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(54) **Title**: ASGPR ANTIBODIES AND USES THEREOF

(57) **Abstract**: The present invention generally relates to antibodies specific for asialoglycoprotein receptor (ASGPR) and their use for selectively delivering effector moieties that influence cellular activity. In addition, the present invention relates to polynucleotides encoding such antibodies, and vectors and host cells comprising such polynucleotides. The invention further relates to methods for producing the antibodies of the invention, and to methods of using them in the treatment of disease.

ASGPR ANTIBODIES AND USES THEREOF

Field of the Invention

The present invention generally relates to antibodies specific for asialoglycoprotein receptor (ASGPR) and their use for selectively delivering effector moieties that influence cellular activity. In addition, the present invention relates to polynucleotides encoding such antibodies, and vectors and host cells comprising such polynucleotides. The invention further relates to methods for producing the antibodies of the invention, and to methods of using them in the treatment of disease.

Background

Asialoglycoprotein receptor (ASGPR) is a transmembrane receptor composed of two subunits, H1 and H2. The subunits are believed to oligomerize throught their extracellular stalk regions. ASGPR is a member of the C-type lectin family (calcium-ion dependent lectin) and mediates the endocytosis and degradation of a wide variety of desialylated glycoproteins. ASGPR is selectively expressed on liver parenchymal cells (hepatocytes), which makes it an attractive target for liver-specific therapies. Many liver diseases, e.g. hepatitis, liver cirrhosis or hepatocellular carcinoma (HCC), can be caused directly or indirectly by viral infection, such as hepatitis virus B (HBV) or C (HCV) infection. Chronic infection with HCV is one of the major causes of cirrhosis and HCC. Similarly, chronic HBV infection accounts for 5-10% of chronic liver disease and cirrhosis in the US. Approved therapies for HBV and HCV infection include interferons (IFN), such as interferon alpha. However, side effects have hampered development and widespread use of these therapies in many cases. Such IFN-associated side effects are thought to be due in part to induction of interferon-stimulated genes (ISGs) in peripheral blood cells following systemic exposure to IFN. Hence, to minimize side effects associated with IFN therapy for liver diseases, and also to augment the antiviral effect of conventional interferons, it is desirable to selectively deliver IFN to the liver. ASGPR has been recognized as potential target molecule on hepatocytes for such selective delivery. For example, WO 92/22310 describes an approach for targeting interferon to the liver by conjugation of recombinant IFN to an asialoglycoprotein. In a similar approach, an interferon molecule itself has been modified to

produce asialo-interferon for binding to ASGPR (Eto and Takahashi, Nat Med 5, 577-581 (1999)) More recently, an approach based on anti-ASGPR single variable domain (dAb) antibodies has been described (WO 2011/086143).

However, none of these approaches has been shown to be clinically successful so far, and there remains a need for improved targeting molecules for selective delivery of therapeutic molecules, e.g. interferon, to the liver. The antibodies of the present invention combine several advantageous properties, which make them particularly suitable for targeting effector moieties such as interferons to ASGPR-expressing cells, e.g. for the treatment of liver diseases.

Summary of the Invention

In one aspect, the invention provides an antibody capable of specific binding to asialoglycoprotein receptor (ASGPR), wherein the antibody comprises a) the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14; b) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2; c) the heavy chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 6; d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 10; e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 18; f) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22; g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 26; h) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 30; i) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 32; j) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 34; or k) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22.

In a particular embodiment, the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14. In another particular embodiment, the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2.

In a further aspect, the invention provides an antibody capable of specific binding to ASGPR, wherein the antibody competes for binding to an epitope of ASGPR with an antibody comprising the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14. In one embodiment, said antibody recognizes an epitope in the stalk region of ASGPR. In one embodiment, said antibody is an affinity matured variant of the antibody comprising the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14. In one embodiment, said antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 16, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 14. In one embodiment, said antibody comprises the light chain variable region sequence of SEQ ID NO: 14 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. In one embodiment, said antibody comprises the heavy chain variable region sequence of SEQ ID NO: 16 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions.

In still a further aspect, the invention provides a an antibody capable of specific binding to ASGPR, wherein the antibody competes for binding to an epitope of ASGPR with an antibody comprising the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2. In one embodiment, said antibody recognizes an epitope in the carbohydrate recognition domain (CRD) of ASGPR. In one embodiment, said antibody is an affinity matured variant of the antibody comprising the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2. In one embodiment, said antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 4, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 2. In one embodiment, said antibody comprises the light chain variable region sequence of SEQ ID NO: 2 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. In one embodiment, said antibody comprises the heavy chain variable region sequence of SEQ ID NO: 4 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. In one embodiment, said antibody comprises a) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 36; b) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 38; c) the heavy

chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 40; d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 42; e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 44; f) the heavy
5 chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 46; or g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 48.

In one embodiment, the antibody of the invention is capable of specific binding to human and cynomolgus monkey ASGPR. In one embodiment, the antibody binds to human ASGPR with an
10 dissociation constant (K_D) of smaller than 1 μM, particularly smaller than 100 nM, more particularly smaller than 1 nM, when measured as Fab fragment by Surface Plasmon Resonance (SPR). In one embodiment, the antibody binds to human ASGPR with a K_D of smaller than 1 μM, particularly smaller than 500 nM, more particularly smaller than 100 nM or even smaller than 10 nM, when measured as IgG_1 by fluoresecence resonance energy transfer (FRET). In one
15 embodiment, the antibody does not compete with a natural ligand of ASGPR for binding to ASGPR. In a specific embodiment, said natural ligand of ASGPR is asialofetuin. In one embodiment, the antibody does not detectably bind to CLEC10A, particularly human CLEC10A. In one embodiment, the antibody does not specifically bind to cells lacking ASGPR expression, particularly human cells, more particularly human blood cells. In one embodiment, the antibody
20 is internalized into a cell expressing ASGPR upon binding of the antibody to ASGPR on the surface of said cell. In a specific embodiment, the antibody is recycled back to the surface of said cell at about the same rate as it is internalized into said cell. In one embodiment, the antibody does not significantly induce downregulation of ASGPR expression at the surface of a cell upon binding of the antibody to ASGPR on the surface of said cell.

25 In one embodiment, the antibody of the invention is a human antibody. In one embodiment, the antibody comprises a human Fc region, particularly an IgG Fc region, more particularly an IgG_1 Fc region. In one embodiment, the antibody is a full-length antibody. In one embodiment, the antibody is an IgG class antibody, particularly an IgG_1 subclass antibody. In one embodiment, the antibody comprises in the Fc region a modification reducing binding affinity of the antibody
30 to an Fc receptor, particularly an Fcγ receptor. In a specific embodiment, said Fc receptor is an activating Fc receptor. In a further specific embodiment, said Fc receptor is selected from the group of FcγRIIIa (CD16a), FcγRI (CD64), FcγRIIa (CD32), and FcαRI (CD89). In a more

specific embodiment, said Fc receptor is FcγRIIIa, particularly human FcγRIIIa. In one embodiment, the antibody comprises an amino acid substitution in the Fc region at a position selected from P329, L234 and L235 (EU numbering). In one embodiment, the antibody comprises the amino acid substitutions P329G, L234A and L235A in the Fc region (EU 5 numbering). In a further embodiment, the antibody comprises in the Fc region a modification promoting heterodimerization of two non-identical antibody heavy chains. In a specific embodiment, said modification is a knob-into-hole modification, comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the two antibody heavy chains. In one embodiment, the antibody comprises a modification within the interface 10 between the two antibody heavy chains in the CH3 domain, wherein i) in the CH3 domain of one heavy chain, an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance ("knob") within the interface in the CH3 domain of one heavy chain which is positionable in a cavity ("hole") within the interface in the CH3 domain of the other heavy chain, and ii) in the CH3 domain of the other heavy chain, an 15 amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity ("hole") within the interface in the second CH3 domain within which a protuberance ("knob") within the interface in the first CH3 domain is positionable. In one embodiment, the antibody comprises the amino acid substitution T366W and optionally the amino acid substitution S354C in one of the antibody heavy chains, and the amino acid 20 substitutions T366S, L368A, Y407V and optionally Y349C in the other one of the antibody heavy chains.

In one aspect, the invention provides an antibody capable of specific binding to ASGPR according to any of the above embodiments, wherein an effector moiety is attached to the antibody. In one embodiment, not more than one effector moiety is attached to the antibody. In 25 one embodiment said effector moiety is a cytokine molecule. In one embodiment, said cytokine molecule is fused at its amino-terminal amino acid to the carboxy-terminal amino acid of one of the antibody heavy chains, optionally through a peptide linker. In one embodiment, said cytokine molecule is a human cytokine. In one embodiment, said cytokine molecule is an interferon molecule. In a specific embodiment, said interferon molecule is interferon alpha, particularly 30 human interferon alpha, more particularly human interferon alpha 2 (see SEQ ID NO: 138) or human interferon alpha 2a (see SEQ ID NO: 139). In one embodiment, wherein the cytokine molecule is an interferon molecule, the antibody has anti-viral activity in cells expressing ASGPR on their surface. In a specific embodiment, the antibody has no anti-viral activity in cells

not expressing significant levels of ASGPR on their surface. In a further specific embodiment, said anti-viral activity is selected from inhibition of viral infection, inhibition of virus replication, inhibition of cell killing and induction of interferon-stimulated genes.

The invention further provides a polynucleotide encoding the antibody of the invention. Further provided is a vector, particularly an expression vector, comprising the polynucleotide of the invention. In another aspect, the invention provides a host cell comprising the polynucleotide or the vector of the invention. The invention also provides a method for producing an antibody of the invention, comprising the steps of (i) culturing the host cell of the invention under conditions suitable for expression of said antibody, and (ii) recovering said antibody. Also provided is an antibody capable of specific binding to ASGPR, produced by said method.

In one aspect, the invention provides a pharmaceutical composition comprising the antibody of the invention and a pharmaceutically acceptable carrier. The antibody or the pharmaceutical composition of the invention is also provided for use as a medicament, and for use in the treatment or prophylaxis of a liver disease, specifically a viral infection, more specifically hepatitis virus infection, particularly hepatitis B virus (HBV) infection. The antibody or the pharmaceutical composition of the invention is also provided for use in the treatment or prophylaxis of cancer, specifically liver cancer, more specifically hepatocellular carcinoma (HCC). Further provided is the use of the antibody of the invention for the manufacture of a medicament for the treatment of a disease in an individual in need thereof, and a method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the antibody of the invention in a pharmaceutically acceptable form. In one embodiment, said disease is a liver disease. In a more specific embodiment, said liver disease is a viral infection. In an even more specific embodiment, said liver disease is hepatitis virus infection, particularly HBV infection. In another embodiment, said disease is cancer. In a more specific embodiment, said cancer is liver cancer. In an even more specific embodiment, said liver cancer is hepatocellular carcinoma (HCC). In one embodiment, said individual is a mammal, particularly a human. In a further aspect, the antibody of the invention is provided for targeting a cell expressing ASGPR in an individual. Also provided is a method for targeting a cell expressing ASGPR in an individual, comprising administering to said individual a composition comprising the antibody of the invention in a pharmaceutically acceptable form. In one embodiment, said cell is a liver cell, particularly a hepatocyte. In one embodiment, said individual is a mammal, particularly a human.

Brief Description of the Drawings

Figure 1. Schematic diagram of the generated antigen constructs. The nucleotide sequences of all antigens were fused to the C-terminal end of a human-derived IgG_1 Fc sequence. The ASGPR1- and CLEC10A-derived CRDs were fused to a sequence encoding an Fc(hole) fragment and co-expressed with a sequence encoding an Fc(knob) fragment resulting in a monomeric display per Fc dimer. From left to right: Fc-CRD (ASGPR1), Fc-stalk (ASGPR1), Fc-stalk-CRD (ASGPR1), Fc-CRD (CLEC10A), Fc-stalk (CLEC10A), Fc-stalk-CRD (CLEC10A). Thick, curved line: $(G_4S)_3$ linker; thick, straight line: Xa and IgAse cleavage site.

Figure 2. Schematic overview of the generation of the generic Fab library randomized in the CDR3 regions of the heavy and the light chain. In a first step, three PCR fragments were generated which were then fused by (splicing by overlapping extension; SOE) PCR. The final fragment was gel-purified, digested with *Nco*I / *Nhe*I alongside with similarly treated acceptor phagemid, ligated and transformed into bacteria. PCR1 (A, B, C): (1) LMB3; (2) (A) VI_3_19_L3r_V / (B) VI_3_19_L3r_HV / (C) VI_3_19_L3r_HLV. PCR2: (3) RJH80; (4) DP47CDR3_ba (mod). PCR3 (A, B, C): (5) (A) DP47 v4 4 / (B) DP47 v4 6 / (C) DP47 v4 8; (6) fdseqlong.

Figure 3. Binding analysis of selected anti-human ASGPR H1-specific clones to HepG2 cells as human IgG_1 antibodies. Antibody concentration was 30 μg/ml. An isotype control antibody served as a negative control.

Figure 4. FRET analysis on transiently transfected cells expressing a transmembrane ASGPR H1-SNAP tag fusion protein labeled with terbium. Analysis was done by adding antibodies at a concentration ranging from 50-0.39 nM followed by addition of an anti-humanFc-d2 (final 200 nM per well) as acceptor molecule. Specific FRET signal was measured after 3 h and KD values were calculated (KD_{51A12} = 200 nM, KD_{R7F12} = 22 nM, KD_{R9E10} = 6.2 nM, KD_{R5C2} = 5.9 nM, KD_{4F3} = 4.5 nM).

Figure 5 and 6. Competition of asialofetuin, a natural ligand for ASGPR, and anti-ASGPR H1 antibodies. HepG2 cells were pre-incubated with labeled asialofetuin before indicated antibodies were added in a dilution row to the cells. Binding of both components to the cells was analyzed by FACS analysis. (A) antibody detection; (B) asialofetuin detection.

Figure 7. Internalization analysis of the two anti-human ASGPR H1 antibody clones 51A12 and 4F3 as IgGs. (A) antibodies were incubated with HepG2 cells at 4°C to prevent internalization, and washed at 4°C before the cells were cultured in pre-warmed medium and incubated at 37°C for up to 120 min. Samples were taken at indicated time points, labeled with the secondary

antibody on ice and fixed using PFA. (B) Same steps were performed as described under (A) but antibodies were incubated with the cells at 37°C allowing ASGPR to internalize. (C) Same steps were performed as described under (A), but using directly FITC-labeled antibodies. Cell surface-bound antibodies were detected using PE-conjugated anti-Fc antibody. (D) Same experiment as under (C) but showing FITC signal, representing both surface-exposed and internalized antibodies.

Figure 8. Randomization strategy of the LCDR3 region of clone 51A12. Shown are (A) the LCDR3 protein sequence of the parental clone 51A12, (B) the LCDR3 protein sequence of the plasmid serving as a template for the library without cysteines and glycosylation sequence, and (C) the randomized positions in LCDR3. During generation of the library, trinucleotide primers allow to exclude triplets encoding cysteines or amino acids contributing to the formation of a glycosylation site.

Figure 9. Schematic overview of the generation of the affinity maturation library randomized in LCDR3 of the template 51A12 (A82G, C112S, C113S, S116A) (SEQ ID NO: 33). In a first step, two PCR fragments were generated which were then fused by (SOE) PCR. The final fragment was gel-purified, digested with *Nco*I / *Pst*I alongside with similarly treated acceptor phagemid, ligated and transformed into bacteria. PCR1: (1) LMB3, (2) LCDR3rev. PCR2: (3) LCDR3rand, (4) fdseqlong.

Figure 10. Binding analysis of affinity-matured 51A12-derived clones to HepG2 cells as human Fab fragments. Fab concentrations of 10, 3.3, and 1.1 μg/ml were used. The parental clone 51A12 (SEQ ID NOs 2 and 4), 51A12 (S116A) (SEQ ID NOs 4 and 30), a clone devoid of the glycosylation sequence, and 51A12 (A82G, C112S, C113S, S116A) (SEQ ID NOs 4 and 34), the template clone for the affinity maturation library, served as controls.

Figure 11. Binding analysis of affinity-matured 51A12-derived clones to HepG2 cells as human IgG_1 antibodies. Concentrations in a dilution row ranging from 0.01 to 20 μg/ml were used. The parental clone 51A12 (SEQ ID NOs 2 and 4) served as a control (A). Binding analysis to Hela cells at a concentration of 10 μg/ml was used as a negative control (B).

Figure 12. Schematic diagram of the generated antibody-cytokine conjugates. The gene encoding interferon-α2a was fused to the C terminal end of an ASGPR H1-specific antibody heavy chain comprising a knob modification. While bivalent ASGPR binding of the antibody-cytokine protein was achieved by co-expression of the corresponding ASGPR H1-specific heavy chain comprising a hole modification and the light chain ((A), 2:1 valency), expression of a Fc(hole) fragment sequence resulted in a monomeric antibody-cytokine conjugate with only one ASGPR

H1-specific binding site per molecule ((B), 1:1 valency). Small black dots: modification preventing FcγR binding (for example L234A L235A P329G). Large black dots: modification promoting heterodimerization (for example knob-into-hole).

Figures 13-19. Purification and analytical characterization of selected antibody-IFNα immunoconjugates (Fig. 13: 51A12 kih IgG-IFNα; Fig. 14: 4F3 kih IgG-IFNα; Fig. 15: 51A12 (C7) kih IgG-IFNα; Fig. 16: 51A12 (C1) kih IgG-IFNα; Fig. 17: 51A12 (E7) kih IgG-IFNα; Fig. 18: isotype control kih IgG-IFNα; Fig. 19: monovalent 51A12 kih IgG-IFNα). The purification method involved an affinity step (protein A) (A) followed by size exclusion chromatography (Superdex 200, GE Healthcare) (B). The final product was analyzed and characterized by analytical size exclusion chromatography (Superdex 200 column) (C) and microfluidic protein analysis (Caliper) or SDS-PAGE (D).

Figure 20. Binding selectivity of ASGPR-specific IgG kih IFNα fusion constructs 51A12 (A) and 4F3 (B). HepG2, primary human hepatocytes, Huh-7 cells, A549 cells, Hela cells, and 293T cells were incubated with 1 µg/ml 51A12-IFNα (A) or 4F3-IFNα (B) for 45 min on ice. After three washes, cells were stained with secondary goat anti-human IgG antibody on ice for 30 min, and the cells were washed three times before being analyzed using a Calibur flow cytometer. Binding to human PBMC was performed by using 1 µg/ml of directly labeled 51A12 IgG kih IFNα (A) and 4F3 IgG kih IFNα (B) using the Zenon® R-Phycoerythrin Human IgG Labeling Kit according to manufacturer's instructions, isotype IgG kih IFNα and a CD81 mAb were used as negative and positive controls, respectively.

Figure 21. Binding saturation curves of ASGPR mAb 4F3-IFNα on primary human hepatocytes and HepG2 cells. Binding saturation of ASGPR mAb 4F3-IFNα on human hepatocytes derived from three different donors (A-C) and HepG2 cells (D). Cells were incubated with serially diluted 4F3 IgG kih IFNα for 45 min on ice. After three washes, cells were stained with secondary goat anti-human IgG antibody on ice for 30 min, and washed again three times before being analyzed using a Calibur flow cytometer.

Figure 22. Analysis of the surface-exposed levels of ASGPR over time in presence of specific antibodies. HepG2 cells were incubated with either ASGPR-specific clone 51A12 IgG or the corresponding monovalent or bivalent antibody-cytokine conjugate. Cell samples were taken after up to 5 hrs and binding of the IgG constructs to ASGPR was measured by detection of either the antibody (A) or the cytokine (B). An anti-CD20 antibody (GA101) was used as a negative control.

Figure 23. Rapid internalization of clone 51A12 antibody-cytokine conjugate. Alexa488-labeled 51A12 IgG kih IFNα construct was incubated with HepG2 cells and ASGPR-mediated internalization of the construct was recorded by confocal microscopy for 1 h over 10 stacks (z-level). Binding of the antibody-cytokine conjugate on the cell surface occurs in clusters rather than homogenous distribution (A). Once bound to the cell surface, the conjugate internalizes very rapidly in vesicles which are transported into the cell body (B, single cell surrounded). Vesicles are then recycled back to the apical side of the cell (not shown).

Figure 24. Antiviral activity of ASGPR mAb-IFNα molecules and other control IFN molecules in EMCV CPE (A) and HCV replicon (B) assays. (A) Hela cells were pretreated with serially diluted IFN molecules for 3 h before adding EMCV virus. Cells were cultured for 24 h, and cell viability was measured by adding CellTiter Glo. (B) Huh-7 2209 replicon cells were treated with serially diluted IFN molecules and luciferase activity was measured after 3 days.

Figure 25. ISG induction by 51A12-IFNα in various hepatic and non-hepatic cells. Hepatic cells (primary hepatocytes (B) and HepG2 (A)) and non-hepatic cells (human PBMC (D) and Hela (C)) were treated with various serially diluted IFNα molecules for 6 h, total RNA was extracted and TaqMan RT-PCR was used to quantify ISG MX1 (A, C) and Rsad2 (B, D) gene expression. Data shown are from three or more experiments.

Figure 26. Sustained ISG induction by 51A12-IFNα and 4F3-IFNα in primary human hepatocytes (PHH) (B) and Huh7 (A) cells. Primary human hepatocytes (PHH) and Huh7 cells were treated with serially diluted IFNα molecules for 6 h (left) and 72 h (right), total RNA was extracted and TaqMan RT-PCR was used to quantify ISG MX1 (A) and Rsad2 (B) gene expression.

Figure 27. Representative ISG expression in monkey liver samples. Monkey liver samples from the four dose groups collected at different time points were analyzed by microarray. Expression of four representative ISG genes is shown in a 3D graph. The four dose groups are indicated. For each dose group, from left to right, bars represent ISG fold induction at day -5, day 2, day 4, and day 8 of the 3 monkeys.

Figure 28. Blood and liver gene expression heatmaps (IFN module M3.1). mRNA microarray analysis was performed on blood PBMC and liver biopsy samples, and their IFNα response was analyzed with gene modules determined from blood transcriptomics studies (Chaussabel et al. (2008), Immunity 29, 150-64). In panel (A), the fold-change expression values from baseline (see inset) for the genes of the interferon module M3.1 were plotted in heatmap form for both blood and liver samples using the R statistics package (www.r-project.org). Non-supervised

hierarchical clustering of the liver interferon-induced genes reveals a highly induced subset (dashed rectangle) in the 10 μg/kg dose of 51A12 but not the isotype-IFNα compound at days 1 and 3. (B) This subset of genes was plotted for both liver and blood. Non-supervised hierarchical clustering this subset reveals a differential pattern of expression between blood and liver where
5 the upper half of the heatmap shows induction in liver for 51A12 but not isotype-IFNα at the 10 μg/kg dose and in the lower half, induction in blood only for isotype-IFNα at the high dose.

