are transported in vesicles from the ER to the Golgi apparatus and then to the plasma membrane. ER-resident proteins are returned to the ER due to their COOH-terminal retention signal, KDEL. Synthetic fusion of a retention sequence to a protein, which originally does not have such a sequence, leads to the inhibition of its secretion and to its localization in the ER. For example, recombinant human IL-6 carrying a retention signal accumulated within the ER and therefore retained IL-6-R in the ER. This led to a complete loss of cell surface expression of IL-6-R on IL-6-KDEL-expressing cells (69). Here, we constructed an anti-vIL-6 antibody (MAV) carrying the ER retention signal (KDEL). Antibody retention in the ER was demonstrated by transfection of COS-7 cells with a plasmid encoding MAV-KDEL. Cotransfection with vIL-6 resulted in the intracellular binding of vIL-6 to the antibody and in the retention of vIL-6/MAV-KDEL in the ER. Using this cell model system, secretion of vIL-6 was completely eliminated. Recently, a model of an intracellular signaling mechanism of vIL-6 was reported (43), and this model is further supported by our data. It was shown that only a small proportion of synthesized vIL-6 is secreted and that intracellular signal activation via a vIL-6-gp130 interaction in the ER is predominant. A fusion of vIL-6 to the ER retention signal KDEL abolished the secretion of vIL-6 but not signaling; therefore, the MAV-KDEL antibody might have an effect by not only specifically blocking vIL-6 secretion but also by neutralizing vIL-6-induced signaling from within the cell. In the present study, the blockade of vIL-6 secretion from cells cotransfected with MAV-KDEL cDNA was demonstrated, and it was shown that this retention led to a subsequent reduction of STAT3 phosphorylation, indicating

that vIL-6 retention within the ER abrogates vIL-6 signaling. For a targeted gene therapeutic approach of HHV-8-associated diseases, the anti-vIL-6 antibody MAV fused to the endoplasmic retention sequence KDEL (MAV-KDEL) will be useful. In this case, an expression plasmid coding for MAV-KDEL would have to be introduced into HHV-8-infected cells. Another strategy was recently presented by Cohen-Saidon et al., who described the direct delivery of single-chain Fv-transacting transcriptional activator fusion proteins into living cells. The HIV transacting transcriptional activator protein can enter and leave cells in an unconventional manner (16) and has therefore been used as a molecular tool for the delivery of proteins into living cells.

In summary, we have selected the first human recombinant antibody against vIL-6 encoded by HHV-8, which might be a promising candidate for the therapy of HHV-8-associated diseases. As described in this work, the antibody specifically recognizes and binds to native vIL-6. In vivo, MAV reduces the effect of viral IL-6 by the inhibition of vIL-6-induced signaling from inside and outside of a cell and inhibits the proliferation of the PEL-derived BCBL-1 cell line. Based on this work, a large variety of additional fusion proteins may demonstrate the potential of recombinant antibody engineering for generating novel therapeutic strategies.

ACKNOWLEDGMENTS

We thank Stefanie Schnell and Ingrid Pfort for excellent technical assistance. The expert help of Marie-Luise Kruse (University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany) with the immunofluorescence analysis is gratefully acknowledged. The help of Karl-Josef Kallen during the initial phase of the project is gratefully acknowledged. We thank Greg Winter (University of Cambridge, United Kingdom) for the generous gift of the phage display libraries.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to S.R.-J. (SFB415, project C6).

REFERENCES

- Aasland, D., B. Oppmann, J. Grötzinger, S. Rose-John, and K.-J. Kallen. 2002. The upper cytokine binding module and the Ig-like domain of the leukemia inhibitory factor (LIF) receptor are sufficient for a functional LIFR complex. *J. Mol. Biol.* **315**:637–646.
- Aasland, D., B. Schuster, J. Grötzinger, S. Rose-John, and K. J. Kallen. 2003. Analysis of the leukemia inhibitory factor receptor functional domains by chimeric receptors and cytokines. *Biochemistry* **42**:5244–5252.
- Aoki, Y., E. S. Jaffe, Y. Chang, K. Jones, J. Teruya-Feldstein, P. S. Moore, and G. Tosato. 1999. Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. *Blood* **93**:4034–4043.
- Aoki, Y., M. Narazaki, T. Kishimoto, and G. Tosato. 2001. Receptor engagement by viral interleukin-6 encoded by Kaposi sarcoma-associated herpesvirus. *Blood* **98**:3042–3049.