Detailed Description of the Invention

Definitions

Terms are used herein as generally used in the art, unless otherwise defined in the following.
10 "Asialoglycoprotein receptor", abbreviated as ASGPR, refers to any native ASGPR from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed ASGPR as well as any form of ASGPR that results from processing in the cell. The term also encompasses naturally occurring variants of ASGPR, e.g.,
15 splice variants or allelic variants. In one embodiment, the antibody of the invention is capable of specific binding to human ASGPR, particularly human ASGPR H1, more particularly the extracellular domain of human ASGPR H1.
The amino acid sequence of human ASGPR H1 (also known as CLEC4H1) is shown in UniProt (www.uniprot.org) accession no. P07306 (version 131), or NCBI (www.ncbi.nlm.nih.gov/)
20 RefSeq NP_001662.1. The extracellular domain (ECD) of human ASGPR H1 extends from amino acid position 62 to 291. The nucleotide and amino acid sequences of a human ASGPR H1 ECD fused to a human Fc region is shown in SEQ ID NOs 129 and 130, respectively. The ASGPR H1 ECD comprises the stalk region, which extends from amino acid position 62 of the full sequence to around amino acid position 160 (SEQ ID NOs 123 and 124 show nucleotide and
25 amino acid sequences of a human ASGPR H1 stalk region fused to a human Fc region), and the carbohydrate recognition domain (CRD), which extends from around amino acid position 161 of the full sequence to around amino acid position 278 (SEQ ID NOs 117 and 118 show nucleotide and amino acid sequences of a human ASGPR H1 CRD region fused to a human Fc region).
In one embodiment, the antibody is also capable of binding to cynomolgus ASGPR, particularly
30 cynomolgus ASGPR H1, more particularly the extracellular domain of cynomolgus ASGPR H1. The sequence of cynomolgus ASGPR H1 is shown in NCBI GenBank accession no.

EHH57654.1. SEQ ID NOs 131 and 132 show the nucleotide and amino acid sequences, respectively, of a cynomolgus ASGPR H1 ECD fused to a human Fc region.

By “human CLEC10A” is meant the protein described in UniProt accession no. Q8IUN9 (version 86), particularly the extracellular domain of said protein which extends from amino acid position 61 to amino acid position 316 of the full sequence. SEQ ID NOs 133 and 134 show the nucleotide and amino acid sequences, respectively, of a human CLEC10A ECD fused to a human Fc region.

As used herein, the term “conjugate” refers to a fusion polypeptide molecule that includes one effector moiety and a further peptide molecule, particularly an antibody. A fusion protein of an antibody and an effector moiety is referred to as an “immunoconjugate”. An (immune)conjugate as referred to herein is a fusion protein, i.e. the components of the (immune)conjugate are linked to each other by peptide-bonds, either directly or through peptide linkers.

An “epitope” is a region of an antigen that is bound by an antibody. The term refers to a site (e.g. a contiguous stretch of amino acids or a conformational configuration made up of different regions of non-contiguous amino acids) on a polypeptide macromolecule to which an antibody binds, forming an antibody-antigen complex.

An “antibody that competes for binding to an epitope” with a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

By "specific binding" is meant that the binding is selective for the antigen and can be discriminated from unwanted or non-specific interactions. The ability of an antibody to bind to a specific antigen can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. Surface Plasmon Resonance (SPR) technique (analyzed on a BIAcore instrument) (Liljeblad et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). In one embodiment, the extent of binding of an antibody to an unrelated protein is less than about 10% of the binding of the antibody to the antigen as measured, e.g. by SPR. In certain embodiments, an antibody that binds to the antigen has a dissociation constant (K_D) of $\leq 1\ \mu M$, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, ≤ 0.1 nM, ≤ 0.01 nM, or ≤ 0.001 nM (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g. from 10^{-9} M to 10^{-13} M).

"Affinity" or "binding affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g. an antibody) and its binding partner (e.g. an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g. antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D), which is the ratio of dissociation and association rate constants (k_{off} and k_{on}, respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by common methods known in the art, including those described herein. A particular method for measuring affinity is Surface Plasmon Resonance (SPR).

"Reduced binding", for example reduced binding to an Fc receptor, refers to a decrease in affinity for the respective interaction, as measured for example by SPR. For clarity the term includes also reduction of the affinity to zero (or below the detection limit of the analytic method), i.e. complete abolishment of the interaction. Conversely, "increased binding" refers to an increase in binding affinity for the respective interaction.

By "internalization" is meant the removal of a molecule from the surface from a cell by uptake of said molecule into the intracellular space. A particular form of internalization is receptor-mediated endocytosis, which occurs upon binding of a ligand or antibody to a cell surface (membrane-spanning) receptor, by inward budding of plasma membrane vesicles containing the receptor and bound ligand or antibody. Internalization can be assessed using art-known techniques. A method based on determination of protein levels on the cell surface by FACS is described in the Examples hereinbelow.

The term "recycling" as used herein refers to the re-appearance of a molecule on the surface of a cell after previous internalization of said molecule into said cell. Recycling implies that the molecule is not degraded within the cell upon internalization. If recycling occurs at the same rate as internalization, a dynamic steady state is reached wherein the number of molecules on the cell surface stays essentially constant. Recycling can be detected by techniques well known in the art, e.g. by determination of protein levels on the cell surface by FACS or using (confocal) microscopy methods as described in the Examples hereinbelow.

By "downregulation" is meant the reduction of the copy number of a certain protein, e.g. a cell surface receptor, within or at the surface of a cell. Downregulation as used herein particularly refers to a reduction in the copy number of a cell surface protein present at the cell surface, e.g. by internalization and/or degradation, or reduced expression. Downregulation of protein levels

can be detected by various methods established in the art, including e.g. Western blot (for overall protein levels) or FACS (for surface protein levels).

As used herein, the term "effector moiety" refers to a molecule, particularly a polypeptide molecule (e.g. a protein or glycoprotein), that influences cellular activity, for example, through signal transduction or other cellular pathways. Accordingly, the effector moiety can be associated with receptor-mediated signaling that transmits a signal from outside the cell membrane to modulate a response in a cell bearing one or more receptors for the effector moiety. In one embodiment, an effector moiety can elicit a cytotoxic response in cells bearing one or more receptors for the effector moiety. In another embodiment, an effector moiety can elicit a proliferative response in cells bearing one or more receptors for the effector moiety. In another embodiment, an effector moiety can elicit differentiation in cells bearing receptors for the effector moiety. In another embodiment, an effector moiety can alter expression (i.e. upregulate or downregulate) of an endogenous cellular protein in cells bearing receptors for the effector moiety. Non-limiting examples of effector moieties include small molecules, cytokines, growth factors, hormones, enzymes, substrates, and cofactors. The effector moiety can be associated with the antibody in a variety of configurations.

The term “attached” includes linkage by any kind of interaction, including chemical or peptide bonds.

“Fused” refers to components that are linked by peptide bonds, either directly or via one or more peptide linkers.

As used herein, the term “cytokine” refers to a molecule that mediates and/or regulates a biological or cellular function or process (e.g. immunity, inflammation, and hematopoiesis). The term “cytokine” as used herein includes lymphokines, chemokines, monokines, and interleukins. Examples of cytokines include, but are not limited to, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β, TGF-β, TNF-α, and TNF-β. Particular cytokines are interferons (IFN), particularly IFN-α. In particular embodiments the cytokine is a human cytokine. The sequences of particular cytokines, human IFNα2 and IFNα2a, are shown in SEQ ID NOs 138 and 139, respectively.

“Interferon-stimulated genes” (ISGs) refers to genes the expression of which in a cell can be stimulated by contacting said cell with an interferon molecule, particularly an IFNα molecule. Typically, ISGs comprise a recognition sequence (e.g. an interferon-stimulated response element (ISRE)) to which one or more interferon-activated signaling molecules (e.g. STATs) can bind, thereby leading to enhanced expression of the ISG. Examples of ISGs include MX1 (myxovirus

restistance 1, also known as interferon-induced protein p78), RSAD2 (radical S-adenosyl methionine domain containing 2, also known as cytomegalovirus-induced gene 5), HRASLS2 (HRAS-like suppressor 2), IFIT1 (interferon-induced protein with tetratricopeptide repeats 1), and IFITM2 (interferone-induced transmembrane protein 2).

5 As used herein, the term "single-chain" refers to a molecule comprising amino acid monomers linearly linked by peptide bonds. In one embodiment, the effector moiety is a single-chain peptide molecule. Non-limiting examples of single-chain effector moieties include cytokines, growth factors, hormones, enzymes, substrates, and cofactors. When the effector moiety is a cytokine and the cytokine of interest is normally found as a multimer in nature, each subunit of 10 the multimeric cytokine is sequentially encoded by the single-chain of the effector moiety. Accordingly, non-limiting examples of useful single-chain effector moieties include GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β, TGF-β, TNF-α, and TNF-β.

As used herein, the term "effector moiety receptor" refers to a polypeptide molecule capable of 15 binding specifically to an effector moiety. Where an effector moiety specifically binds to more than one receptor, all receptors that specifically bind to the effector moiety are "effector moiety receptors" for that effector moiety. For example, where IFNα is the effector moiety, the effector moiety receptor that binds to an IFNα molecule (e.g. an antibody fused to IFNα) is the IFNα receptor 1 or 2 (see UniProt accession no. P17181 (version 121) and NCBI RefSeq NP_000620.2 20 for human IFNα receptor 1, and UniProt accession no. P48551 (version 131) and NCBI RefSeqs NP_997467.1 & NP_997468.1 for human IFNα receptor 2).

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they 25 exhibit the desired antigen-binding activity.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$, diabodies, linear antibodies, single-chain antibody molecules (e.g. scFv), and single-domain antibodies. For 30 a review of certain antibody fragments, see Hudson et al., Nat Med 9, 129-134 (2003). For a review of scFv fragments, see e.g. Plückthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$

fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046. Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., Nat Med 9, 129-134 (2003); and Hollinger et al., Proc Natl Acad Sci USA 90,
5 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., Nat Med 9, 129-134 (2003). Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see e.g. U.S. Patent No. 6,248,516 B1). Antibody fragments can
10 be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. E. coli or phage), as described herein.

The terms “full length antibody”, “intact antibody”, and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native
15 antibody structure or having heavy chains that contain an Fc region as defined herein.

“Native antibodies” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG class antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two light chains and two heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable
20 heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3), also called a heavy chain constant region. Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a light chain constant domain (CL), also called a light chain constant region. The heavy chain of an antibody may be assigned to one of five types, called α (IgA), δ (IgD), ε
25 (IgE), γ (IgG), or μ (IgM), some of which may be further divided into subtypes, e.g. γ_1 (IgG_1), γ_2 (IgG_2), γ_3 (IgG_3), γ_4 (IgG_4), α_1 (IgA_1) and α_2 (IgA_2). The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain. An IgG class antibody essentially consists of two Fab fragments and an Fc domain, linked via the immunoglobulin hinge region.

30 As used herein, “Fab fragment” refers to an antibody fragment comprising a light chain fragment comprising a VL domain and a constant domain of a light chain (CL), and a VH domain and a first constant domain (CH1) of a heavy chain.

The "class" of an antibody or immunoglobulin refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See, e.g. Kindt et al., Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity.

The term "hypervariable region" or "HVR", as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the complementarity determining regions (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. Exemplary hypervariable loops occur at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, J. Mol. Biol. 196, 901-917 (1987)). Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of L1, 50-56 of L2, 89-97 of L3, 31-35B of H1, 50-65 of H2, and 95-102 of H3 (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)). With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, a-CDR-L3, a-CDR-H1, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of L1, 50-55 of L2, 89-96 of L3, 31-35B of H1, 50-58 of H2, and 95-102 of H3 (see Almagro and Fransson, Front. Biosci. 13, 1619-1633 (2008)). Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g. FR residues) are numbered herein according to Kabat et al., supra (refered to as "Kabat numbering").

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

5 An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The term "parent antibody" herein refers to an antibody that serves as a starting point or basis for 10 the preparation of an antibody variant. In one embodiment, the parent antibody is a humanized or human antibody.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition 15 of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g. 20 containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates 25 the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic 30 animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

The term "Fc domain" or "Fc region" herein is used to define a C-terminal region of an antibody heavy chain that contains at least a portion of the constant region. The term includes native

sequence Fc regions and variant Fc regions. An IgG Fc region comprises an IgG CH2 and an IgG CH3 domain. The "CH2 domain" of a human IgG Fc region usually extends from an amino acid residue at about position 231 to an amino acid residue at about position 340. In one embodiment, a carbohydrate chain is attached to the CH2 domain. The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain. The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e. from an amino acid residue at about position 341 to an amino acid residue at about position 447 of an IgG). The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" ("knob") in one chain thereof and a corresponding introduced "cavity" ("hole") in the other chain thereof; see US Patent No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to promote heterodimerization of two non-identical antibody heavy chains as herein described. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

The term "effector functions" refers to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), cytokine secretion, immune complex-mediated antigen uptake by antigen presenting cells, down regulation of cell surface receptors (e.g. B cell receptor), and B cell activation.

An "activating Fc receptor" is an Fc receptor that following engagement by an Fc region of an antibody elicits signaling events that stimulate the receptor-bearing cell to perform effector functions. Activating Fc receptors include FcγRIIIa (CD16a), FcγRI (CD64), FcγRIIa (CD32), and FcαRI (CD89). A particular activating Fc receptor is human FcγRIIIa (see UniProt accession no. P08637 (version 141)).

The term "peptide linker" refers to a peptide comprising one or more amino acids, typically about 2-20 amino acids. Peptide linkers are known in the art or are described herein. Suitable,

non-immunogenic linker peptides include, for example, $(G_4S)_n$, $(SG_4)_n$ or $G_4(SG_4)_n$ peptide linkers. “n” is generally a number between 1 and 10, typically between 2 and 4.

A “modification promoting heterodimerization” is a manipulation of the peptide backbone or the post-translational modifications of a polypeptide, e.g. an antibody heavy chain, that reduces or prevents the association of the polypeptide with an identical polypeptide to form a homodimer. A modification promoting heterodimerization as used herein particularly includes separate modifications made to each of two polypeptides desired to form a dimer, wherein the modifications are complementary to each other so as to promote association of the two polypeptides. For example, a modification promoting heterodimerization may alter the structure or charge of one or both of the polypeptides desired to form a dimer so as to make their association sterically or electrostatically favorable, respectively. Heterodimerization occurs between two non-identical polypeptides, such as two antibody heavy chains wherein further components attached to each of the heavy chains (e.g. effector moiety) are not the same. In the antibodies according to the present invention, the modification promoting heterodimerization is in the Fc domain, particularly in the CH3 domain. In some embodiments the modification promoting heterodimerziation comprises an amino acid mutation, specifically an amino acid substitution. In a particular embodiment, the modification promoting heterodimerization comprises a separate amino acid mutation, specifically an amino acid substitution, in each of the two antibody heavy chains.

A “knob-into-hole modification” refers to a modification within the interface between two antibody heavy chains in the CH3 domain, wherein i) in the CH3 domain of one heavy chain, an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance (“knob”) within the interface in the CH3 domain of one heavy chain which is positionable in a cavity (“hole”) within the interface in the CH3 domain of the other heavy chain, and ii) in the CH3 domain of the other heavy chain, an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity (“hole”) within the interface in the second CH3 domain within which a protuberance (“knob”) within the interface in the first CH3 domain is positionable. In one embodiment, the “knob-into-hole modification” comprises the amino acid substitution T366W and optionally the amino acid substitution S354C in one of the antibody heavy chains, and the amino acid substitutions T366S, L368A, Y407V and optionally Y349C in the other one of the antibody heavy chains.

An amino acid “substitution” refers to the replacement in a polypeptide of one amino acid with another amino acid. In one embodiment, an amino acid is replaced with another amino acid having similar structural and/or chemical properties, e.g., conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. For example, amino acid substitutions can also result in replacing one amino acid with another amino acid having different structural and/or chemical properties, for example, replacing an amino acid from one group (e.g., polar) with another amino acid from a different group (e.g., basic). Amino acid substitutions can be generated using genetic or chemical methods well known in the art. Genetic methods may include site-directed mutagenesis, PCR, gene synthesis and the like. It is contemplated that methods of altering the side chain group of an amino acid by methods other than genetic engineering, such as chemical modification, may also be useful. Various designations may be used herein to indicate the same amino acid substitution. For example, a substitution from proline at position 329 of the Fc domain to glycine can be indicated as 329G, G329, G_{329}, P329G, or Pro329Gly.

“Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer

program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code.
5 The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary. In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A
10 that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total
15 number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.
20 "Polynucleotide" or "nucleic acid" as used interchangeably herein, refers to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated
25 nucleotides and their analogs. A sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may comprise modification(s) made after synthesis, such as conjugation to a label.
The term "vector" as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating
30 nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

The terms “host cell”, “host cell line”, and “host cell culture” are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein. A host cell is any type of cellular system that can be used to generate the antibodies of the present invention. Host cells include cultured cells, *e.g.* mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue.

An “effective amount” of an agent refers to the amount that is necessary to result in a physiological change in the cell or tissue to which it is administered.

A "therapeutically effective amount" of an agent, e.g. a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. A therapeutically effective amount of an agent for example eliminates, decreases, delays, minimizes or prevents adverse effects of a disease.

An “individual” or “subject” is a mammal. Mammals include, but are not limited to, domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g. humans and non-human primates such as monkeys), rabbits, and rodents (e.g. mice and rats). Particularly, the individual or subject is a human.

The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of a disease in the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing

occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

Antibodies of the invention

The invention provides novel antibodies, particularly monoclonal antibodies, that bind to the asialoglycoprotein receptor (ASGPR).

In a first aspect, the invention provides an antibody capable of specific binding to asialoglycoprotein receptor (ASGPR), wherein the antibody comprises a) the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14; b) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2; c) the heavy chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 6; d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 10; e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 18; f) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22; g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 26; h) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 30; i) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 32; j) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 34; or k) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22.

A particular antibody according to the invention comprises the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14. This antibody clone is designated 4F3. Another particular antibody according to the invention comprises the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2. This antibody is designated 51A12.

The invention provides antibodies which are capable of specific binding to ASGPR and compete for binding to an epitope of ASGPR with antibody 4F3. In one embodiment, such an antibody

binds to the same epitope as 4F3. The 4F3 antibody recognizes an epitope in the stalk region of ASGPR. Accordingly, in one embodiment, such an antibody recognizes an epitope in the stalk region of ASGPR. Also contemplated by the invention are affinity matured variants of the 4F3 antibody. In one embodiment, such an antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 16, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 14. In one embodiment, such an antibody comprises the light chain variable region sequence of SEQ ID NO: 14 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. In one embodiment, such an antibody comprises the heavy chain variable region sequence of SEQ ID NO: 16 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. Variants of the 4F3 antibody may also comprise a heavy chain variable region which is identical to the heavy chain variable region of 4F3, together with a variant light chain variable region, or *vice versa*.

The invention further provides antibodies which are capable of specific binding to ASGPR and compete for binding to an epitope of ASGPR with antibody 51A12. In one embodiment, such and antibody binds to the same epitope as 51A12. The 51A12 antibody recognizes and epitope in the carbohydrate recognition domain (CRD) of ASGPR. Accordingly, in one embodiment, such an antibody recognizes an epitope in the CRD of ASGPR. Also contemplated by the invention are affinity matured variants of the 51A12 antibody, particularly variants obtained by randomization of the light chain CDR3 or 51A12. In one embodiment, such an antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 4, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 2. In one embodiment, such an antibody comprises the light chain variable region sequence of SEQ ID NO: 2 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. In one embodiment, such an antibody comprises the heavy chain variable region sequence of SEQ ID NO: 4 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions. Variants of the 51A12 antibody may also comprise a heavy chain variable region which is identical to the heavy chain variable region of 51A12, together with a variant light chain variable region, or *vice versa*. In one embodiment, such an antibody comprises a) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 36; b) the heavy chain variable region sequence

of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 38; c) the heavy chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 40; d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 42; e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 44; f) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 46; or g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 48.

Preferably, the antibodies of the invention are human antibodies, i.e. the antibodies comprise human variable and constant regions. In one embodiment, the antibodies comprise a human Fc region, particularly a human IgG Fc region, more particularly a human IgG_1 Fc region. Particular antibodies according to the invention are full-length antibodies, particularly full-length IgG class antibodies, more particularly full-length IgG_1 subclass antibodies. Alternatively, the antibodies may be antibody fragments. In one embodiment, the antibodies are Fab fragments or scFv fragments. In some embodiments, the antibodies comprise a Fab fragment and an Fc region, particularly a human IgG Fc region, more particularly a human IgG_1 Fc region, linked by an immunoglobulin hinge region, particularly a human IgG hinge region, more particularly a human IgG_1 hinge region. Specifically, the antibodies may comprise a Fab fragment and an Fc region, linked by an immunoglobulin hinge region, wherein no further Fab fragment is present. In such embodiments, the antibodies are essentially full-length antibodies, lacking one Fab fragment.

Fc regions comprised in the antibodies of the invention may comprise various modifications, as compared to a native Fc region.

While the Fc domain confers to the antibodies favorable pharmacokinetic properties, including a long serum half-life which contributes to good accumulation in the target tissue and a favorable tissue-blood distribution ratio, it may at the same time lead to undesirable targeting of the antibodies to cells expressing Fc receptors rather than to the preferred antigen-bearing cells. Moreover, the co-activation of Fc receptor signaling pathways may lead to cytokine release which, particularly in antibodies having an effector moiety (e.g. a cytokine) attached, results in excessive activation of cytokine receptors and severe side effects upon systemic administration. Therefore, in some embodiments, the antibodies comprise in the Fc region a modification reducing binding affinity of the antibody to an Fc receptor, particularly an Fcγ receptor, as compared to a corresponding antibody comprising an unmodified Fc region. Binding to Fc

receptors can be easily determined e.g. by ELISA, or by Surface Plasmon Resonance (SPR) using standard instrumentation such as a BIAcore instrument (GE Healthcare) and Fc receptors such as may be obtained by recombinant expression. A specific illustrative and exemplary embodiment for measuring binding affinity is described in the following. According to one embodiment, Binding affinity to an Fc receptor is measured by surface plasmon resonance using a BIACORE® T100 machine (GE Healthcare) at 25°C with ligand (Fc receptor) immobilized on CM5 chips. Briefly, carboxymethylated dextran biosensor chips (CM5, GE Healthcare) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Recombinant ligand is diluted with 10 mM sodium acetate, pH 5.5, to 0.5-30 μg/ml before injection at a flow rate of 10 μl/min to achieve approximately 100-5000 response units (RU) of coupled protein. Following the injection of the ligand, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, three- to five-fold serial dilutions of antibody (range between ~0.01 nM to 300 nM) are injected in HBS-EP+ (GE Healthcare, 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20, pH 7.4) at 25°C at a flow rate of approximately 30-50 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE ® T100 Evaluation Software version 1.1.1) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_D) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen et al., J Mol Biol 293, 865-881 (1999). Alternatively, binding affinity antibodies to Fc receptors may be evaluated using cell lines known to express particular Fc receptors, such as NK cells expressing FcγIIIa receptor.

In the modification comprises one or more amino acid mutation that reduces the binding affinity of the antibody to an Fc receptor. Typically, the same one or more amino acid mutation is present in each of the two antibody heavy chains in the Fc domain. In one embodiment said amino acid mutation reduces the binding affinity of the antibody to the Fc receptor by at least 2-fold, at least 5-fold, or at least 10-fold. In embodiments where there is more than one amino acid mutation that reduces the binding affinity of the antibody to the Fc receptor, the combination of these amino acid mutations may reduce the binding affinity of the antibody to the Fc receptor by at least 10-fold, at least 20-fold, or even at least 50-fold. In one embodiment the antibody exhibits less than 20%, particularly less than 10%, more particularly less than 5% of the binding affinity to an Fc receptor as compared to an antibody comprising an unmodified Fc domain.

In one embodiment the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc receptor is an Fcγ receptor, more specifically an FcγRIIIa, FcγRI or FcγRIIa receptor. Preferably,

binding to each of these receptors is reduced. In some embodiments binding affinity to a complement component, specifically binding affinity to C1q, is also reduced. In one embodiment binding affinity to neonatal Fc receptor (FcRn) is not reduced. Substantially similar binding to FcRn, i.e. preservation of the binding affinity of the antibody to said receptor, is achieved when
5 the antibody exhibits greater than about 70% of the binding affinity of an unmodified form of the antibody to FcRn. Antibodies of the invention may exhibit greater than about 80% and even greater than about 90% of such affinity. In one embodiment the amino acid mutation is an amino acid substitution. In one embodiment the antibody comprises an amino acid substitution in the Fc region at position P329 (EU numbering). In a more specific embodiment the amino acid
10 substitution is P329A or P329G, particularly P329G. In one embodiment the antibody comprises a further amino acid substitution in the Fc region at a position selected from S228, E233, L234, L235, N297 and P331. In a more specific embodiment the further amino acid substitution is S228P, E233P, L234A, L235A, L235E, N297A, N297D or P331S. In a particular embodiment the antibody comprises amino acid substitutions in the Fc region at positions P329, L234 and
15 L235. In a more particular embodiment the antibody comprises the amino acid mutations L234A, L235A and P329G (LALA P329G). This combination of amino acid substitutions almost completely abolishes Fcγ receptor binding of a human IgG antibody, as described in PCT patent application no. PCT/EP2012/055393, incorporated herein by reference in its entirety. PCT patent application no. PCT/EP2012/055393 also describes methods of preparing such modified
20 antibodies and methods for determining its properties such as Fc receptor binding or effector functions.

Antibodies comprising modifications in the Fc region can be prepared by amino acid deletion, substitution, insertion or modification using genetic or chemical methods well known in the art. Genetic methods may include site-specific mutagenesis of the encoding DNA sequence, PCR,
25 gene synthesis, and the like. The correct nucleotide changes can be verified for example by sequencing.

Antibodies which comprise modifications reducing Fc receptor binding generally have reduced effector functions, particularly reduced ADCC, as compared to corresponding unmodified antibodies. In some embodiments the antibodies have reduced ADCC. In specific embodiments
30 the reduced ADCC is less than 20% of the ADCC induced by a corresponding antibody comprising an unmodified Fc region. Effector function of an antibody can be measured by methods known in the art. Examples of *in vitro* assays to assess ADCC activity of a molecule of interest are described in U.S. Patent No. 5,500,362; Hellstrom et al. Proc Natl Acad Sci USA 83,

7059-7063 (1986) and Hellstrom et al., Proc Natl Acad Sci USA 82, 1499-1502 (1985); U.S. Patent No. 5,821,337; Bruggemann et al., J Exp Med 166, 1351-1361 (1987). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA); and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI)). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g. in a animal model such as that disclosed in Clynes et al., Proc Natl Acad Sci USA 95, 652-656 (1998). In some embodiments binding of the antibody to a complement component, specifically to C1q, is also reduced. Accordingly, complement-dependent cytotoxicity (CDC) may also be reduced. C1q binding assays may be carried out to determine whether the antibody is able to bind C1q and hence has CDC activity. See e.g. C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J Immunol Methods 202, 163 (1996); Cragg et al., Blood 101, 1045-1052 (2003); and Cragg and Glennie, Blood 103, 2738-2743 (2004)).

In addition to the antibodies described hereinabove and in PCT patent application no. PCT/EP2012/055393, antibodies with reduced Fc receptor binding and/or effector function also include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

IgG_4 antibodies exhibit reduced binding affinity to Fc receptors and reduced effector functions as compared to IgG_1 antibodies. Hence, in some embodiments, the antibodies of the invention are IgG_4 subclass antibodies, particularly human IgG_4 subclass antibodies. In one embodiment the IgG_4 antibody comprises amino acid substitutions in the Fc region at position S228, specifically the amino acid substitution S228P. To further reduce its binding affinity to an Fc receptor and/or its effector function, in one embodiment the IgG_4 antibody comprises an amino acid substitution at position L235, specifically the amino acid substitution L235E. In another embodiment, the IgG_4 antibody comprises an amino acid substitution at position P329, specifically the amino acid substitution P329G. In a particular embodiment, the IgG_4 antibody comprises amino acid substitutions at positions S228, L235 and P329, specifically amino acid substitutions S228P,

L235E and P329G. Such modified IgG_4 antibodies and their Fcγ receptor binding properties are described in PCT patent application no. PCT/EP2012/055393, incorporated herein by reference in its entirety.

Antibodies according to the invention may have effector moieties such as cytokines attached. In particular embodiments, the antibodies comprise only one single effector moiety, fused to one of the two antibody heavy chains, thus these antibodies comprise two non-identical polypeptide chains. Similarly, the antibodies of the invention may be full-length antibodies, lacking one of the Fab fragments, hence comprising a full antibody heavy chain and an antibody heavy chain lacking the VH and CH1 domains. Recombinant co-expression of these polypeptides and subsequent dimerization leads to several possible combinations of the two polypeptides, out of which only heterodimers of the two non-identical polypeptides are useful. To improve the yield and purity of such antibodies in recombinant production, it can thus be advantageous to introduce in the Fc region of the antibody a modification which hinders the formation of homodimers of two identical polypeptides (e.g. two polypeptides comprising an effector moiety, or two polypeptides lacking an effector moiety) and/or promotes the formation of heterodimers of a polypeptide comprising an effector moiety and a polypeptide lacking an effector moiety. Accordingly, in some embodiments, the antibodies of the invention comprise in the Fc region a modification promoting heterodimerization of two non-identical antibody heavy chains. The site of most extensive protein-protein interaction between the two heavy chains of a human IgG antibody is in the CH3 domain of the Fc region. Thus, in one embodiment said modification is in the CH3 domain of the Fc region. In a specific embodiment, said modification is a knob-into-hole modification, comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the two antibody heavy chains. The knob-into-hole technology is described e.g. in US 5,731,168; US 7,695,936; Ridgway et al., Prot Eng 9, 617-621 (1996) and Carter, J Immunol Meth 248, 7-15 (2001). Generally, the method involves introducing a protuberance ("knob") at the interface of a first polypeptide and a corresponding cavity ("hole") in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). Hence, in one embodiment, the antibody comprises a modification

within the interface between the two antibody heavy chains in the CH3 domain, wherein i) in the CH3 domain of one heavy chain, an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance ("knob") within the interface in the CH3 domain of one heavy chain which is positionable in a cavity ("hole") within the interface in the CH3 domain of the other heavy chain, and ii) in the CH3 domain of the other heavy chain, an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity ("hole") within the interface in the second CH3 domain within which a protuberance ("knob") within the interface in the first CH3 domain is positionable. The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis. In a specific embodiment a knob modification comprises the amino acid substitution T366W (EU numbering) in one of the two antibody heavy chains, and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V (EU numbering) in the other one of the two antibody heavy chains. In a further specific embodiment, the antibody heavy chain comprising the knob modification additionally comprises the amino acid substitution S354C, and the antibody heavy chain comprising the hole modification additionally comprises the amino acid substitution Y349C. Introduction of these two cysteine residues results in formation of a disulfide bridge between the two antibody heavy chains in the Fc region, further stabilizing the dimer (Carter, J Immunol Methods 248, 7-15 (2001)). In an alternative embodiment a modification promoting heterodimerization of two non-identical antibody heavy chains comprises a modification mediating electrostatic steering effects, e.g. as described in PCT publication WO 2009/089004. Generally, this method involves replacement of one or more amino acid residues at the interface of the two antibody heavy chains by charged amino acid residues so that homodimer formation becomes electrostatically unfavorable but heterodimerization electrostatically favorable. In a particular embodiment wherein the antibody has an effector moiety attached to it, the effector moiety is fused to the amino- or carboxy-terminal amino acid of the antibody heavy chain comprising the knob modification. Without wishing to be bound by theory, fusion of the effector moiety to the knob-containing heavy chain will further minimize the generation of homodimeric antibodies comprising two effector moieties (steric clash of two knob-containing polypeptides). Similarly, in embodiments wherein the antibody comprises a only single Fab fragment fused to an Fc region, the Fab fragment is preferably fused to the heavy chain of the Fc region comprising the knob modification.

The antibodies of the invention combine a number of properties which are particularly advantageous, for example for therapeutic applications. For example, the antibodies are cross-reactive for human and cynomolgus monkey, which enables e.g. *in vivo* studies in cynomolgus monkeys prior to human use. Hence, in one embodiment, the antibody of the invention is capable of specific binding to human and cynomolgus monkey ASGPR. Furthermore, the antibodies of the invention bind ASGPR with particularly strong affinity and/or avidity. In one embodiment, the antibody binds to human ASGPR with an dissociation constant (K_D) of smaller than 1 μM, particularly smaller than 100 nM, more particularly smaller than 1 nM, when measured as Fab fragment by Surface Plasmon Resonance (SPR). A method for measuring binding affinity by SPR is described herein. Specifically, measurement is made at a temperature of 25°C. In one embodiment, affinity (K_D) of antibodies as Fab fragements is measured by SPR using a ProteOn XPR36 instrument (Biorad) at 25°C with biotinylated mono- (avi-Fc-human ASGPR H1 CRD, SEQ ID NO: 118) or bivalent (avi-Fc-human ASGPR H1 stalk-CRD, SEQ ID NO: 130) ASGPR H1 antigens immobilized on NLC chips by neutravidin capture. In an exemplary method, antigens for immobilization are diluted with PBST (10 mM phosphate, 150 mM NaCl pH 7.4, 0.005% Tween-20) to 10 μg/ml, and injected at 30 μl/min at varying contact times, to achieve immobilization levels of 200, 400 or 800 response units (RU) in vertical orientation. Subsequently, analytes (antibodies) are injected. For one-shot kinetics measurements, injection direction is changed to horizontal orientation, and two-fold dilution series of purified Fab fragments (varying concentration ranges between 100 and 6.25 nM) are injected simultaneously at 50, 60 or 100 μl/min along separate channels 1-5, with association times of 150 or 200 s, and dissociation times of 240 or 600 s. Buffer (PBST) is injected along the sixth channel to provide an "in-line" blank for referencing. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) are calculated using a simple one-to-one Langmuir binding model in ProteOn Manager v3.1 software by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_D) is calculated as the ratio k_{off}/k_{on}. Regeneration is performed in horizontal orientation using 10 mM glycine, pH 1.5 at a flow rate of 100 μl/min for a contact time of 30 s. In another embodiment, the antibody binds to human ASGPR with a K_D of smaller than 1 μM, particularly smaller than 500 nM, more particularly smaller than 100 nM or even smaller than 10 nM, when measured as IgG_1 by fluoresecence resonance energy transfer (FRET). A method for measuring binding affinity (or avidity) by FRET is described herein. In one embodiment, the measurement is performed by contacting cells expressing full-length ASGPR protein labeled with a FRET donor molecule with the antibodies, and detection of bound

antibodies by a secondary antibody labeled with a suitable FRET acceptor molecule. In an exemplary method, the DNA sequence encoding for the SNAP Tag (plasmid purchased from Cisbio) is amplified by PCR and ligated into an expression vector, containing the full length human ASGPR H1 sequence (Origene). The resulting fusion protein is comprised of full-length
5 ASGPR H1 with a C-terminal SNAP tag. HEK293 cells are transfected with 10 μg DNA using Lipofectamine 2000 as transfection reagent. After an incubation time of 20 h, cells are washed with PBS and incubated for 1 h at 37°C in LabMed buffer (Cisbio) containing 100 nM SNAP-Lumi4Tb (Cibsio), leading to specific labeling of the SNAP Tag. Subsequently, cells are washed 4 times with LabMed buffer to remove unbound dye. The labeling efficiency is determined by
10 measuring the emission of terbium at 615 nm compared to buffer. Cells can then be stored frozen at -80°C for up to 6 months. Avidity is measured by adding ASGPR-specific antibodies at a concentration ranging from 50-0.39 nM to labeled cells (100 cells per well) followed by addition of anti-human Fc-d2 (Cisbio, final 200 nM per well) as acceptor molecule for the FRET. After an incubation time of 3h at RT the emission of the acceptor dye (665 nm) as well as of the donor
15 dye (615 nm) is determined using a fluorescence Reader (Victor 3, Perkin Elmer). The ratio of acceptor to donor emission is calculated and the ratio of the background control (cells with anti-human Fc-d2) subtracted. Curves can be analysed in GraphPad Prism5 software and K_D values calculated. A further advantage of the antibodies of the invention is that they do not compete for binding to ASGPR with natural ligands of the receptor (asialoglycoproteins such as e.g.
20 asialofetuin), i.e. antibody binding is not affected by the presence of ASGPR ligand and does not interfere with the natural function of ASGPR. In one embodiment, the antibody does not compete with a natural ligand of ASGPR for binding to ASGPR. In a specific embodiment, said natural ligand of ASGPR is asialofetuin. Competition can be measured by methods well known in the art. In one embodiment, competition with a natural ligand of ASGPR is measured by
25 FACS, for example using an ASGPR-expressing cell line, fluoresecently labeled ligand, and detection of bound antibodies with a secondary antibody having a different fluorescent label. In an exemplary method, the hepatocellular carcinoma cell line HepG2 is used. 0.2 mio cells per well in a 96 well round bottom plate are incubated with 40 μl of Alexa488 labeled asialofetuin (from fetal calf serum, Sigma Aldrich #A4781, final concentration 100 μg/ml) at 4°C for 30 min.
30 The binding is performed in the presence of calcium, as ligand binding to ASGPR is calcium dependent. Unbound protein is removed by washing the cells once with HBSS containing 0.1% BSA. Then 40 μl of the anti-ASGPR antibodies (30, 6, and 1.25 μg/ml final concentration) are added to the cells in the presence of 100 μg/ml asialofetuin. Cells are incubated for 30 min at

4°C and unbound protein is removed by washing the cells once. An APC-conjugated AffiniPure goat anti-human IgG Fc gamma fragment-specific secondary F(ab')2 fragment (Jackson ImmunoResearch #109-136-170; working solution 1:50 in HBSS containing 0.1% BSA) is used as an secondary antibody. After 30 min incubation at 4°C unbound secondary antibody is
5 removed by washing. Cells are fixed using 1% PFA and binding of ligand as well as antibodies is analyzed using BD FACS CantoII (Software BD DIVA). A major advantage of the antibodies of the invention is their high specificity for ASGPR. For example, despite their strong binding to human ASGPR H1, the antibodies do not detectably bind to CLEC10A, which was identified as the closest homologue of human ASGPR H1. In one embodiment, the antibody does not
10 detectably bind to CLEC10A, particularly human CLEC10A. Specifically, the antibody does not detectably bind to CLEC10A wherein binding is measured by SPR (as described herein). Moreover, the antibodies bind to cells which do not express ASGPR only to a similar extent as a corresponding untargeted antibody (isotype control). Hence, in one embodiment, the antibody does not specifically bind to cells lacking ASGPR expression, particularly human cells, more
15 particularly human blood cells. Exemplary cells lacking ASGPR expression include Hela cells (a human cell line derived from cervical cancer), A459 human non-small cell lung cancer cells, human embryonic kidney (HEK) cells, and (human) PBMCs. Binding, or lack of binding, to specific cells can easily be determined for example by FACS. Such methods are well established in the art and also described in the Examples hereinbelow. An important feature of anti-ASGPR
20 antibodies are their internalization characteristics. For example, if the antibody is to be used to target an effector moiety to ASGPR-expressing cells, it is desirable for the antibody to be present on the cell surface for a sufficiently long time for activation of effector moiety receptors. The antibodies of the present invention, upon binding to ASGPR, are internalized into the ASGPR-expressing cell, however, they are recycled back to the cell surface without being degraded
25 inside the cell. Hence, in one embodiment, the antibody is internalized into a cell expressing ASGPR upon binding of the antibody to ASGPR on the surface of said cell. In a specific embodiment, the antibody is recycled back to the surface of said cell at about the same rate as it is internalized into said cell. Internalization and recycling of cell surface proteins or antibodies bound thereto can easily be measured by established methods, such as FACS or (confocal)
30 microscopy techniques. In one embodiment, internalization and/or recycling are measured by FACS. For an antibody to have sustained effects, it is important that its target antigen is present at essentially constant levels. Frequently, antibody binding to target antigens induces downregulation of the latter, leading to reduced efficacy of antibodies. However, the antibodies

of the present invention do not have such effect. In one embodiment, the antibody does not significantly induce downregulation of ASGPR expression at the surface of a cell upon binding of the antibody to ASGPR on the surface of said cell. The level of antigen expression at the surface of a cell can easily be determined by established methods such as FACS.

5 **Antibodies with attached effector moieties**

Particularly useful antibodies according to the present invention are antibodies having an effector moiety, e.g. a cytokine, attached. Antibodies fused to an effector moiety such as a cytokine are also referred to as immunoconjugates herein. The antibodies with attached effector moieties can incorporate, singly or in combination, any of the features described hereinabove in relation to the 10 antibodies of the invention.

Accordingly, in one aspect, the invention provides an antibody capable of specific binding to ASGPR according to any of the above embodiments, wherein an effector moiety is attached to the antibody. In one embodiment, not more than one effector moiety is attached to the antibody. The absence of further effector moieties may reduce targeting of the antibody to sites where the 15 respective effector moiety receptor is presented, thereby improving targeting to and accumulation at sites where the actual target antigen of the antibody, ASGPR, is presented. Furthermore, the absence of an avidity effect for the respective effector moiety receptor can reduce activation of effector moiety receptor-positive cells in peripheral blood upon intravenous administration of the antibody. The effector moieties for use in the invention are generally 20 polypeptides that influence cellular activity, for example, through signal transduction pathways. Accordingly, an effector moiety useful in the invention can be associated with receptor-mediated signaling that transmits a signal from outside the cell membrane to modulate a response within the cell. For example, an effector moiety can be a cytokine. In particular embodiments the effector moiety is human. In particular embodiments, the effector moiety is a peptide molecule 25 and is fused to the antibody through peptide bonds (i.e. the antibody and effector moiety form a fusion protein). In one embodiment, the effector moiety is single-chain peptide molecule. In a further embodiment, the effector moiety is fused at its amino-terminal amino acid to the carboxy-terminal amino acid of one of the heavy chains of the antibody, optionally through a peptide linker. Suitable, non-immunogenic peptide linkers include, for example, $(G_4S)_n$, $(SG_4)_n$ or 30 $G_4(SG_4)_n$ peptide linkers. “n” is generally a number between 1 and 10, typically between 2 and 4. In embodiments wherein the antibody comprises a knob-into-hole modification in the Fc region

as described above, it is preferable to fuse the effector moiety to the antibody heavy chain comprising the knob modification.

In one embodiment said effector moiety is a cytokine molecule. Examples of useful cytokines include, but are not limited to, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-21, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β, TGF-β, TNF-α, and TNF-β. In one embodiment, said cytokine molecule is fused at its amino-terminal amino acid to the carboxy-terminal amino acid of one of the antibody heavy chains, optionally through a peptide linker. In one embodiment, said cytokine molecule is a human cytokine. In one embodiment, said cytokine molecule is an interferon molecule. In a specific embodiment, said interferon molecule is interferon alpha, particularly human interferon alpha, more particularly human interferon alpha 2 (see SEQ ID NO: 138) or human interferon alpha 2a (see SEQ ID NO: 139). Interferon alpha is known to have anti-viral activities. Hence, attaching an interferon molecule to an antibodiy of the invention is particularly useful for targeting virus-infected ASGPR-expressing cells. In one embodiment, wherein the cytokine molecule is an interferon molecule, the antibody has anti-viral activity in cells expressing ASGPR on their surface. In one embodiment, said cells are liver cells, particularly hepatocytes, more particularly human hepatocytes. In one embodiment, said anti-viral activitiy is selective. In a specific embodiment, the antibody has no anti-viral activity in cells not expressing significant levels of ASGPR on their surface. In one embodiment, said cells are blood cells, particularly human blood cells. In one embodiment, said anti-viral activity This selectivity of interferon molecules attached to anti-ASGPR antibodies according to the invention is in contrast to untargeted interferon molecules, which do not distinguish between any intended target cells (e.g. hepatocytes) and cells which should not be affected (e.g. blood cells), and is crucial for possible therapeutic use without major toxicity issues. In a further specific embodiment, said anti-viral activity is selected from inhibition of viral infection, inhibition of virus replication, inhibition of cell killing and induction of one or more interferon-stimulated gene. In a specific embodiment, the one or more interferon-stimulated gene is selected from the group of MX1 (myxovirus restistance 1, also known as interferon-induced protein p78), RSAD2 (radical S-adenosyl methionine domain containing 2, also known as cytomegalovirus-induced gene 5), HRASLS2 (HRAS-like suppressor 2), IFIT1 (interferon-induced protein with tetratricopeptide repeats 1), and IFITM2 (interferone-induced transmembrane protein 2). In one embodiment, the induction of one or more interferon-stimulated gene is at least a 1.5-fold, particularly at least a 2-fold, more particularly at least a 5-fold induction on mRNA level, as compared to induction by a corresponding antibody without interferon molecule attached. Gene

induction on mRNA level can be measured by methods well established in the art, including quantitative reverse-transcription (RT) PCR or microarray analysis, as described herein. Inhibition of cell killing can be determined for example by a virus protection assay, wherein cells are preincubated with the test compound, followed by addition of virus and quantification of
5 living cells after incubation. An exemplary such assay is described in the Examples. Madin-Darby bovine kidney (MDBK) cells are pre-incubated with the antibodies and controls for 1-4 h. Vesicular stomatitis virus is then added for additional 16-24 h. At the end of this incubation step, living cells are stained with crystal violet staining solution (0.5%) and quantification of living cells is performed using a microplate reader at 550-600 nm with a reference wavelength of 690
10 nm. An exemplary assay for assessment of virus replication is also provided in the Examples. This assay uses a Huh 7-derived hepatocarcinoma cell line stably transfected with bicistronic hepatitis C virus (HCV) replicon of which the first open reading frame, driven by the HCV IRES, contains the renilla luciferase gene in fusion with the neomycin phosphotransferase gene (NPTII) and the second open reading frame, driven by EMCV IRES, contains the HCV non-structural
15 genes NS3, NS4a, NS4b, NS5A and NS5B derived from the NK5.1 replicon backbone. Cells are cultured at 37°C in a humidified atmosphere with 5% CO_2 in DMEM supplemented with Glutamax™ and 100 mg/ml sodium pyruvate. The medium was further supplemented with 10% (v/v) FBS (v/v) penicillin/streptomycin and 1% (v/v) geneticin. The cells in DMEM containing 5% (v/v) FBS are plated in 96-well plates at 5000 cells/well in 90 μl volume. 24 hours after
20 plating, antibodies (or medium as a control) are added to the cells in 3-fold dilutions over 12 wells (0.01 -2000 pM), in a volume of 10 μl, so that the final volume after addition of antibody is 100 μl. Renilla luciferase reporter signal is read 72 hours after adding antibodies, using the Renilla Luciferase Assay system (Promega, # E2820). The EC_{50} values are calculated as the compound concentration at which a 50% reduction in the level of renilla luciferase reporter is
25 observed as compared to control samples (in the absence of antibody). Dose-response curves and EC_{50} values are obtained by using the XLfit4 program (ID Business Solutions Ltd., Surrey, UK). In a particular embodiment, the antibody according to the invention is a full-length human IgG_1 antibody comprising the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14, said antibody comprising in the Fc region a
30 modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at the N-terminal amino acid to the C-terminal

amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody heavy chains. In a further specific embodiment, said knob modification comprises the amino acid
5 substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 68, SEQ ID NO: 70 and SEQ ID NO: 72, or variants thereof that retain functionality.