- Atreya, R., J. Mudter, S. Finotto, J. Müllberg, T. Jostock, S. Wirtz, M. Schütz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J. F. Schlaak, H. A. Lehr, F. Autschbach, G. Schürmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P. R. Galle, S. Rose-John, and M. F. Neurath. 2000. Blockade of IL-6 transsignaling abrogates established experimental colitis in mice by suppression of the antiapoptotic resistance of lamina propria T cells. *Nat. Med.* **6**:583–588.
- Bass, S., R. Green, and J. A. Wells. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins* **8**:309–314.
- Bazan, J. F. 1990. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* **87**:6934–6938.
- Becker, C., M. C. Fantini, C. Schramm, H. A. Lehr, S. Wirtz, A. Nikolaev, J. Burg, S. Strand, R. Kiesslich, S. Huber, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, P. R. Galle, M. Blessing, S. Rose-John, and M. F. Neurath. 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* **21**:491–501.
- Burger, R., F. Neipel, B. Fleckenstein, R. Savino, G. Ciliberto, J. R. Kalden, and M. Gramatzki. 1998. Human herpesvirus type 8 interleukin-6 homologue is functionally active on human myeloma cells. *Blood* **91**:1858–1863.
- Cannon, J. S., D. Ciuffo, A. L. Hawkins, C. A. Griffin, M. J. Borowitz, G. S. Hayward, and R. F. Ambinder. 2000. A new primary effusion lymphoma-derived cell line yields a highly infectious Kaposi's sarcoma herpesvirus-containing supernatant. *J. Virol.* **74**:10187–10193.
- Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* **332**:1186–1191.
- Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**:1865–1869.
- Chatterjee, M., J. Osborne, G. Bestetti, Y. Chang, and P. S. Moore. 2002. Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* **298**:1432–1435.
- Chen, G. C., and J. T. Yang. 1977. Two-point calibration of circular dichroism with d-10-camphorsulfonic acid. *Anal. Lett.* **10**:1195–1207.
- Chow, D.-C., X.-L. He, A. L. Snow, S. Rose-John, and K. C. Garcia. 2001. Structure of an extracellular gp130-cytokine receptor signalling complex. *Science* **291**:2150–2155.
- Cohen-Saidon, C., H. Nechushtan, S. Kahlon, N. Livni, A. Nissim, and E. Razin. 2003. A novel strategy using single-chain antibody to show the importance of Bcl-2 in mast cell survival. *Blood* **102**:2506–2512.
- Conrad, U., and J. Scheller. 2005. Considerations on antibody-phage display methodology. *Comb. Chem. High Throughput Screen.* **8**:117–126.
- Corte-Real, S., C. Collins, F. Aires da Silva, J. P. Simas, C. F. R. Barbas, Y. Chang, P. Moore, and J. Goncalves. 2005. Intrabodies targeting the Kaposi sarcoma-associated herpesvirus latency antigen inhibit viral persistence in lymphoma cells. *Blood* **106**:3797–3802.
- Darnell, J. E., I. M. Kerr, and G. R. Stark. 1994. JAK-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**:1415–1421.
- Dela Cruz, C. S., Y. Lee, S. R. Viswanathan, A. S. El-Guindy, J. Gerlach, S. Nikiforow, D. Shedd, L. Gradoville, and G. Miller. 2004. N-linked glycosylation is required for optimal function of Kaposi's sarcoma herpesvirus-encoded, but not cellular, interleukin 6. *J. Exp. Med.* **199**:503–514.
- Fischer, M., J. Goldschmidt, C. Peschel, K. J. Kallen, J. P. J. Brakenhoff, A. Wollmer, J. Grötzinger, and S. Rose-John. 1997. A designer cytokine with high activity on human hematopoietic progenitor cells. *Nat. Biotechnol.* **15**:142–145.
- Gearing, D. P., S. F. Ziegler, M. R. Comeau, D. Friend, B. Thoma, D. Cosman, L. Park, and B. Mosley. 1994. Proliferative responses and binding properties of hematopoietic cells transfected with low-affinity receptors for leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **91**:1119–1123.
- Grötzinger, J., T. Kernebeck, K.-J. Kallen, and S. Rose-John. 1999. IL-6 type cytokine receptor complexes: hexamer or tetramer or both? *Biol. Chem.* **380**:803–813.