10 In another particular embodiment, the antibody according to the invention is a full-length human IgG_1 antibody comprising the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2, said antibody comprising in the Fc region a modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the
15 antibody heavy chains and a hole modification in the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at the N-terminal amino acid to the C-terminal amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody
20 heavy chains. In a further specific embodiment, said knob modification comprises the amino acid substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 50, SEQ ID NO: 52 and SEQ
25 ID NO: 54, or variants thereof that retain functionality.

In still another particular embodiment, the antibody according to the invention is a full-length human IgG_1 antibody comprising the heavy chain variable region sequence of SEQ ID NO: 4 and a light chain variable region sequence selected from the group of SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46 and SEQ ID NO:
30 48, said antibody comprising in the Fc region a modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at

the N-terminal amino acid to the C-terminal amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody heavy chains. In a further specific embodiment, said knob modification comprises the amino acid substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 52, SEQ ID NO: 54 and a polypeptide sequence selected from the group of SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106 and SEQ ID NO: 108, or variants thereof that retain functionality.

In a further embodiment, the antibody of the invention comprises the polypeptide sequences of SEQ ID NO: 92, SEQ ID NO: 52 and SEQ ID NO: 54, or variants thereof that retain functionality. In another embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 94, SEQ ID NO: 52 and SEQ ID NO: 54, or variants thereof that retain functionality. In still a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 56, SEQ ID NO: 58 and SEQ ID NO: 60, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 62, SEQ ID NO: 64 and SEQ ID NO: 66, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 74, SEQ ID NO: 76 and SEQ ID NO: 78, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 80, SEQ ID NO: 82 and SEQ ID NO: 84, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 86, SEQ ID NO: 88 and SEQ ID NO: 90, or variants thereof that retain functionality.

In an alternative embodiment, the antibody according to the invention is a full-length human IgG_1 antibody lacking one of the two Fab fragments and comprising the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14, said antibody comprising in the Fc region a modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the antibody heavy chains and a hole modification in

the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at the N-terminal amino acid to the C-terminal amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody heavy chains. In a further specific embodiment, said knob modification comprises the amino acid substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 68, SEQ ID NO: 70 and SEQ ID NO: 116, or variants thereof that retain functionality.

In another alternative embodiment, the antibody according to the invention is a full-length human IgG_1 antibody lacking one of the two Fab fragments and comprising the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2, said antibody comprising in the Fc region a modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at the N-terminal amino acid to the C-terminal amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody heavy chains. In a further specific embodiment, said knob modification comprises the amino acid substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 50, SEQ ID NO: 52 and SEQ ID NO: 116, or variants thereof that retain functionality.

In still another alternative embodiment, the antibody according to the invention is a full-length human IgG_1 antibody lacking one of the two Fab fragments and comprising the heavy chain variable region sequence of SEQ ID NO: 4 and a light chain variable region sequence selected from the group of SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46 and SEQ ID NO: 48, said antibody comprising in the Fc region a

modification reducing binding affinity of the antibody to FcγRIIIa and a knob-into-hole modification, said knob-into-hole modification comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the antibody heavy chains, and said antibody having an IFNα2 molecule fused at the N-terminal amino acid to the C-terminal amino acid of one of the antibody heavy chains through a peptide linker. In a specific embodiment, said modification reducing binding affinity of the antibody to FcγRIIIa comprises the amino acid substitutions L234A, L235A and P329G (EU numbering) in each of the antibody heavy chains. In a further specific embodiment, said knob modification comprises the amino acid substitution T366W and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V. In still a further specific embodiment, said IFNα2 molecule is fused to the antibody heavy chain comprising the knob modification. In an even more specific embodiment, said antibody comprises the polypeptide sequences of SEQ ID NO: 52, SEQ ID NO: 116 and a polypeptide sequence selected from the group of SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106 and SEQ ID NO: 108, or variants thereof that retain functionality.

In a further embodiment, the antibody of the invention comprises the polypeptide sequences of SEQ ID NO: 92, SEQ ID NO: 52 and SEQ ID NO: 116, or variants thereof that retain functionality. In another embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 94, SEQ ID NO: 52 and SEQ ID NO: 116, or variants thereof that retain functionality. In still a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 56, SEQ ID NO: 58 and SEQ ID NO: 116, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 62, SEQ ID NO: 64 and SEQ ID NO: 116, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 74, SEQ ID NO: 76 and SEQ ID NO: 116, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 80, SEQ ID NO: 82 and SEQ ID NO: 116, or variants thereof that retain functionality. In a further embodiment, the antibody according to the invention comprises the polypeptide sequences of SEQ ID NO: 86, SEQ ID NO: 88 and SEQ ID NO: 116, or variants thereof that retain functionality.

Antibodies of the invention include those that have sequences that are at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequences set forth in SEQ ID NOs

2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 and 116, including functional fragments or variants thereof. The invention also encompasses antibodies comprising these sequences with conservative amino acid substitutions.

5 **Polynucleotides**

The invention further provides polynucleotides encoding an antibody as described herein or an antigen binding portion thereof.

Polynucleotides of the invention include those that are at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequences set forth in SEQ ID NOs 1, 3, 5, 7, 9, 11, 10 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109 and 117, including functional fragments or variants thereof.

The polynucleotides encoding antibodies of the invention may be expressed as a single polynucleotide that encodes the entire antibody or as multiple (e.g., two or more) 15 polynucleotides that are co-expressed. Polypeptides encoded by polynucleotides that are co-expressed may associate through, e.g., disulfide bonds or other means to form a functional antibody. For example, the light chain portion of an antibody may be encoded by a separate polynucleotide from the heavy chain portion of the antibody. When co-expressed, the heavy chain polypeptides will associate with the light chain polypeptides to form the antibody. In 20 another example, the heavy chain portion of the antibody comprising an effector moiety could be encoded by a separate polynucleotide from the other heavy chain portion of the antibody. When co-expressed, the heavy chain polypeptides will associate to form a functional antibody (together with the light chain polypeptide(s)).

In one embodiment, the present invention is directed to a polynucleotide encoding an antibody or 25 an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes a variable region sequence as shown in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 or 48. In another embodiment, the present invention is directed to a polynucleotide encoding an antibody or an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes a polypeptide sequence as shown 30 in SEQ ID NO 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 116. In another embodiment, the invention is further directed to a polynucleotide encoding an antibody or an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%,

98%, or 99% identical to a nucleic acid sequence shown SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109 or 117. In another embodiment, the invention is directed to a polynucleotide encoding an antibody or an antigen binding portion thereof, wherein the polynucleotide comprises a nucleic acid sequence shown in SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109 or 117. In another embodiment, the invention is directed to a polynucleotide encoding an antibody or antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes a variable region sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence of SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 or 48. In another embodiment, the invention is directed to a polynucleotide encoding an antibody or an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes a polypeptide sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence of SEQ ID NO 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 116. The invention encompasses a polynucleotide encoding an antibody or an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes the variable region sequences of SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 or 48 with conservative amino acid substitutions. The invention also encompasses a polynucleotide encoding an antibody of the invention or an antigen binding portion thereof, wherein the polynucleotide comprises a sequence that encodes the polypeptide sequences of SEQ ID NO 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 116 with conservative amino acid substitutions.

In certain embodiments the polynucleotide or nucleic acid is DNA. In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA). RNA of the present invention may be single stranded or double stranded.

Recombinant methods

Antibodies of the invention may be obtained, for example, by solid-state peptide synthesis (e.g. Merrifield solid phase synthesis) or recombinant production. For recombinant production one or more polynucleotide encoding the antibody (fragment), e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such

polynucleotide may be readily isolated and sequenced using conventional procedures. In one embodiment a vector, preferably an expression vector, comprising one or more of the polynucleotides of the invention is provided. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of an antibody (fragment) along with appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, N.Y. (1989); and Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, N.Y (1989). The expression vector can be part of a plasmid, virus, or may be a nucleic acid fragment. The expression vector includes an expression cassette into which the polynucleotide encoding the antibody (fragment) (i.e. the coding region) is cloned in operable association with a promoter and/or other transcription or translation control elements. As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' untranslated regions, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g. on a single vector, or in separate polynucleotide constructs, e.g. on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, e.g. a vector of the present invention may encode one or more polypeptides, which are post- or co-translationally separated into the final proteins via proteolytic cleavage. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions, either fused or unfused to a polynucleotide encoding the antibody (fragment) of the invention, or variant or derivative thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain. An operable association is when a coding region for a gene product, e.g. a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of

the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein. A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions, which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (e.g. the immediate early promoter, in conjunction with intron-A), simian virus 40 (e.g. the early promoter), and retroviruses (such as, e.g. Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit â-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as inducible promoters (e.g. promoters inducible tetracyclins). Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from viral systems (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence). The expression cassette may also include other features such as an origin of replication, and/or chromosome integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal repeats (ITRs).

Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention. For example, if secretion of the antibody is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding an antibody of the invention or an antigen binding portion thereof. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a

signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, *e.g.* an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of
5 the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β-glucuronidase. Exemplary amino acid sequences of secretory signal peptides are shown in SEQ ID NOs 135-137.

10 DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the antibody may be included within or at the ends of the antibody (fragment) encoding polynucleotide.

In a further embodiment, a host cell comprising one or more polynucleotides of the invention is provided. In certain embodiments a host cell comprising one or more vectors of the invention is
15 provided. The polynucleotides and vectors may incorporate any of the features, singly or in combination, described herein in relation to polynucleotides and vectors, respectively. In one such embodiment a host cell comprises (e.g. has been transformed or transfected with) a vector comprising a polynucleotide that encodes (part of) an antibody of the invention. As used herein, the term "host cell" refers to any kind of cellular system which can be engineered to generate the
20 antibodies of the invention or fragments thereof. Host cells suitable for replicating and for supporting expression of antibodies are well known in the art. Such cells may be transfected or transduced as appropriate with the particular expression vector and large quantities of vector containing cells can be grown for seeding large scale fermenters to obtain sufficient quantities of the antibody for clinical applications. Suitable host cells include prokaryotic microorganisms,
25 such as E. coli, or various eukaryotic cells, such as Chinese hamster ovary cells (CHO), insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation is not needed. After expression, the polypeptide may be isolated from the bacterial cell paste in a soluble fraction and can be further purified. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for
30 polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been “humanized”, resulting in the production of a polypeptide with a partially or fully human glycosylation pattern. See Gerngross, Nat Biotech 22, 1409-1414 (2004), and Li et al., Nat Biotech 24, 210-215 (2006). Suitable host cells for the expression of (glycosylated)

polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures can also be utilized as hosts. See e.g. US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham et al., J Gen Virol 36, 59 (1977)), baby hamster kidney cells (BHK), mouse sertoli cells (TM4 cells as described, e.g., in Mather, Biol Reprod 23, 243-251 (1980)), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HELA), canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumor cells (MMT 060562), TRI cells (as described, e.g., in Mather et al., Annals N.Y. Acad Sci 383, 44-68 (1982)), MRC 5 cells, and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including dhfr$^-$ CHO cells (Urlaub et al., Proc Natl Acad Sci USA 77, 4216 (1980)); and myeloma cell lines such as YO, NS0, P3X63 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki and Wu, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003). Host cells include cultured cells, e.g., mammalian cultured cells, yeast cells, insect cells, bacterial cells and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is a eukaryotic cell, preferably a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., Y0, NS0, Sp20 cell). Standard technologies are known in the art to express foreign genes in these systems. Cells expressing a polypeptide comprising either the heavy or the light chain of an antibody, may be engineered so as to also express the other of the antibody chains such that the expressed product is an antibody that has both a heavy and a light chain.

In one embodiment, a method of producing an antibody according to the invention is provided, wherein the method comprises culturing a host cell comprising a polynucleotide encoding the antibody, as provided herein, under conditions suitable for expression of the antibody, and recovering the antibody from the host cell (or host cell culture medium).

Where an antibody is fused to an effector moiety, these components are genetically fused to each other. Antibodies can be designed such that its components are fused directly to each other or indirectly through a linker sequence. The composition and length of the linker may be determined in accordance with methods well known in the art and may be tested for efficacy. Additional sequences may also be included to incorporate a cleavage site to separate the individual components of the fusion if desired, for example an endopeptidase recognition sequence.

In certain embodiments the antibodies of the invention comprise at least an antibody variable region capable of binding to ASGPR. Variable regions can form part of and be derived from naturally or non-naturally occurring antibodies and fragments thereof. Methods to produce polyclonal antibodies and monoclonal antibodies are well known in the art (see e.g. Harlow and Lane, "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory, 1988). Non-naturally occurring antibodies can be constructed using solid phase-peptide synthesis, can be produced recombinantly (e.g. as described in U.S. patent No. 4,186,567) or can be obtained, for example, by screening combinatorial libraries comprising variable heavy chains and variable light chains (see e.g. U.S. Patent. No. 5,969,108 to McCafferty).

Any animal species of antibody can be used in the invention. Non-limiting antibodies useful in the present invention can be of murine, primate, or human origin. If the antibody is intended for human use, a chimeric form of antibody may be used wherein the constant regions of the antibody are from a human. A humanized or fully human form of the antibody can also be prepared in accordance with methods well known in the art (see e. g. U.S. Patent No. 5,565,332 to Winter). Humanization may be achieved by various methods including, but not limited to (a) grafting the non-human (e.g., donor antibody) CDRs onto human (e.g. recipient antibody) framework and constant regions with or without retention of critical framework residues (e.g. those that are important for retaining good antigen binding affinity or antibody functions), (b) grafting only the non-human specificity-determining regions (SDRs or a-CDRs; the residues critical for the antibody-antigen interaction) onto human framework and constant regions, or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, Front Biosci 13, 1619-1633 (2008), and are further described, e.g., in Riechmann et al., Nature 332, 323-329 (1988); Queen et al., Proc Natl Acad Sci USA 86, 10029-10033 (1989); US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Jones et al., Nature 321, 522-525 (1986); Morrison et al., Proc Natl Acad Sci 81,

6851-6855 (1984); Morrison and Oi, Adv Immunol 44, 65-92 (1988); Verhoeyen et al., Science 239, 1534-1536 (1988); Padlan, Molec Immun 31(3), 169-217 (1994); Kashmiri et al., Methods 36, 25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, Mol Immunol 28, 489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., Methods 36, 43-60 (2005) (describing "FR shuffling"); and Osbourn et al., Methods 36, 61-68 (2005) and Klimka et al., Br J Cancer 83, 252-260 (2000) (describing the "guided selection" approach to FR shuffling). Particular antibodies according to the invention are human antibodies. Human antibodies and human variable regions can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, Curr Opin Pharmacol 5, 368-74 (2001) and Lonberg, Curr Opin Immunol 20, 450-459 (2008). Human variable regions can form part of and be derived from human monoclonal antibodies made by the hybridoma method (see e.g. Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Human antibodies and human variable regions may also be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge (see e.g. Lonberg, Nat Biotech 23, 1117-1125 (2005). Human antibodies and human variable regions may also be generated by isolating Fv clone variable region sequences selected from human-derived phage display libraries (see e.g., Hoogenboom et al. in Methods in Molecular Biology 178, 1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001); and McCafferty et al., Nature 348, 552-554; Clackson et al., Nature 352, 624-628 (1991)). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. A detailed description of the preparation of antibodies by phage display can be found in the Examples.

In certain embodiments, the antibodies of the present invention are engineered to have enhanced binding affinity according to, for example, the methods disclosed in PCT publication WO 2011/020783 (see Examples relating to affinity maturation) or U.S. Pat. Appl. Publ. No. 2004/0132066, the entire contents of which are hereby incorporated by reference. The ability of the antibody of the invention to bind to a specific antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. surface plasmon resonance technique (Liljeblad, et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). Competition assays may be used to identify an antibody that competes with a reference antibody for binding to a particular antigen, e.g. an antibody that competes with the 51A12 antibody for binding to

ASGPR. In certain embodiments, such a competing antibody binds to the same epitope (e.g. a linear or a conformational epitope) that is bound by the reference antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols", in Methods in Molecular Biology vol. 66 (Humana Press, Totowa,
5 NJ). In an exemplary competition assay, immobilized antigen (e.g. ASGPR) is incubated in a solution comprising a first labeled antibody that binds to the antigen (e.g. 51A12 antibody) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to the antigen. The second antibody may be present in a hybridoma supernatant. As a control, immobilized antigen is incubated in a solution comprising the first labeled antibody but
10 not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to the antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to
15 the antigen. See Harlow and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Antibodies prepared as described herein may be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography, size exclusion chromatography, and the like. The actual conditions used to
20 purify a particular protein will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity etc., and will be apparent to those having skill in the art. For affinity chromatography purification an antibody, ligand, receptor or antigen can be used to which the antibody binds. For example, for affinity chromatography purification of antibodies of the invention, a matrix with protein A or protein G may be used. Sequential Protein A or G affinity
25 chromatography and size exclusion chromatography can be used to isolate an antibody essentially as described in the Examples. The purity of the antibody can be determined by any of a variety of well known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like. For example, the heavy chain fusion proteins expressed as described in the Examples were shown to be intact and properly assembled as demonstrated by
30 reducing SDS-PAGE (see e.g. Figure 13-19).

Compositions, formulations, and routes of administration

In a further aspect, the invention provides pharmaceutical compositions comprising any of the antibodies provided herein, e.g., for use in any of the below therapeutic methods. In one

embodiment, a pharmaceutical composition comprises any of the antibodies provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises any of the antibodies provided herein and at least one additional therapeutic agent, e.g., as described below.

5 Further provided is a method of producing an antibody of the invention in a form suitable for administration in vivo, the method comprising (a) obtaining an antibody according to the invention, and (b) formulating the antibody with at least one pharmaceutically acceptable carrier, whereby a preparation of antibody is formulated for administration *in vivo*.

Pharmaceutical compositions of the present invention comprise a therapeutically effective 10 amount of one or more antibody dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that are generally non-toxic to recipients at the dosages and concentrations employed, i.e. do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical 15 composition that contains at least one antibody and optionally an additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required 20 by FDA Office of Biological Standards or corresponding authorities in other countries. Preferred compositions are lyophilized formulations or aqueous solutions. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, buffers, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.* antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, antioxidants, proteins, drugs, 25 drug stabilizers, polymers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the 30 therapeutic or pharmaceutical compositions is contemplated.

The composition may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. Antibodies of the present invention (and any additional therapeutic

agent) can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrasplenically, intrarenally, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularally, orally, topically, locally, by inhalation (e.g. aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (e.g. liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference). Parenteral administration, in particular intravenous injection, is most commonly used for administering polypeptide molecules such as the antibodies of the invention.

Parenteral compositions include those designed for administration by injection, e.g. subcutaneous, intradermal, intralesional, intravenous, intraarterial intramuscular, intrathecal or intraperitoneal injection. For injection, the antibodies of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the antibodies may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. Sterile injectable solutions are prepared by incorporating the antibodies of the invention in the required amount in the appropriate solvent with various of the other ingredients enumerated below, as required. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein. Suitable pharmaceutically acceptable carriers

include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Aqueous injection suspensions may contain compounds which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, dextran, or the like. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl cleats or triglycerides, or liposomes.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (18th Ed. Mack Printing Company, 1990). Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g. films, or microcapsules. In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

In addition to the compositions described previously, the antibodies may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example,

the antibodies may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions comprising the antibodies of the invention may be manufactured by means of conventional mixing, dissolving, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the proteins into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The antibodies may be formulated into a composition in a free acid or base, neutral or salt form. Pharmaceutically acceptable salts are salts that substantially retain the biological activity of the free acid or base. These include the acid addition salts, e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

Therapeutic Methods and Compositions

Any of the antibodies provided herein may be used in therapeutic methods.

For use in therapeutic methods, antibodies of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

In one aspect, antibodies of the invention for use as a medicament are provided. In further aspects, antibodies of the invention for use in treating a disease are provided. In certain embodiments, antibodies of the invention for use in a method of treatment are provided. In one embodiment, the invention provides an antibody as described herein for use in the treatment of a disease in an individual in need thereof. In certain embodiments, the invention provides an antibody for use in a method of treating an individual having a disease comprising administering

to the individual a therapeutically effective amount of the antibody. In certain embodiments the disease to be treated is a liver disease. Exemplary liver diseases include hepatits, cirrhosis, or liver cancer such as hepatocellular carcinoma. In a particular embodiment the disease is a viral infection, particularly a hepatitis virus infection, more particularly HBV infection. In another
5 particular embodiment the disease is cancer, particularly liver cancer, more particularly hepatocellular carcinoma (HCC). In certain embodiments the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-viral agent if the disease to be treated is a viral infection or an anti-cancer agent if the disease to be treated is cancer. An “individual” according to any of the
10 above embodiments is a mammal, preferably a human.

In a further aspect, the invention provides for the use of an antibody of the invention in the manufacture or preparation of a medicament for the treatment of a disease in an individual in need thereof. In one embodiment, the medicament is for use in a method of treating a disease comprising administering to an individual having the disease a therapeutically effective amount
15 of the medicament. In certain embodiments the disease to be treated is a liver disease. In a particular embodiment the disease is a viral infection, particularly a hepatitis virus infection, more particularly HBV infection. In other embodiments the disease to be treated is cancer. In a particular embodiment the disease is liver cancer, particularly hepatocellular carcinoma (HCC). In one embodiment, the method further comprises administering to the individual a
20 therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-viral agent if the disease to be treated is a viral infection or an anti-cancer agent if the disease to be treated is cancer. An “individual” according to any of the above embodiments may be a mammal, preferably a human.

In a further aspect, the invention provides a method for treating a disease in an individual,
25 comprising administering to said individual a therapeutically effective amount of an antibody of the invention. In one embodiment a composition is administered to said individual, comprising antibody of the invention in a pharmaceutically acceptable form. In certain embodiments the disease to be treated is a liver disease. In a particular embodiment the disease is a viral infection, particularly a hepatitis virus infection, more particularly HBV infection. In other embodiments
30 the disease to be treated is cancer. In a particular embodiment, the disease is liver cancer, particularly hepatocellular carcinoma (HCC). In certain embodiments the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-viral agent if the disease to be treated is a viral

infection or an anti-cancer agent if the disease to be treated is cancer. An "individual" according to any of the above embodiments may be a mammal, preferably a human.