- Grötzinger, J., G. Kurapkat, A. Wollmer, M. Kalai, and S. Rose-John. 1997. The family of the IL-6-type cytokines: specificity and promiscuity of the receptor complexes. *Protein Struct. Function Genet.* **27**:96–109.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
- Hoischen, S. H., P. Vollmer, P. März, S. Özbek, K. Götz, T. Jostock, T. Geib, J. Müllberg, S. Mechttersheimer, M. Fischer, J. Grötzinger, P. R. Galle, and S. Rose-John. 2000. Human herpesvirus 8 interleukin-6 homologue triggers gp130 on neuronal and hematopoietic cells. *Eur. J. Biochem.* **267**:3604–3612.
- Honda, M., S. Yamamoto, M. Cheng, K. Yasukawa, H. Suzuki, T. Saito, Y. Osugi, T. Tokunaga, and T. Kishimoto. 1992. Human soluble IL-6 receptor: its detection and enhanced release by HIV infection. *J. Immunol.* **148**:2175–2180.
- Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**:61–68.
- Hurst, S. M., T. S. Wilkinson, R. M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, S. Rose-John, G. M. Fuller, N. Topley, and S. A. Jones. 2001. Control of leukocyte infiltration during inflammation: IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment. *Immunity* **14**:705–714.
- Jones, K. D., Y. Aoki, Y. Chang, P. S. Moore, R. Yarchoan, and G. Tosato. 1999. Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. *Blood* **94**:2871–2879.
- Jostock, T., J. Müllberg, S. Özbek, R. Atreya, G. Blinn, N. Voltz, M. Fischer, M. F. Neurath, and S. Rose-John. 2001. Soluble gp130 is the natural inhibitor of soluble IL-6R transsignaling responses. *Eur. J. Biochem.* **268**:160–167.
- Klouché, M., S. Bhakdi, M. Hemmes, and S. Rose-John. 1999. Novel path of activation of primary human smooth muscle cells: upregulation of gp130 creates an autocrine activation loop by IL-6 and its soluble receptor. *J. Immunol.* **163**:4583–4589.
- Klouché, M., N. Brockmeyer, C. Knabbe, and S. Rose-John. 2002. Human herpesvirus 8 derived viral interleukin-6 induces PTX3 expression in Kaposi's sarcoma cells. *AIDS* **16**:9–18.
- Kohfeldt, E., P. Maurer, C. Vannahme, and R. Timpl. 1997. Properties of the extracellular calcium binding module of the proteoglycan testican. *FEBS Lett.* **414**:557–561.

35. Kollet, O., R. Aviram, J. Chebath, H. ben-Hur, A. Nagler, L. Shultz, M. Revel, and T. Lapidot. 1999. The soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro maintenance and proliferation of human CD34(+)/CD38(-/low) cells capable of repopulating severe combined immunodeficiency mice. *Blood* **94**:923-931.
36. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.
37. Luppi, M., P. Barozzi, A. Maiorana, R. Trovato, R. Marasca, M. Morselli, K. Cagossi, and G. Torelli. 1999. Expression of cell-homologous genes of human herpesvirus-8 in human immunodeficiency virus-negative lymphoproliferative diseases. *Blood* **94**:2931-2933.
38. Mackiewicz, A., H. Schooltink, P. C. Heinrich, and S. Rose-John. 1992. Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins. *J. Immunol.* **149**:2021-2027.
39. März, P., J.-C. Cheng, R. A. Gadiant, P. Patterson, T. Stoyan, U. Otten, and S. Rose-John. 1998. Sympathetic neurons can produce and respond to interleukin-6. *Proc. Natl. Acad. Sci. USA* **95**:3251-3256.
40. März, P., K. Heese, B. Dimitriades-Schmutz, S. Rose-John, and U. Otten. 1999. Role of interleukin-6 and soluble IL-6 receptor in region specific induction of astrocytic differentiation and neurotrophin expression. *Glia* **26**:191-200.
41. McCafferty, J., A. D. Griffiths, G. Winter, and D. J. Chiswell. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**:552-554.
42. McMahan, C. J., J. L. Slack, B. Mosley, D. Cosman, S. D. Lupton, L. L. Brunton, C. E. Grubin, J. M. Wignall, N. A. Jenkins, C. I. Brannan, N. G. Copeland, K. Huebner, C. M. Croce, L. A. Cannizzaro, D. Benjamin, S. K. Dower, M. K. Spriggs, and J. E. Sims. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J.* **10**:2821-2832.