The antibodies of the invention are also useful as diagnostic reagents. The binding of an antibody to an antigenic determinant can be readily detected by a label attached to the antibody or by
5 using a labeled secondary antibody specific for the antibody of the invention.

In some embodiments, an effective amount of an antibody of the invention is administered to a cell. In other embodiments, a therapeutically effective amount of an antibody of the invention is administered to an individual for the treatment of disease.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention
10 (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the route of administration, the body weight of the patient, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous or concurrent therapeutic interventions, the patient's clinical history and response to the antibody, and the discretion of the
15 attending physician. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

20 The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1 mg/kg – 10 mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the
25 factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.005 mg/kg to about 10 mg/kg. In other non-limiting examples, a dose may also comprise from about 1 μg/kg body weight, about 5 μg/kg body weight, about 10 μg/kg body weight, about 50
30 μg/kg body weight, about 100 μg/kg body weight, about 200 μg/kg body weight, about 350 μg/kg body weight, about 500 μg/kg body weight, about 1 mg/kg body weight, about 5 mg/kg body weight, about 10 mg/kg body weight, about 50 mg/kg body weight, about 100 mg/kg body weight, about 200 mg/kg body weight, about 350 mg/kg body weight, about 500 mg/kg body

weight, to about 1000 mg/kg body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg body weight to about 100 mg/kg body weight, about 5 μg/kg body weight to about 500 mg/kg body weight etc., can be administered, based on the numbers described above. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The antibodies of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the antibodies of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays, such as cell culture assays. A dose can then be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data, *e.g.,* animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the antibodies which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 50 mg/kg/day, typically from about 0.5 to 1 mg/kg/day. Therapeutically effective plasma levels may be achieved by administering multiple doses each day. Levels in plasma may be measured, for example, by HPLC.

In cases of local administration or selective uptake, the effective local concentration of the antibodies may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

A therapeutically effective dose of the antibodies described herein will generally provide therapeutic benefit without causing substantial toxicity. Toxicity and therapeutic efficacy of an

antibodies can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used to determine the LD_{50} (the dose lethal to 50% of a population) and the ED_{50} (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index,
5 which can be expressed as the ratio LD_{50}/ED_{50}. Antibodies that exhibit large therapeutic indices are preferred. In one embodiment, the antibody according to the present invention exhibits a high therapeutic index. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages suitable for use in humans. The dosage lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage
10 may vary within this range depending upon a variety of factors, e.g., the dosage form employed, the route of administration utilized, the condition of the subject, and the like. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see, e.g., Fingl et al., 1975, in: The Pharmacological Basis of Therapeutics, Ch. 1, p. 1, incorporated herein by reference in its entirety).
15 The attending physician for patients treated with antibodies of the invention would know how and when to terminate, interrupt, or adjust administration due to toxicity, organ dysfunction, and the like. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the
20 condition to be treated, with the route of administration, and the like. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency will also vary according to the age, body weight, and response of the individual patient.

Other agents and treatments

25 The antibodies of the invention may be administered in combination with one or more other agents in therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent. The term "therapeutic agent" encompasses any agent administered to treat a symptom or disease in an individual in need of such treatment. Such additional therapeutic agent may comprise any active ingredients suitable for the particular
30 indication being treated, preferably those with complementary activities that do not adversely affect each other. In certain embodiments, an additional therapeutic agent is an anti-viral agent. In other embodiments, an additional therapeutic agent is an anti-cancer agent.

Such other agents are suitably present in combination in amounts that are effective for the purpose intended. The effective amount of such other agents depends on the amount of antibody used, the type of disorder or treatment, and other factors discussed above. The antibodies are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate compositions), and separate administration, in which case, administration of the antibody of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant.

Articles of manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Examples

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Recombinant DNA techniques

5 Standard methods were used to manipulate DNA as described in Sambrook, J. et al, Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory press, Cold spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

General information regarding the nucleotide sequences of human immunoglobulin light and 10 heavy chains is given in: Kabat, E.A. et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242.

DNA sequencing

DNA sequences were determined by double strand sequencing

Gene synthesis

15 Desired gene segments, where required, were either generated by PCR using appropriate templates or were synthesized at Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR products by automated gene synthesis. In cases where no exact gene sequence was available, oligonucleotide primers were designed based on sequences from closest homologues and the genes were isolated by RT-PCR from RNA originating from the appropriate 20 tissue. The gene segments flanked by singular restriction endonuclease cleavage sites were cloned into standard cloning / sequencing vectors. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. Gene segments were designed with suitable restriction sites to allow subcloning into the respective expression vectors. 25 All constructs were designed with a 5'-end DNA sequence coding for a leader peptide which targets proteins for secretion in eukaryotic cells. SEQ ID NOs 135-137 give exemplary leader peptides.

Cloning of antigen expression vectors

The amplified DNA fragments encoding the antigen of interest were inserted in frame into a 30 mammalian recipient vector downstream of a human IgG_1 Fc coding fragment serving as

solubility- and purification tag (Figure 1). Expression of antigen-Fc fusions with a wild type Fc sequence (SEQ ID NOs 123, 125, 127, 129, 131, 133) resulted in homodimeric molecules (avi-Fc-human ASGPR H1 stalk: SEQ ID NO: 124, avi-Fc-cynomolgus ASGPR H1 stalk: SEQ ID NO: 126, , avi-Fc-human ASGPR H1 stalk CRD: SEQ ID NO: 130, avi-Fc-cynomolgus ASGPR
5 H1 stalk CRD: SEQ ID NO: 132). Protein CLEC10A was identified as the closest homologue to ASGPR H1 and the constructs avi-Fc-human CLEC10A stalk (SEQ ID NO: 128) and avi-Fc-human CLEC10A stalk CRD (SEQ ID NO: 134) were expressed for testing the specificity of the selected binders. In order to express the antigen in a monomeric state, the DNA fragment was fused to an Fc part containing the “hole” mutations (SEQ ID NOs 117, 119) and was co-
10 expressed with an Fc-“knob” (SEQ ID NO: 121) counterpart (Fc-human ASGPR H1 CRD: SEQ ID NOs 118 and 122, avi-Fc-human CLEC10A CRD: SEQ ID NOs 120 and 122). The antigen expression was generally driven by an MPSV promoter and transcription was terminated by a synthetic polyA signal sequence located downstream of the CDS. In addition, all constructs contained an N-terminal Avi tag allowing specific biotinylation during co-expression with Bir A
15 biotin ligase. In addition to the expression cassette, each vector contained an EBV oriP sequence for autonomous replication in EBV-EBNA expressing cell lines.

Production and purification of antigens and antibodies

Both antigens and antibodies were transiently transfected into HEK 293 cells, stably expressing the EBV-derived protein EBNA. A simultaneously co-transfected plasmid encoding biotin ligase
20 Bir A allowed Avi tag-specific biotinylation *in vivo*. The proteins were then purified using a protein A column followed by gel filtration.

Generation of a generic lambda Fab-library

A generic lambda antibody library in the Fab-format was generated on the basis of human germline genes using the following V-domain pairings: Vl3_19 lambda light chain with VH3_23
25 heavy chain resulting in a DP47-lambda library. The library was randomized in CDR3 of the light chain (L3) and CDR3 of the heavy chain (H3) and was assembled from three fragments by “splicing by overlapping extension” (SOE) PCR. Fragment 1 comprises the 5’ end of the antibody gene including randomized L3, fragment 2 is a central constant fragment spanning from the end of L3 to the beginning of H3, whereas fragment 3 comprises randomized H3 and the 3’
30 portion of the Fab fragment. The following primer combinations were used to generate library fragments for library: fragment 1 (LMB3 (SEQ ID NO: 146) - Vl_3_19_L3r primers (SEQ ID NOs 143-145)), fragment 2 (RJH80 (SEQ ID NO: 148) - DP47CDR3_ba (mod) (SEQ ID NO:

149)), fragment 3 (DP47-v4 primers (SEQ ID NO: 140-142) - fdseqlong (SEQ ID NO: 147) (Table 1). PCR parameters for production of library fragments were 5 min initial denaturation at 94°C, 25 cycles of 60 sec 94°C, 60 sec 55°C, 60 sec 72°C and terminal elongation for 10 min at 72°C. For assembly PCR, using equimolar ratios of the 3 fragments as template, parameters were 3 min initial denaturation at 94°C and 5 cycles of 60 s 94°C, 60 sec 55°C, 120 sec 72°C. At this stage, outer primers were added and additional 20 cycles were performed prior to a terminal elongation for 10 min at 72°C (Figure 2). After assembly of sufficient amounts of full length randomized Fab fragments, they were digested with *Nco*I / *Nhe*I alongside with similarly treated acceptor phagemid vector. 15 µg of Fab library insert were ligated with 13.3 µg of phagemid vector. Purified ligations were used for 60 transformations resulting in 1.5 x 10^9 transformants. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections.

Table 1. Sequences of primers used for the generation of the generic lambda library.

SEQ ID NO	NAME	SEQUENCE
140	DP47-v4-4	CGAGGACACGGCCGTATATTACTGTGCG-5-1–2-2-3-4-GAC-TAC- TGGGGCCAAGGAACCCTGGTCACCGTCTCG 1 : G/D=20, E/V/S=10, A/P/R/L/T/Y=5%; 2 : G/Y/S=15, A/D/T/R/P/L/V/N/W/F/I/E=4,6%; 3 : G/A/Y=20, P/W/S/D/T=8%; 4 : F=46, L/M=15, G/I/Y=8%; 5 : K=70, R=30%
141	DP47-v4-6	CGAGGACACGGCCGTATATTACTGTGCG-5-1–2-2-2-2-3-4-GAC-TAC- TGGGGCCAAGGAACCCTGGTCACCGTCTCG 1 : G/D=20, E/V/S=10, A/P/R/L/T/Y=5%; 2 : G/Y/S=15, A/D/T/R/P/L/V/N/W/F/I/E=4,6%; 3 : G/A/Y=20, P/W/S/D/T=8%; 4 : F=46, L/M=15, G/I/Y=8%; 5 : K=70, R=30%
142	DP47-v4-8	CGAGGACACGGCCGTATATTACTGTGCG-5-1–2-2-2-2-2-2-3-4-GAC-TAC- TGGGGCCAAGGAACCCTGGTCACCGTCTCG 1 : G/D=20, E/V/S=10, A/P/R/L/T/Y=5%; 2 : G/Y/S=15, A/D/T/R/P/L/V/N/W/F/I/E=4,6%; 3 : G/A/Y=20, P/W/S/D/T=8%; 4 : F=46, L/M=15, G/I/Y=8%; 5 : K=70, R=30%
143	Vl_3_19_L3r_V	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC V***HV*** A***TT*** A***CC*** G***CT*** A***CT*** A***TC*** A***CG*** GGAGTTACAGTAATAGTCAGCCTCATCTTCCGC underlined: 60% original base and 40% randomization as M bolded and italic: 60% original base and 40% randomization as N
144	Vl_3_19_L3r_HV	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC C***MM*** A***TG*** A***TT*** A***CC*** G***CT*** A***CT*** A***TC*** A***CG*** GGAGTTACAGTAATAGTCAGCCTCATCTTCCGC underlined: 60% original base and 40% randomization as M bolded and italic: 60% original base and 40% randomization as N
	Vl_3_19_L3	GGACGGTCAGCTTGGTCCCTCCGCCGAATAC R***HM*** V***WG***

145	r_HLV	A***TG*** A***TT*** A***CC*** G***CT*** A***CT*** A***TC*** A***CG*** GGAGTTACAGTAATAGTCAGCCTCATCTTC CGC underlined: 60% original base and 40% randomization as M bolded and italic: 60% original base and 40% randomization as N
146	LMB3	CAGGAAACAGCTATGACCATGATTAC
147	fdseqlong	GACGTTAGTAAATGAATTTTCTGTATGAGG
148	RJH80	TTCGGCGGAGGGACCAAGCTGACCGTCC
149	DP47CDR3_ ba (mod)	CGCACAGTAATATACGGCCGTGTCC

Selection of anti-human ASGPR H1 binders from a generic lambda Fab library

Selections against the complete or fragments of the extracellular domain (ECD) of human ASGPR H1 were carried out using HEK293-expressed monomeric or dimeric human ASGPR protein fragments fused to the Fc-portion of a human IgG1 antibody (SEQ ID NO: 118, 120, 124, 126, 128, 130, 132, 134). While ASGPR H1 CRD and CLEC10A CRD were expressed as monomeric Fc fusions using the Fc „knob-into-hole" format (only one Fc carrying a C-terminally fused CRD), all stalk fragments and total ECDs were expressed as homodimeric Fc fusion proteins (Figure 1). The antigens were enzymatically biotinylated by co-expression of the biotin ligase Bir A via an N-terminal avi-tag. Panning rounds were performed in solution according to the following pattern: (1) Preclearing of ~10^{12} phagemid particles using human IgG_1 coated at 10 µg/ml onto NUNC maxisorp plates to avoid Fc-binders, (2) binding of non-Fc binding phagemid particles from the supernatant of the pre-clearing reaction to 100 nM biotinylated antigen protein for 0.5 h in a total volume of 1 ml, (3) capture of biotinylated antigen and attached specifically binding phage by addition of 5.4 x 10^7 streptavidin-coated magnetic beads for 10 min, (4) washing of beads using 5x 1 ml PBS/Tween-20 and 5x 1 ml PBS, (5) elution of phage particles by addition of 1 ml 100 mM triethylamine (TEA) for 10 min and neutralization by addition of 500 µl 1M Tris/HCl pH 7.4, (6) Re-infection of log-phase *E. coli* TG1 cells with the phage particles in the supernatant, infection with helperphage VCSM13 and subsequent PEG/NaCl precipitation of phagemid particles to be used in subsequent selection rounds. Selections were carried out over 3-5 rounds using either constant or decreasing (from 10^{-7} M to 2 x 10^{-9} M) antigen concentrations. In round 2, capture of antigen-phage complexes was performed using neutravidin plates instead of streptavidin beads. Specific binders were identified by ELISA as follows: 100 µl of 50 nM biotinylated human Fc-stalk-CRD, Fc-CRD, or Fc-stalk per well were coated on neutravidin plates. Fab-containing bacterial supernatants were added and binding Fabs were detected via their Flag-tags by using an anti-Flag/HRP secondary antibody.

Clones exhibiting significant signals over background were short-listed for sequencing (SEQ ID NOs 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27) and further analyses.

Purification of Fabs

Fabs from bacterial cultures (protein sequence of variable domains listed as SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28) were purified for the exact analysis of the kinetic parameters. For each clone, a 500 ml culture was inoculated with bacteria harboring the corresponding phagemid and induced with 1 mM IPTG at an OD_{600} 0.9. Afterwards, the cultures were incubated at 25°C overnight and harvested by centrifugation. After the incubation of the resuspended pellet for 20 min in 25 ml PPB buffer (30 mM Tris-HCl pH 8, 1 mM EDTA, 20% sucrose), bacteria were centrifuged again and the supernatant was harvested. This incubation step was repeated once with 25 ml of a 5 mM $MgSO_4$ solution. The supernatants of both incubation steps were pooled, filtered and loaded on an IMAC column (His gravitrap, GE Healthcare). Subsequently, the column was washed with 40 ml washing buffer (500 mM NaCl, 20 mM imidazole, 20 mM NaH_2PO_4 pH 7.4). After the elution (500 mM NaCl, 500 mM imidazole, 20 mM NaH_2PO_4 pH 7.4) the eluate was re-buffered using PD10 columns (GE Healthcare). The kinetic parameters of the purified Fabs were then studied by SPR-analysis (Proteon XPR36, Biorad) in a dilution row that ranged from 200 nM to 6.25 nM.

Affinity-determination by SPR

Affinity (K_D) of selected Fab clones was measured by surface plasmon resonance using a ProteOn XPR36 instrument (Biorad) at 25°C with biotinylated mono- (avi-Fc-human ASGPR H1 CRD, SEQ ID NO: 118) or bivalent (avi-Fc-human ASGPR H1 stalk-CRD, SEQ ID NO: 130) ASGPR H1 antigens immobilized on NLC chips by neutravidin capture. Immobilization of recombinant antigens (ligand): Antigens were diluted with PBST (10 mM phosphate, 150 mM NaCl pH 7.4, 0.005% Tween-20) to 10 μg/ml, then injected at 30 μl/min at varying contact times, to achieve immobilization levels of 200, 400 or 800 response units (RU) in vertical orientation. Injection of analytes: For one-shot kinetics measurements, injection direction was changed to horizontal orientation, two-fold dilution series of purified Fab (varying concentration ranges between 100 and 6.25 nM) were injected simultaneously at 50, 60 or 100 μl/min along separate channels 1-5, with association times of 150 or 200 s, and dissociation times of 240 or 600 s. Buffer (PBST) was injected along the sixth channel to provide an "in-line" blank for referencing. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were calculated using a simple one-to-one Langmuir binding model in ProteOn Manager v3.1 software by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation

constant (K_D) was calculated as the ratio k_{off}/k_{on}. Regeneration was performed in horizontal orientation using 10 mM glycine, pH 1.5 at a flow rate of 100 µl/min for a contact time of 30 s. Two clones, 51A12 (SEQ ID NO: 002 and 004) and 52C4 (SEQ ID NO: 006 and 008), were found to be specific to the ASGPR H1 CRD. Remarkably, clone 51A12 revealed an affinity in
5 the subnanomolar range. Clones 5A4 (SEQ ID NO: 010 and 012), 4F3 (SEQ ID NO: 014 and 016), R5C2 (SEQ ID NO: 018 and 020), R9E10 (SEQ ID NO: 022 and 024), and R9E10 (SEQ ID NO: 026 and 028) were raised either against the stalk region of ASGPR H1 or the interface between the stalk and CRD. The affinity to their corresponding human and cynomolgus epitopes was similar. In contrast, no binding to avi-Fc-human CLEC10A stalk CRD (SEQ ID NO: 134)
10 was detected, demonstrating the high specificity of these binders. Interestingly, clone 5A4 demonstrated strong binding to the stalk antigen but not to stalk-CRD. The kinetic and thermodynamic data of all measurements are summarized in Table 2.

Table 2. Kinetic and thermodynamic parameters of anti-ASGPR H1 Fabs.

Antibody clone	huASGPR H1 stalk ka (1/Ms) kd (1/s) **KD (M)**	cyASGPR H1 stalk ka (1/Ms) kd (1/s) **KD (M)**	huASGPR H1 stalk CRD ka (1/Ms) kd (1/s) **KD (M)**	cyASGPR H1 stalk CRD ka (1/Ms) kd (1/s) **KD (M)**	hu/cyASGPR H1 CRD ka (1/Ms) kd (1/s) **KD (M)**	huCLEC10A stalk CRD ka (1/Ms) kd (1/s) **KD (M)**
52C4					1.39×10^{5} 3.96×10^{-3} $\mathbf{2.86 \times 10^{-8}}$	no binding
51A12					1.10×10^{5} 6.28×10^{-5} $\mathbf{5.70 \times 10^{-10}}$	no binding
5A4	2.44×10^{5} 1.05×10^{-3} $\mathbf{4.30 \times 10^{-9}}$	3.72×10^{5} 1.40×10^{-3} $\mathbf{3.76 \times 10^{-9}}$				no binding
4F3	1.51×10^{5} 4.45×10^{-3} $\mathbf{2.95 \times 10^{-8}}$	1.43×10^{5} 4.80×10^{-3} $\mathbf{3.36 \times 10^{-8}}$	2.24×10^{5} 4.08×10^{-3} $\mathbf{1.82 \times 10^{-8}}$	1.69×10^{5} 3.65×10^{-3} $\mathbf{2.16 \times 10^{-8}}$		no binding
R9E10			5.86×10^{5} 2.11×10^{-3} $\mathbf{3.60 \times 10^{-9}}$	4.60×10^{5} 1.99×10^{-3} $\mathbf{4.34 \times 10^{-9}}$		no binding
R7E12			2.94×10^{5} 2.68×10^{-3} $\mathbf{9.12 \times 10^{-9}}$	2.47×10^{5} 2.36×10^{-3} $\mathbf{9.55 \times 10^{-9}}$		no binding
R5C2			4.23×10^{5} 1.12×10^{-3} $\mathbf{2.65 \times 10^{-9}}$	3.34×10^{5} 1.14×10^{-3} $\mathbf{3.41 \times 10^{-9}}$		no binding

Cloning of variable antibody domains into expression vectors

All Fabs demonstrating specific binding to their corresponding antigen by SPR were converted into an IgG_1 / lambda antibody. Therefor, the PCR-amplified DNA fragments of heavy and light chain v-domains were inserted in frame into either the human IgG_1 constant heavy chain or the 5 human constant lambda light chain containing respective recipient mammalian expression vector. The antibody expression was driven by an MPSV promoter and transcription was terminated by a synthetic polyA signal sequence located downstream of the CDS. In addition to the expression cassette each vector contained an EBV oriP sequence for autonomous replication in EBV-EBNA expressing cell lines.

10 **Binding analysis of the antibodies to HepG2 cells**

Binding of human IgG_1 anti-ASGPR antibodies to the hepatocellular carcinoma cell line HepG2 was measured by FACS. Briefly, 0.2 mio cells per well in a 96 well round bottom plate were incubated in 300 µl with the anti-ASGPR antibodies at a concentration of 30 µg/ml for 30 min at 4°C. Unbound antibody was removed by washing the cells with PBS containing 0.1% BSA. 15 Bound antibodies were detected with FITC-conjugated AffiniPure goat anti-human IgG Fc gamma fragment-specific secondary F(ab')2 fragment (Jackson ImmunoResearch #109-096-098; working solution 1:20 in PBS, 0.1% BSA). After 30 min incubation at 4°C unbound antibody was removed by washing and cells were fixed using 1% PFA. Cells were analyzed using BD FACS CantoII (Software BD DIVA) (Figure 3). All antibodies showed strong binding to the 20 HepG2 cells.

Fluorescence Resonance Energy Transfer assay

The avidity of the IgGs to their epitope on ASGPR-expressing cells was determined by Fluorescence Resonance Energy Transfer (FRET) analysis. For this analysis, the DNA sequence encoding for the SNAP Tag (plasmid purchased from Cisbio) was amplified by PCR and ligated 25 into an expression vector, containing the full length human ASGPR H1 sequence (Origene). The resulting fusion protein was comprised of full-length ASGPR H1 with a C-terminal SNAP tag. HEK293 cells were transfected with 10 µg DNA using Lipofectamine 2000 as transfection reagent. After an incubation time of 20 h, cells were washed with PBS and incubated for 1 h at 37°C in LabMed buffer (Cisbio) containing 100 nM SNAP-Lumi4Tb (Cibsio), leading to 30 specific labeling of the SNAP Tag. Subsequently, cells were washed 4 times with LabMed buffer to remove unbound dye. The labeling efficiency was determined by measuring the emission of terbium at 615 nm compared to buffer. Cells were then stored frozen at -80°C for up to 6 months.

Avidity was measured by adding ASGPR-specific antibodies at a concentration ranging from 50-0.39 nM to labeled cells (100 cells per well) followed by addition of anti-humanFc-d2 (final 200 nM per well) as acceptor molecule for the FRET. After an incubation time of 3h at RT the emission of the acceptor dye (665 nm) as well as of the donor dye (615 nm) was determined using a fluorescence Reader (Victor 3, Perkin Elmer). The ratio of acceptor to donor emission was calculated and the ratio of the background control (cells with anti-huFc-d2) subtracted. Curves were analysed in GraphPad Prism5 and K_D values calculated (Figure 4). While clone 4F3 shows the lowest affinity to ASGPR H1 stalk-CRD measured by SPR (Table 2), binding intensity as an IgG to the cell surface is driven by strong avidity making 4F3 to the clone with the strongest binding intensity at low concentrations. In contrast, clone 51A12 which binds to the CRD shows a significantly weaker binding intensity at low antibody concentrations to cells than to the purified antigen in SPR studies.

Binding competition with a natural ASGPR ligand

Competition of the ASGPR antibodies with a desialylated glycoprotein such as asialofetuin as a natural ligand for ASGPR was analyzed using the hepatocellular carcinoma cell line HepG2. 0.2 mio cells per well in a 96 well round bottom plate were incubated with 40 µl of Alexa488 labeled asialofetuin (from fetal calf serum, Sigma Aldrich #A4781, final concentration 100 µg/ml) at 4°C for 30 min. The binding was performed in the presence of calcium, as ligand binding to ASGPR is calcium dependent. Unbound protein was removed by washing the cells once with HBSS containing 0.1% BSA. Then 40 µl of the anti-ASGPR antibodies (30, 6, and 1.25 µg/ml final concentration) were added to the cells in the presence of 100 µg/ml asialofetuin. Cells were incubated for 30 min at 4°C and unbound protein was removed by washing the cells once. An APC-conjugated AffiniPure goat anti-human IgG Fc gamma fragment-specific secondary F(ab')2 fragment (Jackson ImmunoResearch #109-136-170; working solution 1:50 in HBSS containing 0.1% BSA) was used as an secondary antibody. After 30 min incubation at 4°C unbound secondary antibody was removed by washing. Cells were fixed using 1% PFA and analyzed using BD FACS CantoII (Software BD DIVA). Analysis of both the CRD-specific and the stalk-CRD-specific antibodies revealed that antibodies bind to ASGPR H1 independently of the presence of the asialofetuin and vice versa, and no binding competition takes place. (Figure 5 and 6).

Internalization study

Uptake of desialylated glycoproteins into liver cells after binding to ASGPR is known to occur very rapidly. During this receptor-mediated endocytosis, the lumen of the endosome becomes acidic allowing the receptor-ligand complexes to dissociate. While the ligand is targeted for degradation in lysosomes, ASGPR was shown to recycle back to the cell surface. In order to
5 analyze the retention time of the antibodies on the cell surface, internalization of the ASGPR-antibody complex was analyzed using the hepatocellular carcinoma cell line HepG2. ASGPR-positive HepG2 cells were shifted in cell culture medium to 4°C in order to inhibit internalization. After 45 min incubation with the antibodies (30 µg/ml) on a shaker at 4°C, unbound antibodies were removed by washing twice with cold PBS and cells were re-suspended and cultured in pre-
10 warmed medium at 37°C to re-activate the cellular metabolism including receptor-mediated endocytosis. One aliquot was taken immediately and stored on ice which represents time point zero. Remaining cells were incubated at 37°C and after 5, 15, 30 and 120 min additional samples were taken and washed with cold PBS to stop further internalization. Cell surface-bound antibodies were detected using PE-conjugated AffiniPure goat anti-human IgG Fc gamma-
15 specific secondary F(ab')2 antibody Fragment (Jackson ImmunoResearch #109-116-170, working solution 1:50). After 30 min incubation at 4°C unbound antibody was removed by washing with PBS containing 0.1% BSA. Cells were fixed using 1% PFA and analyzed using BD FACS CantoII (Software BD DIVA). Figure 7A shows exemplary cell surface exposed antibody levels of clones 4F3 and 51A12. Interestingly, extracellular antibody signal decreased
20 significantly (up to 60% signal decrease) during the first 30 min but then decrease delayed for the rest of the time course. This result indicates that antibodies are internalized very efficiently but then eventually recycle back to the cell surface leading to a dynamic steady state condition of constant internalization and recycling. In order to support this hypothesis, the same experiment was performed, but incubation of the cells with the antibodies was performed in cell culture
25 medium for 45 min at 37°C. These conditions allow receptor-antibody complexes to be formed and be internalized during the entire incubation time, eventually leading to a steady state of constant endocytosis and recycling. Afterwards, unbound antibody was removed by washing twice with warm PBS and cells were re-suspended in warm medium. One sample was taken immediately and stored on ice which represents time point zero. Remaining cells were incubated
30 at 37°C and after 5, 15, 30 and 120 min additional samples were taken and washed with cold PBS to stop further internalization. Detection of surface-exposed antibodies was performed as described above. FACS analysis revealed that the decrease of the signal intensity was less pronounced during the time course of the experiment after antibody incubation at 37°C than 4°C

suggesting that incubation of the antibodies at 37°C yields in an equilibrium of internalization and recycling of the antibody-receptor complex (Figure 7B). In order to further endorse the hypothesis of constant internalization and recycling, internalization of ASGPR H1-specific antibodies was further analyzed using a set of directly FITC-labeled antibodies. As before, 5 labeled antibodies were incubated with HepG2 cells at 4°C allowing the antibodies to bind to ASGPR H1 but not to internalize. After 45 min incubation with the antibodies (30 μg/ml) on a shaker at 4°C, unbound antibodies were removed by washing twice with cold PBS and cells were re-suspended and cultured in pre-warmed medium at 37°C to re-activate the cellular metabolism including receptor-mediated endocytosis. A cell aliquot was taken after 0, 5, 15, 30 and 120 min 10 and washed with cold PBS to stop further internalization. Cell surface-bound antibodies were detected using PE-conjugated AffiniPure goat anti-human IgG Fc gamma-specific secondary F(ab')2 antibody Fragment (Jackson ImmunoResearch #109-116-170, working solution 1:50). As seen before, the detection level of surface-exposed decreased significantly during the first 30 min before it stabilized (Figure 7C). However, detection of the IgGs by FITC signal, 15 representing both surface-exposed and internalized antibodies, revealed that the total amount of antibody stayed constant over time (Figure 7D). This result strongly supports the finding that antibodies are in a dynamic steady state condition of constant internalization and recycling.

Generation of a clone 51A12-based L3 affinity library

Analysis of the antibody sequences revealed two hot spots in the CDR3 region of the 51A12 20 light chain, namely two adjacent cysteines and a glycosylation site (Figure 8). For the generation of 51A12-derived clones without cysteines and glycosylation, a maturation library randomized in LCDR3 was generated. The sequence of clone 51A12 (A82G, C112S, C113S, S116A) (SEQ ID NO: 33) was used as a template for the randomization. Triplets encoding positions "RDISSNRAVRN" were randomized throughout the segment. For the generation of the library, 25 a DNA portion resulting from a two-fragment overlap PCR product was cloned into the phage vector. For the generation of fragment 1, the primer combination LCDR3 rand (SEQ ID NO: 151) and fdseqlong (SEQ ID NO: 147) (Table 1 and 3) were used, using clone 51A12 (A82G, C112S, C113S, S116A) as a template. Amplification conditions included an initial 5 min 94°C incubation step followed by 25 cycles, each consisting of a 30 sec 94°C denaturation, a 30 sec 30 60°C annealing, and a 90 sec 72°C elongation step, followed by a final 10 min 72°C elongation step. The resulting fragment was purified on an agarose gel. Fragment 2 was generated with the primer combination LCDR3rev (SEQ ID NO: 150) and LMB3 (SEQ ID NO: 146) (Table 1 and 3). Amplification conditions included an initial 5 min 94°C incubation step followed by 25

cycles, each consisting of a 30 sec 94°C denaturation, a 30 sec 60°C annealing, and a 30 sec 72°C elongation step, followed by a final 10 min 72°C elongation step. For the assembly of both fragments, equimolar amounts of fragment 1 and 2 were used. Amplification conditions included an initial 5 min 94°C incubation step followed by 5 cycles without primers, each cycle consisting of a 1 min 94°C denaturation, a 1 min 60°C annealing, and a 120 sec 72°C elongation step. After the addition of the outer primers LMB3 and fdseqlong, 20 additional cycles were performed using the same parameters. At the end, a final 10 min 72°C incubation step was performed. Both, the resulting gel-purified DNA fragment and clone 51A12 (A82G, C112S, C113S, S116A) (SEQ ID NO: 33) were digested with NcoI/PstI (Figure 9). For generation of the library, ligation was performed with 10 μg insert and 30 μg vector. Purified ligation was transformed into TG1 bacteria by electroporation resulting in 3 x 10^9 transformants. Phagemid particles displaying the Fab library were rescued and purified by PEG/NaCl purification to be used for selections.

Table 3. Sequences of primers used for the generation of the L3 affinity maturation library.

SEQ ID	NAME	SEQUENCE
150	LCDR3rev	GGAGTTACAGTAATAGTCAGCCTC
151	LCDR3 rand	GAGGCTGACTATTACTGTAACTCC 1-2-3-4-5-6-7-8-9-10-11 TTCGGCGGAGGGACCAAGCTGACCGTC 1: 50% R, 3.1% Rest (no S, T, C); 2: 50% D, 2.8% Rest (no C); 3: 50% I, 2.8% Rest (no C); 4: 50% S, 2.8% Rest (no C) ; 5: 50% S, 2.8% Rest (no C); 6: 50% N, 2.8% Rest (no C); 7: 50% R, 2.8% Rest (no C); 8: 50% A, 3.1% Rest (no S, T, C); 9: 50% V, 2.8% Rest (no C); 10: 50% R, 2.8% Rest (no C); 11: 50% N, 2.8% Rest (no C)

Selection of affinity matured 51A12-derived clones without cysteines and glycosylation site

Generation of affinity-matured 51A12-derived Fabs without cysteines and glycosylation site within LCDR3 was carried out by phage display using standard protocols (Silacci et al. (2005), Proteomics 5, 2340-50). In the first panning round, selection was carried out in solution according to the following procedure: (1) binding of ~10^{12} phagemid particles to 10 nM biotinylated Fc-CRD for 0.5 h in a total volume of 1 ml, (2) capture of biotinylated Fc-CRD and specifically bound phage particles by addition of 5.4 x 10^7 streptavidin-coated magnetic beads for 10 min, (3) washing of beads using 5 x 1 ml PBS/Tween-20 and 5 x 1 ml PBS, (4) elution of phage particles by addition of 1 ml 100 mM TEA for 10 min and neutralization by adding 500 μl 1M Tris/HCl pH 7.4, (5) re-infection of exponentially growing *E. coli* TG1 bacteria, and (6) infection with helperphage VCSM13 and subsequent PEG/NaCl precipitation of phagemid

particles to be used in subsequent selection rounds. Selections were carried out over three rounds using decreasing (from 10 x 10^{-9} M to 0.5 x 10^{-9} M) antigen concentrations. In round 2 and 3, capture of antigen-phage complexes was performed using neutravidin plates instead of streptavidin beads. In addition, neutravidin plates were washed for 3 h in 2 l PBS. Specific
5 binders were identified by ELISA as follows: 100 µl of 50 nM biotinylated Fc-CRD per well were coated on neutravidin plates. Fab-containing bacterial supernatants were added and binding Fabs were detected via their Flag-tags by using an anti-Flag/HRP secondary antibody. ELISA-positive clones were bacterially expressed as soluble Fab fragments in 96-well format and supernatants were subjected to a kinetic screening experiment by SPR-analysis using Proteon
10 XPR36. Clones expressing Fabs with the highest affinity constants were identified and the light chains of the corresponding phagemids were sequenced (51A12_C1, SEQ ID NO: 35; 51A12_C7, SEQ ID NO: 37; 51A12_E7, SEQ ID NO: 39; 51A12_H3, SEQ ID NO: 41; 51A12_A6, SEQ ID NO: 43; 51A12_D1, SEQ ID NO: 45; 51A12_H6, SEQ ID NO: 47). All clones were devoid of any critical amino acids in the CDR3 region of the light chain.

15 **Affinity determination of the 51A12-based affinity matured clones by SPR**

Affinity (K_D) of purified 51A12-derived Fab fragments consisting of the parental heavy chain (SEQ ID NO: 4) and the affinity-matured light chains (51A12_C1, SEQ ID NO: 36; 51A12_C7, SEQ ID NO: 38; 51A12_E7, SEQ ID NO: 40; 51A12_H3, SEQ ID NO: 42; 51A12_A6, SEQ ID NO: 44; 51A12_D1, SEQ ID NO: 46; 51A12_H6, SEQ ID NO: 48) was measured by surface
20 plasmon resonance using a ProteOn XPR36 instrument (Biorad) at 25°C with biotinylated mono- (avi-Fc-human ASGPR H1 CRD, SEQ ID NO: 118) or bivalent (avi-Fc-human ASGPR H1 stalk-CRD, SEQ ID NO: 130) ASGPR H1 antigens immobilized on NLC chips by neutravidin capture. Immobilization of recombinant antigens (ligand): Antigens were diluted with PBST (10 mM phosphate, 150 mM NaCl pH 7.4, 0.005% Tween-20) to 10 µg/ml, then injected at 30
25 µl/min at varying contact times, to achieve immobilization levels of 200, 400 or 800 response units (RU) in vertical orientation. Injection of analytes: For one-shot kinetics measurements, injection direction was changed to horizontal orientation, two-fold dilution series of purified Fab (varying concentration ranges between 12.5 and 0.78 nM) were injected simultaneously at 100 µl/min along separate channels 1-5, with association times of 150 or 200 s, and dissociation
30 times of 3600 s. Buffer (PBST) was injected along the sixth channel to provide an “in-line” blank for referencing. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were calculated using a simple one-to-one Langmuir binding model in ProteOn Manager v3.1 software by simultaneously fitting the association and dissociation sensorgrams. The equilibrium

dissociation constant (K_D) was calculated as the ratio k_{off}/k_{on}. Regeneration was performed in horizontal orientation using 10mM glycine, pH 1.5 at a flow rate of 100 µl/min for a contact time of 30 s. While most of the selected clones showed similar affinities like the parental clone, clone 51A12_A6 (SEQ ID NO: 44) showed a significantly improved affinity (Table 4).

5 **Table 4.** Kinetic and thermodynamic parameters of affinity-matured anti-ASGPR1 Fabs.

ASGPR CRD-specific binders	human / cyno ASGPR1 CRD		
	ka(1/Ms)	kd(1/s)	KD(M)
51A12	1.10E+05	6.28E-05	**5.70E-10**
51A12 A82G S116A	1.27E+05	1.60E-04	**1.25E-09**
51A12 S116A	1.31E+05	1.78E-4	**1.43E-09**
51A12 _C1	1.74E+05	4.19E-05	**2.41E-10**
51A12_ E7	2.15E+05	9.64E-05	**4.48E-10**
51A12 _H3	1.63E+05	8.30E-05	**5.10E-10**
51A12_A6	3.26E+05	2.61E-05	**8.01E-11**
51A12_C7	1.99E+05	4.67E-05	**2.35E-10**
51A12_D1	4.00E+05	8.85E-05	**2.21E-10**
51A12_H6	0.86E+05	2.79E-05	**3.25E-10**

Binding analysis of the affinity matured 51A12 derivatives to HepG2 cells

Binding of the selected affinity matured 51A12 derivatives to the ASGPR-positive hepatocellular carcinoma cell line HepG2 was measured by FACS. As a negative control, the ASGPR-negative
10 cell line Hela was used. 0.2 mio cells per well in a 96 well round bottom plate were incubated in 300 µl with either purified Fab fragments (1.1, 3.3 and 10 µg/ml) or human IgG_1-converted antibodies (0.01, 0.04, 0.1, 0.4, 1.1, 3.3 and 10 µg/ml) for 30 min at 4°C. Unbound molecules were removed by washing the cells with PBS containing 0.1% BSA. Bound molecules were detected with either a FITC-conjugated AffiniPure goat anti-human F(ab')2 fragment-specific
15 secondary F(ab')2 fragment (Jackson Immuno Research Lab #109-096-097) or a FITC-conjugated AffiniPure goat anti-human IgG Fc gamma fragment-specific secondary F(ab')2 fragment (Jackson ImmunoResearch #109-096-098; working solution 1:20 in PBS, 0.1% BSA). After 30 min incubation at 4°C unbound antibody was removed by washing and cells were fixed using 1% PFA. Cells were analyzed using BD FACS CantoII (Software BD DIVA). Analysis of

the Fab binding to HepG2 cells revealed strong binding of all clones (Figure 10). Variant 51A12_A6 (SEQ ID NO: 44) was the strongest binder in both SPR analysis and the cell binding study. Binding analysis of the clone variants as IgG_1-converted antibodies to HepG2 cells resulted in a similarly strong binding pattern for all clones (Figure 11A) while binding to Hela
5 cells at the highest antibody concentration was very weak or not detectable (Figure 11B), underlining the specificity of these clone variants.

Generation of IgG-IFNα DNA constructs

DNA sequences encoding ASGPR H1-targeted IgG-IFNα fusion proteins were generated based on the ASGPR H1-antibodies 51A12, 51A12 (S116A), 51A12 (A82G, S116A), 52C4, 5A4, 4F3,
10 R5C2, R9E10, R7E12, 51A12_C1, 51A12_C7, 51A12_E7, 51A12_H3, 51A12_A6, 51A12_D1 and 51A12_H6 wherein one Interferon-α2a (IFNα) was fused to the C-terminus of one heterodimeric heavy chain as shown in Figure 12A. Targeting to the liver hepatocytes where ASGPR H1 is selectively expressed is achieved via the bivalent antibody Fab region (avidity effect). Heterodimerization resulting in the presence of a single IFNα is achieved by application
15 of the knob-into-hole (kih) technology. In order to minimize the generation of homodimeric IgG-cytokine fusions, the cytokine was fused to the C-terminus (with deletion of the C-terminal Lys residue) of the knob-containing IgG heavy chain via a $(G_4S)_3$ linker. The antibody-cytokine fusion has IgG-like properties. To reduce FcγR binding/effector function and prevent FcR co-activation, P329G L234A L235A (LALA) mutations were introduced in the Fc domain.
20 However, FcRn binding is not impaired. The DNA sequences encoding these immunoconjugates are given in SEQ ID NOs 49, 51 and 53 (51A12), SEQ ID NOs 55, 57 and 59 (52C4), SEQ ID NOs 93, 51 and 53 (51A12 A82G, S116A), SEQ ID NOs 91, 51 and 53 (51A12, S116A), SEQ ID NOs 61, 63 and 65 (5A4), SEQ ID NOs 67, 69 and 71 (4F3), SEQ ID NOs 73, 75 and 77 (R5C2), SEQ ID NOs 79, 81 and 83 (R9E19), SEQ ID NOs 85, 87 and 89 (R7E12), SEQ ID
25 NOs 95, 51 and 53 (51A12_C1), SEQ ID NOs 97, 51 and 53 (51A12_C7), SEQ ID NOs 99, 51 and 53 (51A12_E7), SEQ ID NOs 101, 51 and 53 (51A12_H3), SEQ ID NOs 103, 51 and 53 (51A12_A6), SEQ ID NOs 105, 51 and 53 (51A12_D1), SEQ ID NOs 107, 51 and 53 (51A12_H6). In addition, an alternative hole- heavy chain was created where both VH and CH1 domains were deleted (SEQ ID NO: 115). The resulting Fc fragment was able to hetero-dimerize
30 with the full-length knob heavy chain leading to a monovalent antibody with a single cytokine fusion (Figure 12B). As a negative control for functional assays, corresponding DNA constructs encoding a control DP47GS / DPL16 non-targeted IgG-IFNa protein wherein the IgG does not

bind to a specified target was generated. The DNA sequence of this isotype immunoconjugate is given in SEQ ID NOs 109, 111 and 113.

Expression and purification of the antibody-cytokine constructs

Immunoconjugates were produced by co-transfecting exponentially growing HEK293-EBNA cells with the mammalian expression vectors using calcium phosphate-transfection. Alternatively, HEK293-EBNA cells growing in suspension were transfected by polyethylenimine (PEI) with the respective expression vectors. Subsequently, the IgG-cytokine fusion proteins were purified from the supernatant by a method composed of one affinity step (protein A) followed by size exclusion chromatography (Superdex 200, GE Healthcare). The protein A column (HiTrap ProtA, GE Healthcare) was equilibrated in 20 mM sodium phosphate, 20 mM sodium citrate pH 7.5. After loading of the supernatant, the column was first washed with 20 mM sodium phosphate, 20 mM sodium citrate, pH 7.5 and subsequently washed with 13.3 mM sodium phosphate, 20 mM sodium citrate, 500 mM sodium chloride, pH 5.45. The IgG-cytokine fusion protein was eluted with 20 mM sodium citrate, 100 mM sodium chloride, 100 mM glycine, pH 3. Fractions were neutralized, pooled, and purified by size exclusion chromatography (HiLoad 16/60 Superdex 200, GE Healthcare) in final formulation buffer (25 mM potassium phosphate, 125 mM sodium chloride, 100 mM glycine pH 6.7 or 20 mM histidine, 140 mM NaCl, pH 6.0). The protein concentration of purified protein samples was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and molecular weight of immunoconjugates were analyzed by SDS-PAGE or Caliper in the presence and absence of a reducing agent (5 mM 1,4-dithiotreitol). The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instructions (4-20% Tris-glycine gels or 3-12% Bis-Tris). The aggregate content of immunoconjugate samples was analyzed using a Superdex 200 10/300GL analytical size-exclusion column (GE Healthcare) in 2 mM MOPS, 150 mM NaCl, 0.02% NaN_3, pH 7.3 running buffer at 25°C. A summary of the analytical data is shown for selected clones in Figure 13 (51A12 kih IgG IFNα, SEQ ID NOs 50, 52, 54), Figure 14 (4F3 kih IgG IFNα, SEQ ID NOs 68, 70, 72), Figure 15 (51A12_C1 kih IgG IFNα, SEQ ID NO: 96, 52, 54), Figure 16 (51A12_E7 kih IgG IFNα, SEQ ID NO: 100, 52, 54), Figure 17 (51A12_C7 kih IgG IFNα, SEQ ID NO: 98, 52, 54), Figure 18 (untargeted kih IgG IFNα, SEQ ID NO: 110, 112, 114) and Figure 19 (monovalent 51A12 kih IgG IFNα, SEQ ID NO: 50, 52, 116).