43. Meads, M. B., and P. G. Medveczky. 2004. Kaposi's sarcoma-associated herpesvirus encoded viral interleukin-6 is secreted and modified differently than human interleukin-6: evidence for a unique autocrine signaling mechanism. *J. Biol. Chem.* **279**:51793-51803.
44. Molden, J., Y. Chang, Y. You, P. S. Moore, and M. A. Goldsmith. 1997. A Kaposi's sarcoma-associated herpesvirus-encoded cytokine homolog (vIL-6) activates signaling through the shared gp130 receptor subunit. *J. Biol. Chem.* **272**:19625-19631.
45. Moore, P. S., C. Boshoff, R. A. Weiss, and Y. Chang. 1996. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* **274**:1739-1744.
46. Müllberg, J., T. Geib, T. Jostock, S. H. Hoischen, P. Vollmer, N. Voltz, D. Heinz, P. R. Galle, M. Klouche, and S. Rose-John. 2000. IL-6-receptor independent stimulation of human gp130 by viral IL-6. *J. Immunol.* **164**:4672-4677.
47. Nador, R. G., E. Cesarman, A. Chadburn, D. B. Dawson, M. Q. Ansari, J. Sald, and D. M. Knowles. 1996. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood* **88**:645-656.
48. Neipel, F., J. C. Albrecht, A. Ensser, Y. Q. Huang, J. J. Li, A. E. F. Kien, and B. Fleckenstein. 1997. Human herpesvirus 8 encodes a homolog of interleukin-6. *J. Virol.* **71**:839-842.
49. Nishi, J., and I. Maruyama. 2000. Increased expression of vascular endothelial growth factor (VEGF) in Castleman's disease: proposed pathomechanism of vascular proliferation in the affected lymph node. *Leuk. Lymphoma* **38**:387-394.
50. Novick, D., H. Engelmann, D. Wallach, and M. Rubinstein. 1989. Soluble cytokine receptors are present in normal human urine. *J. Exp. Med.* **170**:1409-1414.
51. Nowell, M. A., P. J. Richards, S. Horiuchi, N. Yamamoto, S. Rose-John, N. Topley, A. S. Williams, and S. A. Jones. 2003. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *J. Immunol.* **171**:3202-3209.
52. Oksenhendler, E., E. Boulanger, L. Galicier, M. Q. Du, N. Dupin, T. C. Diss, R. Hamoudi, M. T. Daniel, F. Agbalika, C. Boshoff, J. P. Clauvel, P. G. Isaacson, and V. Meignin. 2002. High incidence of Kaposi sarcoma-associated herpesvirus-related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. *Blood* **99**:2331-2336.
53. O'Leary, J., M. Kennedy, D. Howells, I. Silva, V. Uhlmann, K. Luttich, S. Biddolph, S. Lucas, J. Russell, N. Bermingham, M. O'Donovan, M. Ring, C. Kenny, M. Sweeney, O. Sheils, C. Martin, S. Picton, and K. Gatter. 2000. Cellular localisation of HHV-8 in Castleman's disease: is there a link with lymph node vascularity? *Mol. Pathol.* **53**:69-76.
54. O'Shannessy, D. J., M. Brigham-Burke, K. K. Sonesson, P. Hensley, and I. Brooks. 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of non-linear least squares analysis methods. *Anal. Biochem.* **212**:457-468.
55. Palacios, R., and M. Steinmetz. 1985. IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. *Cell* **41**:727-734.
56. Parravicini, C., E. Lauri, L. Baldini, A. Neri, F. Poli, G. Sirchia, M. Moroni, M. Galli, and M. Corbellino. 1997. Kaposi's sarcoma-associated herpesvirus infection and multiple myeloma. *Science* **278**:1969-1970.
57. Parravicini, C., M. Corbellino, M. Paulli, U. Magrini, M. Lazzarino, P. S. Moore, and Y. Chang. 1997. Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. *Am. J. Pathol.* **151**:1517-1522.
58. Pelham, R. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* **15**:483-486.
59. Peters, M., S. Jacobs, M. Ehlers, P. Vollmer, J. Müllberg, E. Wolf, G. Brem, K. H. Meyer zum Büschenfelde, and S. Rose-John. 1996. The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6. *J. Exp. Med.* **183**:1399-1406.
60. Peters, M., A. Müller, and S. Rose-John. 1998. Interleukin-6 and soluble interleukin-6 receptor: direct stimulation of gp130 and hematopoiesis. *Blood* **92**:3495-3504.