Affinity-determination of IgG-IFNα immunoconjugates to ASGPR H1 by SPR

The ASGPR H1 binding activity of clones 51A12 and 52C4 used as examaplary IgG-IFNα immunoconjugates was determined and compared to the corresponding unmodified IgG antibodies by surface plasmon resonance (SPR) on a ProteOn XPR36 instrument (Biorad). Biotinylated avi-Fc human ASGPR H1 CRD antigen was immobilized on NLC chips by
5 neutravidin capture. Immobilization of recombinant antigens (ligand): Antigens were diluted with PBST (10 mM phosphate, 150 mM sodium chloride pH 7.4, 0.005% Tween-20) to 10 μg/ml, then injected at 30 μl/min at varying contact times, to achieve immobilization levels of 400 response units (RU) in vertical orientation. Injection of analytes: For one-shot kinetics measurements, injection direction was changed to horizontal orientation, two-fold dilution series
10 of purified IgGs, mono- and bivalent antibody-cytokine fusions (varying concentration ranges between 50 and 3.25 nM) were injected simultaneously at 50 μl/min along separate channels 1-5, with association times of 120 or 200 s, and dissociation times of 300 s. Buffer (PBST) was injected along the sixth channel to provide an "in-line" blank for referencing. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were calculated using a simple one-to-one
15 Langmuir binding model in ProteOn Manager v3.1 software by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_D) was calculated as the ratio k_{off}/k_{on}. Regeneration was performed in horizontal orientation using 10mM glycine, pH 1.5 at a flow rate of 100 μl/min for a contact time of 30 s. The data show that – within the error of the method – the affinity (monovalent display) and avidity (dimeric display)
20 for human ASGPR H1 is retained for both clone 51A12-based (SEQ ID NOs 50, 52, 54)and clone 52C4-based (SEQ ID NOs 56, 58, 60) immunoconjugate (Table 5).

Table 5. Kinetic and thermodynamic parameters of the monovalent and bivalent binding formats of clone 51A12 and 52C4 to ASGPR H1.

Name of the binder	**# of binding arms**	**human / cyno ASGPR1 CRD**		
		k_{on} (1/Ms)	**k_{off} (1/s)**	**K_D(M)**
51A12 IgG	2	3.5×10^{5}	7.34×10^{-5}	**2.1×10^{-10}**
51A12 kih IgG-IFNα	2	6.74×10^{5}	15.7×10^{-5}	**2.33×10^{-10}**
51A12 Fab	1	1.10×10^{5}	6.28×10^{-5}	**5.71×10^{-10}**
monovalent 51A12 kih IgG-IFNα	1	2.45×10^{5}	13.9×10^{-5}	**5.68×10^{-10}**
52C4 IgG	2	5.57×10^{5}	49.9×10^{-5}	**0.89×10^{-9}**

52C4 kih IgG-IFNα	2	4.26 x 10^5	49E x 10^{-5}	**1.15 x 10^{-9}**
52C4 Fab	1	1.39 x 10^5	396 x 10^{-5}	**28.6 x 10^{-9}**
monovalent 52C4 kih IgG-IFNα	1	1.14 x 10^5	302 x 10^{-5}	**26.5 x 10^{-9}**

Binding of IgG-IFNα immunoconjugates to ASGPR-positive and -negative cells

In order to characterize the specificity of the antibody conjugates, antibody-cytokine conjugates were incubated with both ASGPR-positive and negative cells and specific binding was measured by FACS analysis. For this, primary human hepatocytes (from 3 donors; purchased from Celsis In Vitro Technologies (Baltimore, MD)), Huh-7 cells, HepG2 cells, A549 cells, Hela cells, and 293T cells (each 1 x 10^5) were incubated with 1 µg of ASGPR H1-specific IgG kih IFNα samples for 45 min on ice. After washing, the cells were incubated with a labeled goat anti-human IgG secondary antibody (BD Biosciences, San Diego, CA) for 30 min on ice. After three washes, the stained cells were analyzed by FACS analysis using a Calibur flow cytometer. In all FACS assays, an isotype control conjugate (untargeted kih IgG IFNα, SEQ ID NOs 110, 112, 114) was used to determine the background, which was subtracted from the MFI values for the tested antibodies. Binding analysis to human peripheral blood mononuclear cells (PBMC) was performed by using directly labeled antibody conjugates (Zenon® R-Phycoerythrin Human IgG Labeling Kit, Life Technologies) according to manufacturer's instructions. Binding analysis revealed that clone 51A12 IgG kih IFNα (SEQ ID NOs 50, 52, 54) and 4F3 IgG kih IFNα (SEQ ID NOs 68, 70, 72) showed highly specific binding to ASGPR-positive cells while the signal on ASGPR-negative cells was comparable to the isotype control conjugate (Figure 20). In addition, binding saturation curves of clone 4F3 IgG kih IFNα were analyzed. For this, the antibody-IFNα conjugate was incubated with primary human hepatocytes (from 3 donors) in a dilution row ranging from 0.0001 to 6.7 µg/ml and binding intensity was recorded by FACS analysis. As shown in Figure 21 binding saturation on primary human hepatocytes as well as on the control cell line HepG2 was reached at 0.25-0.74 µg/ml antibody concentrations, and higher antibody concentration did not significantly increase the binding signal further.

Analysis of the surface-exposed ASGPR level on HepG2 cells over time

Uptake of desialylated glycoproteins into liver cells after binding to ASGPR is known to occur very rapidly. During this receptor-mediated endocytosis, the lumen of the endosome becomes acidic allowing the receptor-ligand complexes to dissociate. While the ligand is targeted for

degradation in lysosomes, ASGPR was shown to recycle back to the cell surface. Since receptor binding followed by internalization was shown for several receptors to trigger down-regulation of receptor expression, the levels of surface-exposed ASGPR in the presence of anti ASGPR H1 antibodies was measured over time. For this experiment, HepG2 cells were incubated for up to 5 h with clone 51A12-derived antibodies, either as IgG or as mono- or bivalent antibody-IFNα fusion proteins. As a negative control, an unrelated antibody without binding specificity to HepG2 cells was used (GA101) (all antibodies at 30 μg/ml). During incubation at 37°C, samples were taken after 30, 60, 120, 180, and 300 min and washed with cold PBS. Cell surface bound antibodies were detected using an APC-conjugated goat anti-human IgG Fcg fragment specific F(ab')2 fragment (Jackson Immuno Research Lab, working solution 1:50). After 30 min incubation at 4°C unbound antibody was removed by washing with PBS containing 0.1% BSA. Cells were fixed using 1% PFA and analyzed using BD FACS CantoII (Software BD DIVA). In order to verify the integrity of the antibody-cytokine fusion, the presence of IFNα was also detected. Cells were incubated with a mouse monoclonal antibody against human interferon alpha (MMHA-1, #21105-1, R&D Systems, 5 μg/ml) for 30 min at 4°C. Unbound antibody was removed by washing with PBS containing 0.1% BSA and a FITC-conjugated anti-mouse F(ab')2 Fragment (Serotec, STAR105F; working solution 1:50) was used as secondary antibody. Cells were fixed using 1% PFA and analyzed using BD FACS CantoII (Software BD DIVA). The results shown in Figure 22 demonstrate the constant level of surface-exposed antibody bound to ASGPR without any binding-induced down-regulation of the receptor over the measured time period. Of note, the monomeric IgG-IFNα construct gave the strongest signal, most likely due to the fact that twice the number of monomeric IgG-IFNα molecules can bind per ASGPR complex (Figure 22A).

Confocal microscopy

Three-dimensional and time-resolved analysis of the ASGPR-mediated internalization of the antibody-cytokine fusion constructs was performed by confocal microscopy. For this analysis, HepG2 cells were grown to 50-60% confluency on glass-bottom dishes (Nunc) in a cell incubator. The dishes were then rinsed twice with pre-warmed PBS (37°C) to replace the medium with PBS and quickly placed on the microscope stage (at 37°C, 5% CO_2). For this experiment, clone 51A12 kih IgG IFNα was directly labeled with Alexa488. The labeled construct (20 μg/ml) was added to HepG2 cells directly at the microscope stage. Acquisitions started 5 min after antibody addition using a spinning-disk confocal microscope. Data acquisitions occurred every 3 sec for 1 h (100x magnification) on 10 stacks (z-level) that covered

the entire cell thickness. Binding of the antibody-cytokine construct to surface-exposed ASGPR was not equally distributed but found to be clustered (Figure 23A). Clusters were spread over the whole surface. Time-resolved analysis of this experiment clearly revealed the immediate internalization of the antibody-cytokine fusion construct within minutes (Figure 23B). After internalization of the IgG-cytokine constructs in vesicles, the proteins are then transported back to the surface on the apical side of the cell (data not shown).

Affinity-determination of IgG-IFNα immune conjugates to Interferon-alpha receptor 2 by SPR

The binding activity of IgG-IFNα immune conjugates to the high affinity Interferon-alpha receptor 2 (IFNAR2) was determined and compared to Roferon by surface plasmon resonance (SPR) on a ProteOn XPR36 instrument (Biorad). Commercially available IFNAR2-Fc fusion proteins (R&D Systems) were immobilized in vertical orientation on the sensorchip surface by standard amine coupling. For one-shot kinetics measurements, injection direction was changed to horizontal orientation, two-fold dilution series of purified antibody-cytokine fusions (varying concentration ranges between 50 and 3.25 nM) were injected simultaneously at 50 μl/min along separate channels 1-5, with association times of 120 or 200 s, and dissociation times of 300 s. Buffer (PBST) was injected along the sixth channel to provide an “in-line” blank for referencing. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were calculated using a simple one-to-one Langmuir binding model in ProteOn Manager v3.1 software by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_D) was calculated as the ratio k_{off}/k_{on}. Regeneration was performed in horizontal orientation using 10mM glycine, pH 1.5 at a flow rate of 100 μl/min for a contact time of 30 s. The measured affinity of antibody-cytokine fusion protein was around (k_{on} 1.57 x 10^6 1/Ms; k_{off} 6.15 x 10^{-3} 1/s; K_D 4 nM) and thus comparable to the published affinity of recombinantly produced protein Roferon, indicating that the fusion of IFNα to the C-terminal end of an IgG has no impact on the binding affinity to IFNAR2.

Determination of the antiviral activity of ASGPR mAb-IFNα

In order to analyze the functional activity of IFNα as part of the IgG-cytokine fusion, and to compare it with Roferon, biological activity of the IFNα fusion constructs was tested in a virus protection assay. For this study, MDBK cells were pre-incubated with either Roferon or the antibody-cytokine fusions for 1-4 h. Vesicular stomatitis virus was then added for additional 16-24 h. At the end of this incubation step, living cells were stained with crystal violet staining

solution (0.5%) and quantification of living cells was performed using a microplate reader at 550-600 nm with a reference wavelength of 690 nm. Biological activity of all IgG-cytokine constructs was determined in a full dose-response curve analysis against a standardized Roferon solution with a 4 Parameter-Logistics fitting function. As shown in Table 6, the antibody-IFNα fusion constructs show an activity that corresponds to about 5% of Roferon's activity, independent of the antibody's binding valency. Since the fusion of IFNα to the C-terminal end of an IgG has no impact on the binding affinity to IFNAR2 (shown above) it is likely that the interaction to the low affinity interferon-alpha receptor 1 (IFNAR1) is sterically impaired, ultimately leading to a reduced signaling of the IFNAR holocomplex.

Table 6. Functional IFNα activity of the antibody-cytokine conjugate in comparison to Roferon.

Sample name	Pictogram	Loss (%) compared to Roferon
ASGPR 51A12 kih IFNa		-94.3
monovalentASGPR 51A12 kih IFNa		-94.9
monovalentASGPR 52C4 kih IFNa		-94.8

Antiviral activity of the ASGPR-specific IgG-IFNα conjugates in HCV replicon and EMCV CPE assays

In order to characterize and compare the functional activity of the IgG-IFNα fusion proteins with commercially available Roferon and Pegasys (peginterferon-α2a), antiviral activity was studied using ASGPR-positive (Huh7-2209-3) and ASGPR-negative cells (Hela).

In order to analyze the antiviral activity of the compounds on ASGPR-negative cells, HeLa cells were seeded at 15,000/well in 96-well opaque-walled plate. After overnight culture, the wells were evacuated and 50 μl antibody-cytokine conjugate diluted in EMEM (with 10% FBS) was added. The HeLa cells were pre-treated with the IgG-IFNα constructs for 3 h at 37°C, before the 50 μl EMCV (VR-1762, ATCC) were added into each well (2,000 TCID50/well in EMEM). The viable cells were measured 24 h post infection using the CellTiter-Glo kit (G7572, Promega).

100 μl CellTiter-Glo reagent was added to each well and incubated at room temperature for 10 min with gentle shaking. Then the luminescence signal was recorded by using a Berthold Mithras Luminometer (Berthold Technologies). The results represent percentage of survival cells (Figure 24A) and all EC_{50} values as well as the number of experimental reiterations are
5 summarized in Table 7. On ASGPR-negative Hela cells, the EC50 values for Roferon are up to 75-fold smaller than for other compounds such as 4F3 IgG kih IFNα, indicating that the functional activitiy of this compound is much higher than of the other compounds tested. In contrast, the activity of Pegasys was comparable to those of the ASGPR-specific IgG kih IFNα conjugates.

10 The ASGPR-positive Huh 7-derived hepatocarcinoma cell line 2209-23 was developed by stable transfection of a bicistronic HCV replicon of which the first open reading frame, driven by the HCV IRES, contains the renilla luciferase gene in fusion with the neomycin phosphotransferase gene (NPTII) and the second open reading frame, driven by EMCV IRES, contains the HCV non-structural genes NS3, NS4a, NS4b, NS5A and NS5B derived from the NK5.1 replicon
15 backbone. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in DMEM supplemented with Glutamax™ and 100 mg/ml sodium pyruvate (#10569-010). The medium was further supplemented with 10% (v/v) FBS (#10082-139), 1% (v/v) penicillin/streptomycin (#15140-122) and 1% (v/v) geneticin. All reagents were obtained from Invitrogen.

Huh 7 2209-23 cells in DMEM containing 5% (v/v) Fetal Bovine Serum were plated in 96-well
20 plates at 5000 cells/well in 90 μl volume. 24 hours after plating, antibody-cytokine conjugates (or medium as a control) were added to the cells in 3-fold dilutions over 12 wells (0.01 -2000 pM), in a volume of 10 μl. Final volume after addition of compound was 100 μl. Renilla luciferase reporter signal was read 72 hours after adding compounds, using the Renilla Luciferase Assay system (Promega, # E2820). The EC_{50} values were calculated as the compound
25 concentration at which a 50% reduction in the level of renilla luciferase reporter was observed as compared to control samples (in the absence of compound). Dose-response curves and EC_{50} values were obtained by using the XLfit4 program (ID Business Solutions Ltd., Surrey, UK). Despite the reduced antiviral activity of the antibody-cytokine constructs when exposed to ASGPR-negative cells (Table 6 and Figure 24A), clones 51A12 IgG kih IFNα and 4F3 IgG kih
30 IFNα were more potent in protecting cells from viral infection and multiplication than Roferon when incubated with the ASGPR-positive cell line Huh7 2209-23 (Figure 24B and Table 7). In contrast, the potency of the isotype control (untargeted IgG kih IFNα) was significantly lower, underlining the positive consequence of targeting the IgG kih IFNα conjugates to ASGPR H1.

Table 7. Summary of antiviral activity of various ASGPR H1-specific antibody-IFNα conjugates.

IFNa Molecule	HCV Replicon (Huh-7)			EMCV assay (Hela)		
	Mean EC50 (pM)	STDEV	n=	Mean EC50 (pM)	STDEV	n=
Roferon	0.07	0.03	4	1.8	0.4	6
Pegasys	0.96	0.46	5	61.2	9.4	9
Isotype-IFN	0.59	0.12	3	108.3	5.0	6
51A12-IFN	0.02	0.01	4	68.2	14.4	6
4F3-IFNa	0.04	0.01	4	135.6	29.1	6
R7E12-IFN	0.11	0.04	3	29.2	2.3	6
R9E10-IFN	0.43	0.21	3	49.0	5.6	6
R5C2-IFN	0.54	0.23	3	56.3	4.9	6
5A4-IFN	0.86	0.4	3	58.7	5.8	6

IFNα activity of the ASGPR-specific IgG-IFNα conjugates in hepatic and non-hepatic cells

5 IFNα exerts its antiviral activities through induction of hundreds of IFN-stimulated genes (ISG). To verify the antiviral activities, and further confirm the ASGPR-targeting mediated enhanced IFNα activity, we determined the ISG expression in hepatic and non-hepatic cells. Hepatic cells (primary hepatocytes and HepG2) and non-hepatic cells (human PBMC and Hela) were treated with various serially diluted IFNα molecules for 6 h and total RNA was extracted from cells 10 using 5PRIME RNA extraction kit (#FP2302530, 5PRIME, Gaithersburg, MD).

TaqMan (real-time PCR) assays for ISG genes MX1 and RSAD2 were custom designed. Assays were selected to lie within the Affymetrix probe sequence of interest or within the 3' coding sequence of the reference mRNAs of interest.

All gene expression assays were performed on an ABI PRISM® 7900HT Sequence Detection 15 System (Applied Biosystems). PCR mix consisted of 10 µl PerfeCTa® qPCR FastMix, ROX™ (Quanta), 1 µl TaqMan or 0.06 µl IDT assay, and 2 µl DEPC-treated water (Ambion, Applied Biosystems) for each reaction. cDNA samples were diluted to 10 ng/µl in RNase-free water (Ambion, Applied Biosystems), and 7 µl added to a 384-well optical plate (Applied Biosystems) containing 13 µl pre-distributed assay PCR mix. All samples were queried with one assay for a 20 target gene or one for the endogenous control gene assays, 18S, GAPDH (Rhesus), ACTB (Rhesus) and GUSB (Rhesus) (TaqMan Gene Expression Assays, Applied Biosystems). Each measurement was performed in triplicate. The following PCR conditions were used: 45°C for 2 min, then 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 45 s.

The expression levels of target genes were normalized to the geometric mean of 18S, ACTB, GAPDH and GUSB and represented as relative expression (*E*), $E = 2^{(\Delta Ct)}$, where ΔCt is the difference between reference and target gene cycles at which the amplification exceeds an arbitrary threshold. As shown in Figure 25, isotype IgG kih IFNα control showed reduced ISG
5 induction compared to Roferon, with similar activity to Pegasys (PEG-IFNα) in all cells. In the ASGPR-negative Hela and PBMC cells, clone 51A12 IgG kih IFNα also showed reduced activity, similar to the isotype control. However, in the ASGPR-positive hepatic cells HepG2 and primary human hepatocytes, clone 51A12 IgG kih IFNα showed enhanced IFNα activity compared to the isotype control, to a similar level of Roferon. This result confirms the above-
10 described enhanced antiviral activity of the 51A12 antibody-IFNα conjugate.
In order to understand whether these ASGPR-targeted IFNα molecules have sustained IFNα activity, we monitored ISG expression in Huh-7 and primary hepatocytes for up to 72 h. As shown in Figure 26, both ASGPR-targeting IFNα molecules 51A12 IgG kih IFNα and 4F3 IgG kih IFNα showed sustained ISG induction at 72 h after treatment, while Pegasys and Roferon
15 showed significantly reduced ISG induction at 72 h.

Cynomolgus monkey single dose PK/PD study

Encouraged by *in vitro* results that ASGPR antibody-based targeted IFNα molecules showed reduced IFNα activity in non-hepatic cells and enhanced IFNα activity in hepatic cells (liver-targeted IFNα effects), a cynomolgus monkey study was designed to confirm the liver-targeted
20 IFNα effects *in vivo*. Because the ASGPR-specific clone 51A12 binds to human and monkey ASGPR with identical affinity and human IFNα has similar activity in the monkeys, monkeys can be used as PK/PD models for liver-targeted IFNα proof-of-concept studies. In the monkey study we directly compared 51A12 IgG kih IFNα and isotype IgG kih IFNα control. Both molecules were injected subcutaneously at dosage levels of either 1 or 10 µg/kg, monkey blood
25 and liver biopsy samples were collected before and after dosing, and their PK (pharmacokinetics) and PD (pharmacodynamics) were monitored. The dose groups are listed in Table 8.

Table 8. Twelve cynomolgus monkeys were divided into four groups as shown below.

Group	Dose (µg/kg), compound	No of Animals	Formulation Strength (µg/ml)
1	10 µg/kg, 51A12, SC, Single Dose	3	20
2	1 µg/kg, 51A12, SC, Single Dose	3	2
3	10 µg/kg, iso-control, SC, Single Dose	3	20
4	1 µg/kg, iso-control, SC, Single Dose	3	2

Sample collection, transfer and storage

Blood (approximately 1 ml) was collected from each animal 5 days before dosage and at 2, 6, 12, 24, 48, 72, 96, 168, and 336 hours after injection. Samples for pharmacokinetics were collected into tubes without anticoagulant. Blood for pharmacokinetics was collected prior to the 5 pharmacodynamic blood collections.

For the gene expression study, blood (approximately 2.5 ml) was collected from each animal 5 days before dosage and at 2, 6, 12, 24, 48, 72, 96, 168, and 336 hours after injection. Samples were collected into PAXgene^{TM} blood RNA collection tubes and the tubes were mixed by inverting 8-10 times. The PAXgene^{TM} blood RNA collection tube was the last tube drawn in the 10 phlebotomy sequence (i.e. after the clinical pathology and pharmacokinetic collections).

Liver tissue was collected from two separate locations (at least 25 mg/sample) in the liver via laparoscopic procedure from each animal on day -5, and on day 2, day 4 and day 8. Each tissue sample was excised and immediately placed into separate pre-weighed and labeled cryo-vial tubes and flash-frozen in liquid nitrogen. Because of the need for immediate freezing, the sample 15 vials were not weighed after collection of the liver samples. For day -5 and day 2, the first liver tissue aliquot for each animal was taken from the left lateral lobe of the liver, and the second liver tissue aliquot for each animal was taken from the right lateral lobe of the liver. For day 4 and day 8, the first liver tissue aliquot for each animal was taken from the left medial lobe of the liver, and the second liver tissue aliquot for each animal was taken from the right medial lobe of 20 the liver. Liver biopsy samples were snap-frozen in 2 ml tubes. RNAlater-ICE (P/N 7031, Ambion), pre-chilled on dry ice, was added to frozen tissues and stored at -80°C. Blood samples were received in PAXgene tubes according to the protocol and stored at -80°C prior to processing.

Measurement of clone 51A12 IgG kih IFNα and isotype IgG kih IFNα control in monkey 25 serum samples

Aliquots of cynomolgus monkey serum were analyzed for the dosed compound using a sandwich ELISA assay that uses an anti-IFNα antibody (Lot no. 34495-28, Roche Nutley, NJ, USA) as the capture reagent and HRP-labeled anti-human Fc antibody (Lot no. Wbr72_MM_090602, Roche Diagnostics, Penzberg, Germany) as the detection reagent. After coating of plates with anti-IFNα 30 antibody at room temperature for 1 h, the plates were treated with 2% BSA blocking buffer for 1 h. After washing, HRP-labeled anti-human Fc antibody was added to each well and incubated for 1 h with gentle shaking. After washing, 100 μl/well TMB substrate solution (#11 484 281 001, Roche Diagnostics, Penzberg, Germany) was added for about 20 min. The reaction was then

stopped by adding 50 µl/well 2N HCl. The plates were read within 2 min at 450 nm with reference wavelength of 650 nm. The lower limit of quantitation (LLOQ) of this method was 10 ng/ml. The precision (% CV) and accuracy (% relative error) of the assay met the acceptance criteria. Assay performance, as monitored by the analysis of QC samples analyzed along with the samples, was as shown in Table 9. The serum concentrations are shown in Table 10-13. A single injection of isotype IgG kih IFNα at 10 µg/kg yielded significant exposure in the blood that peaked at around 100 ng/ml for one week. In contrast, at the same dose level, 51A12 IgG kih IFNα was below quantification level at any time point. Both molecules were undetectable in the blood at 1 µg/kg dose level. The PK parameters are summarized in Table 14.