61. Peters, M., P. Schirmacher, J. Goldschmidt, M. Odenthal, C. Peschel, H. P. Dienes, E. Fattori, G. Ciliberto, K. H. Meyer zum Büschenfelde, and S. Rose-John. 1997. Extramedullary expansion of hematopoietic progenitor cells in IL-6/sIL-6R double transgenic mice. *J. Exp. Med.* **185**:755-766.
62. Pflanz, S., L. Hibbert, J. Mattson, R. Rosales, E. Vaisberg, J. F. Bazan, J. H. Phillips, T. K. McClanahan, R. de Waal Malefyt, and R. A. Kastelein. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J. Immunol.* **172**:2225-2231.
63. Raje, N., J. Gong, D. Chauhan, G. Teoh, D. Avigan, Z. Wu, D. Chen, S. P. Treon, I. J. Webb, D. W. Kufe, and K. C. Anderson. 1999. Bone marrow and peripheral blood dendritic cells from patients with multiple myeloma are phenotypically and functionally normal despite the detection of Kaposi's sarcoma herpesvirus gene sequences. *Blood* **93**:1487-1495.
64. Renné, C., K.-J. Kallen, J. Müllberg, T. Jostock, J. Grötzinger, and S. Rose-John. 1998. A new type of cytokine receptor antagonist directly targeting gp130. *J. Biol. Chem.* **273**:27213-27219.
65. Rettig, M. B., H. J. Ma, R. A. Vescio, M. Pold, G. Schiller, D. Belson, A. Savage, C. Nishikubo, C. Wu, J. Fraser, J. W. Said, and J. R. Berenson. 1997. Kaposi's sarcoma-associated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients. *Science* **276**:1851-1854.
66. Rojas, A., C. Almoguera, and J. Jordano. 1999. Transcriptional activation of a heat shock gene promoter in sunflower embryos: synergism between ABI3 and heat shock factors. *Plant J.* **20**:601-610.
67. Romano, M., M. Sironi, C. Toniatti, N. Polentatutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, F. Bussolino, V. Poli, G. Ciliberto, and A. Mantovani. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* **6**:315-325.
68. Rose-John, S., and P. C. Heinrich. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.* **300**:281-290.
69. Rose-John, S., H. Schooltink, H. Schmitz-Van de Leur, J. Müllberg, P. C. Heinrich, and L. Graeve. 1993. Intracellular retention of interleukin-6 abrogates signaling. *J. Biol. Chem.* **268**:22084-22091.
70. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
71. Scheller, J., M. Kovaleva, J. Eichler, K.-J. Kallen, and S. Rose-John. 2004. Development of a monoclonal-antibody-based enzyme linked immunoabsorbent assay for the IL-6/IL-6R interaction and its competitive inhibition. *J. Immunol. Methods* **291**:93-100.
72. Scheller, J., B. Schuster, C. Holscher, T. Yoshimoto, and S. Rose-John. 2005. No inhibition of IL-27 signaling by soluble gp130. *Biochem. Biophys. Res. Commun.* **326**:724-728.
73. Smith, G. P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**:1315-1317.
74. Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. C. Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, and F. Sigaux. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* **86**:1276-1280.
75. Staskus, K. A., R. Sun, G. Miller, P. Racz, A. Jaslowski, C. Metroka, H. Brett-Smith, and A. T. Haase. 1999. Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *J. Virol.* **73**:4181-4187.
76. Taga, T., and T. Kishimoto. 1997. gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* **15**:797-819.
77. Udagawa, N., N. J. Horwood, J. Elliott, A. Mackay, J. Owens, H. Okamura, M. Kurimoto, T. J. Chambers, T. J. Martin, and M. T. Gillespie. 1997. Interleukin-18 (interferon-gamma-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation. *J. Exp. Med.* **185**:1005-1012.
78. Wan, X., H. Wang, and J. Nicholase. 1999. Human herpesvirus 8 interleukin-6 (IL-6) signals through gp130 but has structural and receptor-binding properties distinct from those of human IL-6. *J. Virol.* **73**:8268-8278.
79. Waxman, E., E. Rusinova, C. A. Hasselbacher, G. P. Schwartz, W. R. Laws, and J. B. Ross. 1993. Determination of the tryptophan:tyrosine ratio in proteins. *Anal. Biochem.* **210**:425-428.