Table 9. Analytical performance of clone 51A12 IgG kih IFNα quality control samples in cynomolgus monkey serum.

	Run Date	Curve Number	QC1 30.0 ng/mL	QC2 90.0 ng/mL	QC3 270 ng/mL
	13-Dec-2011	1	24.3	78.8	252
			29.1	95.2	236
	13-Dec-2011	2	*	76.4	251
			26.5	94.7	247
	20-Dec-2011	3	21.7	74.7	245
			28.6	84.3	264
		4	25.4	82.6	288
			28.2	91.2	279
	Mean		26.3	84.7	258
	%CV		10.2	9.6	6.9
	%Rel. Error		-12.3	-5.9	-4.4
	* Deactivated				

Table 10. Serum concentration (ng/ml) of 1 µg/kg isotype IgG kih IFNα.

Time [h]	Subject 1	Subject 2	Subject 3	Mean	S.D.	%CV	n
0	BLQ<10.0	BLQ<10.0	BLQ<10.0				
2	BLQ<10.0	BLQ<10.0	BLQ<10.0				
6	BLQ<10.0	BLQ<10.0	BLQ<10.0				
12	BLQ<10.0	BLQ<10.0	BLQ<10.0				
24	BLQ<10.0	BLQ<10.0	BLQ<10.0				

48	BLQ<10.0	BLQ<10.0	BLQ<10.0				
72	BLQ<10.0	BLQ<10.0	BLQ<10.0				
96	BLQ<10.0	BLQ<10.0	BLQ<10.0				
168	BLQ<10.0	BLQ<10.0	BLQ<10.0				
336	BLQ<10.0	BLQ<10.0	BLQ<10.0				

Table 11. Serum concentration (ng/ml) of 1 µg/kg 51A12 IgG kih IFNα.

Time [h]	Subject 1	Subject 2	Subject 3	Mean	S.D.	%CV	n
0	BLQ<10.0	BLQ<10.0	BLQ<10.0				
2	BLQ<10.0	BLQ<10.0	BLQ<10.0				
6	BLQ<10.0	BLQ<10.0	BLQ<10.0				
12	BLQ<10.0	BLQ<10.0	BLQ<10.0				
24	BLQ<10.0	BLQ<10.0	BLQ<10.0				
48	BLQ<10.0	BLQ<10.0	BLQ<10.0				
72	BLQ<10.0	BLQ<10.0	BLQ<10.0				
96	BLQ<10.0	BLQ<10.0	BLQ<10.0				
168	BLQ<10.0	BLQ<10.0	BLQ<10.0				
336	BLQ<10.0	BLQ<10.0	BLQ<10.0				

Table 12. Serum concentration (ng/ml) of 10 µg/kg 51A12 IgG kih IFNα.

Time [h]	Subject 1	Subject 2	Subject 3	Mean	S.D.	%CV	n
0	BLQ<10.0	BLQ<10.0	BLQ<10.0				
2	BLQ<10.0	BLQ<10.0	BLQ<10.0				
6	BLQ<10.0	BLQ<10.0	BLQ<10.0				
12	BLQ<10.0	BLQ<10.0	BLQ<10.0				
24	BLQ<10.0	BLQ<10.0	BLQ<10.0				
48	BLQ<10.0	BLQ<10.0	BLQ<10.0				
72	BLQ<10.0	BLQ<10.0	BLQ<10.0				

96	BLQ<10.0	BLQ<10.0	BLQ<10.0				
168	BLQ<10.0	BLQ<10.0	BLQ<10.0				
336	BLQ<10.0	BLQ<10.0	BLQ<10.0				

Table 13. Serum concentration (ng/ml) at 10 μg/kg isotype IgG kih IFNα.

Time [h]	Subject 1	Subject 2	Subject 3	Mean	S.D.	%CV	n
0	BLQ<10.0	BLQ<10.0	BLQ<10.0				
2	BLQ<10.0	33.7	12.4	23.1			2
6	49.8	40.9	61.5	50.7	10.3	20.3	3
12	66.4	44.3	67.9	59.5	13.2	22.2	3
24	109	63.1	71.1	81.1	24.5	30.2	3
48	119	99.3	88.3	102	15.6	15.3	3
72	105	88.2	94.0	95.7	8.53	8.9	3
96	124	85.0	95.2	101	20.2	20.0	3
168	78.9	56.5	58.3	64.6	12.4	19.2	3
336	19.6	11.0	BLQ<10.0	15.3			2

Table 14. Cynomolgus monkey serum PK parameters of 10 μg/kg isotype-IFNα.

Parameter	Units	Subject 1	Subject 2	Subject 3	Mean	S.D.	%CV	n
original dose	μg/kg	10	10	10				
C_{max}	ng/ml	124	99	95	106	16	14.8	3
T_{max}	hours	96	48	96	80	28	34.6	3
AUC	ng*hours/ml	25251	18124	13279	18885	6022	31.9	3
AUC interval	hours	(0-336)	(0-336)	(0-168)				
AUC/dose	ng*hours/ml/μg/kg	2525	1812	1328	1888	602	31.9	3
AUC extrap	ng*hours/ml	27615	19253	21839	22903	4281	18.7	3
% AUC extrap	%	8.6	5.9	39	17.9	18.5	104	3
AUC extrap /	ng*hours/ml/μg/kg	2762	1925	2184	2290	428	18.7	3

dose								
$T_{1/2}$	hours	83.6	71.1	102	85.5	15.4	18.0	3

RNA extraction

Total RNA was extracted from 96 liver biopsy samples (48 animals x 2 biopsies per sampling) using the Qiagen RNeasy Mini Kit (P/N 74104) and quantitated on the Nanodrop 8000. Total RNA quality of the liver samples was assessed on the Caliper LabChip GX. RNA from all samples was of sufficient quantity and quality to perform qPCR and microarray-based gene expression measurements.

Total RNA isolation and quantitation from 120 blood samples was performed at Expression Analysis (Raleigh, NC). Total RNA quality of the blood samples was assessed on the Agilent Bioanalyzer 2100. RNA from all samples was of sufficient quantity and quality to perform qPCR and microarray-based gene expression measurements.

Microarray analysis

Two separate protocols were used to convert total RNA into cDNA: Affymetrix GeneChip HT 3' IVT Express (P/N 901225) and NuGEN Ovation RNA Amplification System V2 (P/N 3100-60).

Blood

100 ng total RNA from liver biopsy samples was converted into double-stranded cDNA and amplified RNA (aRNA) using the GeneChip HT 3' IVT Express Kit according to the manufacturer's protocols. The hybridization mix contained 12.5 µg aRNA, 2x Hybridization Mix (P/N 900720), DMSO, 20x Hybridization Controls, and Oligo B2 Controls.

Liver

50 ng total RNA from blood samples was converted to single-stranded cDNA using the NuGEN Ovation RNA Amplification System V2 kit following the manufacturer's protocol. The hybridization mix contained 3 µg SPIA-amplified cDNA, 2x Hybridization Mix (P/N 900720), DMSO, 20x Hybridization Controls, and Oligo B2 Controls.

The hybridization mixes for liver and blood samples were hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. Staining and washing steps were performed as suggested by the manufacturer (Affymetrix). Each hybridized Affymetrix GeneChip array was scanned with a GeneChip Scanner 3000 7G (Agilent/Affymetrix). Image analysis was performed with the Affymetrix GCOS software. The resulting .cel files were assessed using standardized quality

control metrics. Data were normalized and expression value calculation / differential expression was determined using standard data analysis packages.

In Figure 27 the expression of four representative ISGs HRASLS2, ITI44, IFIT1, and IFITM2 in monkey liver samples is graphed. More robust induction of the expression of these four ISGs was found in 51A12 IgG kih IFNα dosed monkeys, in comparison to the isotype IgG kih IFNα dosed monkeys.

IFN gene expression analysis (M3.1 heatmap)

In order to more comprehensively analyze the IFNα stimulated gene expression by IFNα molecules, IFNα response was analyzed with IFN gene modules determined from blood transcriptomics studies (Chaussabel et al. (2008), Immunity 29, 150-64). As shown in Figure 28A, the fold-change expression values from baseline for the genes of the interferon module M3.1 were plotted in heatmap form for both blood and liver samples using the R statistics package (www.r-project.org). Non-supervised hierarchical clustering of the liver interferon-induced genes reveals a highly induced subset (dashed rectangle) in the 10 μg/kg dose of 51A12 but not the isotype IFNα compound at days 1 and 3. Non-supervised hierarchical clustering this subset reveals a differential pattern of expression between blood and liver where some of the genes were more significantly induced in liver by 51A12 but not isotype-IFNα at the 10 μg/kg dose and other genes were more significantly induced in blood by isotype IFNα at the high dose (Figure 28B).

In summary, ASGPR-targeting IFNα molecule 51A12 IgG kih IFNα showed undetectable exposure in the blood, and lower IFNα activity (ISG expression stimulation) in the blood but higher IFNα activity in monkey liver, as compared to isotype IFNα control.

* * *

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Claims

1. An antibody capable of specific binding to asialoglycoprotein receptor (ASGPR), wherein the antibody comprises
   a) the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14;
   b) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2;
   c) the heavy chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 6;
   d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 10;
   e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 18;
   f) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22;
   g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 26;
   h) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 30;
   i) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 32;
   j) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 34; or
   k) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 22.
2. The antibody of claim 1, wherein the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 16 and the light chain variable region sequence of SEQ ID NO: 14.
3. An antibody capable of specific binding to ASGPR, wherein the antibody competes for binding to an epitope of ASGPR with the antibody of claim 2.
4. The antibody of claim 3, wherein the antibody recognizes an epitope in the stalk region of ASGPR.

5. The antibody of claim 3 or 4, wherein the antibody is an affinity matured variant of the antibody of claim 2.
6. The antibody of any one claims 3-5, wherein the antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 16, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 14.
7. The antibody of any one of claims 3-6, wherein the antibody comprises the light chain variable region sequence of SEQ ID NO: 14 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions.
8. The antibody of any one of claims 3-7, wherein the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 16 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions.
9. The antibody of claim 1, wherein the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 2.
10. An antibody capable of specific binding to ASGPR, wherein the antibody competes for binding to an epitope of ASGPR with the antibody of claim 9.
11. The antibody of claim 10, wherein the antibody recognizes an epitope in the carbohydrate recognition domain (CRD) of ASGPR.
12. The antibody of claim 10 or 11, wherein the antibody is an affinity matured variant of the antibody of claim 9.
13. The antibody of any one claims 10-12, wherein the antibody comprises a heavy chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 4, and a light chain variable region sequence that is at least about 96%, 97%, 98% or 99% identical to the sequence of SEQ ID NO: 2.
14. The antibody of any one of claims 10-13, wherein antibody comprises the light chain variable region sequence of SEQ ID NO: 2 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions.
15. The antibody of any one of claims 10-14, wherein the antibody comprises the heavy chain variable region sequence of SEQ ID NO: 4 with one, two, three, four, five, six or seven, particularly two, three, four or five, amino acid substitutions.
16. The antibody of any one of claims 10-14, wherein the antibody comprises
a) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable

region sequence of SEQ ID NO: 36;

b) the heavy chain variable region sequence of SEQ ID NO: 4 and the light chain variable region sequence of SEQ ID NO: 38;

c) the heavy chain variable region sequence of SEQ ID NO: 8 and the light chain variable region sequence of SEQ ID NO: 40;

d) the heavy chain variable region sequence of SEQ ID NO: 12 and the light chain variable region sequence of SEQ ID NO: 42;

e) the heavy chain variable region sequence of SEQ ID NO: 20 and the light chain variable region sequence of SEQ ID NO: 44; or

f) the heavy chain variable region sequence of SEQ ID NO: 24 and the light chain variable region sequence of SEQ ID NO: 46; or

g) the heavy chain variable region sequence of SEQ ID NO: 28 and the light chain variable region sequence of SEQ ID NO: 48.

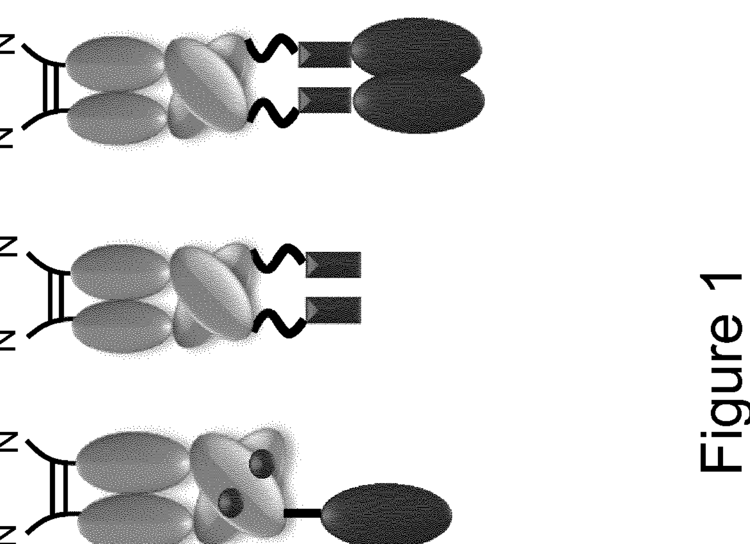
17. The antibody of any one of the preceding claims, wherein the antibody is capable of specific binding to human and cynomolgus monkey ASGPR.
18. The antibody of any one of the preceding claims, wherein the antibody binds to human ASGPR with an dissociation constant (K_D) of smaller than 1 μM, particularly smaller than 100 nM, more particularly smaller than 1 nM, when measured as Fab fragment by Surface Plasmon Resonance (SPR).
19. The antibody of any one of the preceding claims, wherein the antibody binds to human ASGPR with a K_D of smaller than 1 μM, particularly smaller than 500 nM, more particularly smaller than 100 nM or even smaller than 10 nM, when measured as IgG_1 by fluoresecence resonance energy transfer (FRET).
20. The antibody of any one of the preceding claims, wherein the antibody does not compete with a natural ligand of ASGPR for binding to ASGPR.
21. The antibody of claim 20, wherein said natural ligand of ASGPR is asialofetuin.
22. The antibody of any one of the preceding claims, wherein the antibody does not detectably bind to CLEC10A, particularly human CLEC10A.
23. The antibody of any one of the preceding claims, wherein the antibody does not specifically bind to cells lacking ASGPR expression, particularly human cells, more particularly human blood cells.

24. The antibody of any one of the preceding claims, wherein the antibody is internalized into a cell expressing ASGPR upon binding of the antibody to ASGPR on the surface of said cell.
25. The antibody of claim 24, wherein the antibody is recycled back to the surface of said cell at about the same rate as it is internalized into said cell.
26. The antibody of any one of the preceding claims, wherein the antibody does not significantly induce downregulation of ASGPR expression at the surface of a cell upon binding of the antibody to ASGPR on the surface of said cell.
27. The antibody of any one of the preceding claims, wherein the antibody is a human antibody.
28. The antibody of any one of the preceding claims, wherein the antibody comprises a human Fc region, particularly an IgG Fc region, more particularly an IgG_1 Fc region.
29. The antibody of any one of the preceding claims, wherein the antibody is a full-length antibody.
30. The antibody of any one of the preceding claims, wherein the antibody is an IgG class antibody, particularly an IgG_1 subclass antibody.
31. The antibody of any one of claims 28-30, wherein the antibody comprises in the Fc region a modification reducing binding affinity of the antibody to an Fc receptor, particularly an Fcγ receptor.
32. The antibody of claim 31, wherein said Fc receptor is an activating Fc receptor.
33. The antibody of claim 31 or 32, wherein said Fc receptor is selected from the group of FcγRIIIa (CD16a), FcγRI (CD64), FcγRIIa (CD32), and FcαRI (CD89).
34. The antibody of any one of claims 31-33, wherein said Fc receptor is FcγRIIIa, particularly human FcγRIIIa.
35. The antibody of any one of claims 31-34, wherein the antibody comprises an amino acid substitution in the Fc region at a position selected from P329, L234 and L235 (EU numbering).
36. The antibody of any one of claims 31-35, wherein the antibody comprises the amino acid substitutions P329G, L234A and L235A in the Fc region (EU numbering).
37. The antibody of any one of claims 28-36, wherein the antibody comprises in the Fc region a modification promoting heterodimerization of two non-identical antibody heavy chains.

38. The antibody of claim 37, wherein said modification is a knob-into-hole modification, comprising a knob modification in one of the antibody heavy chains and a hole modification in the other one of the two antibody heavy chains.
39. The antibody of any one of claims 28-38, wherein the antibody comprises a modification within the interface between the two antibody heavy chains in the CH3 domain, wherein i) in the CH3 domain of one heavy chain, an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance ("knob") within the interface in the CH3 domain of one heavy chain which is positionable in a cavity ("hole") within the interface in the CH3 domain of the other heavy chain, and ii) in the CH3 domain of the other heavy chain, an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity ("hole") within the interface in the second CH3 domain within which a protuberance ("knob") within the interface in the first CH3 domain is positionable.
40. The antibody of any one of claims 28-39, wherein the antibody comprises the amino acid substitution T366W and optionally the amino acid substitution S354C in one of the antibody heavy chains, and the amino acid substitutions T366S, L368A, Y407V and optionally Y349C in the other one of the antibody heavy chains.
41. The antibody of any one of the preceding claims, wherein an effector moiety is attached to the antibody.
42. The antibody of claim 41, wherein not more than one effector moiety is attached to the antibody.
43. The antibody of claim 41 or 42, wherein said effector moiety is a cytokine molecule.
44. The antibody of claim 43, wherein the cytokine molecule is fused at its amino-terminal amino acid to the carboxy-terminal amino acid of one of the antibody heavy chains, optionally through a peptide linker.
45. The antibody of claim 43 or 44, wherein the cytokine molecule is a human cytokine.
46. The antibody of any one of claims 43-45, wherein the cytokine molecule is an interferon molecule.
47. The antibody of claim 46, wherein said interferon molecule is interferon alpha, particularly human interferon alpha, more particularly human interferon alpha 2 or human interferon alpha 2a.
48. The antibody of claim 46 or 47, wherein the antibody has anti-viral activity in cells expressing ASGPR on their surface.

49. The antibody of claim 48, wherein the antibody has no anti-viral activity in cells not expressing significant levels of ASGPR on their surface.
50. The antibody of claim 48 or 49, wherein said anti-viral activity is selected from inhibition of viral infection, inhibition of virus replication, inhibition of cell killing and induction of interferon-stimulated genes.
51. A polynucleotide encoding the antibody of any one of the preceding claims or an antigen binding portion thereof.
52. A vector, particularly an expression vector, comprising the polynucleotide of claim 51.
53. A host cell comprising the polynucleotide of claim 51 or the vector of claim 52.
54. A method for producing the antibody of any one of claims 1-50, comprising the steps of (i) culturing the host cell of claim 53 under conditions suitable for expression of said antibody, and (ii) recovering said antibody.
55. An antibody capable of specific binding to ASGPR, produced by the method of claim 54.
56. A pharmaceutical composition comprising the antibody of any one of claims 1-50 or 55 and a pharmaceutically acceptable carrier.
57. The antibody of any one of claims 1-50 or 55, or the pharmaceutical composition of claim 56 for use as a medicament.
58. The antibody of any one of claims 1-50 or 55, or the pharmaceutical composition of claim 56 for use in the treatment or prophylaxis of a liver disease.
59. The antibody or the pharmaceutical composition of claim 58, wherein said liver disease is a viral infection.
60. The antibody or the pharmaceutical composition of claim 58 or 59, wherein said liver disease is hepatitis virus infection, particularly hepatitis B virus (HBV) infection.
61. The antibody of any one of claims 1-50 or 55, or the pharmaceutical composition of claim 56 for use in the treatment or prophylaxis of cancer.
62. The antibody of claim 61, wherein said cancer is liver cancer.
63. The antibody of claim 62, wherein said liver cancer is hepatocellular carcinoma (HCC).
64. Use of the antibody of any one of claims 1-50 or 55 for the manufacture of a medicament for the treatment of a disease in an individual in need thereof.
65. The use of claim 64, wherein said disease is a liver disease.
66. The use of claim 65, wherein said liver disease is a viral infection.
67. The use of claim 65 or 66, wherein said liver disease is hepatitis virus infection, particularly HBV infection.

68. The use of claim 64, wherein said disease is cancer.
69. The use of claim 68, wherein said cancer is liver cancer.
70. The use of claim 69, wherein said liver cancer is hepatocellular carcinoma (HCC).
71. The use of any one of claims 64-70, wherein said individual is a mammal, particularly a human.
72. A method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the antibody of any one of claims 1-50 or 55 in a pharmaceutically acceptable form.
73. The method of claim 72, wherein said disease is a liver disease.
74. The method of claim 73, wherein said liver disease is a viral infection.
75. The method of claim 73 or 74, wherein said liver disease is hepatitis virus infection, particularly HBV infection.
76. The method of claim 72, wherein said disease is cancer.
77. The method of claim 76, wherein said cancer is liver cancer.
78. The method of claim 77, wherein said liver cancer is hepatocellular carcinoma (HCC).
79. The method of any one of claims 72-78, wherein said individual is a mammal, particularly a human.
80. The antibody of any one of claims 1-50 or 55 for targeting a cell expressing ASGPR in an individual.
81. The antibody of claim 80, wherein said cell is a liver cell, particularly a hepatocyte.
82. The antibody of claim 80 or 81, wherein said individual is a mammal, particularly a human.
83. A method for targeting a cell expressing ASGPR in an individual, comprising administering to said individual a composition comprising the antibody of any one of claims 1-50 or 55 in a pharmaceutically acceptable form.
84. The method of claim 83, wherein said cell is a liver cell, particularly a hepatocyte.
85. The method of claim 83 or 84, wherein said individual is a mammal, particularly a human.
86. The invention as described hereinbefore.



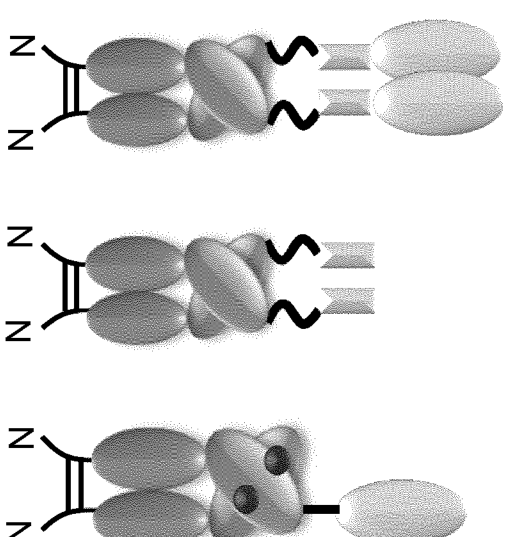
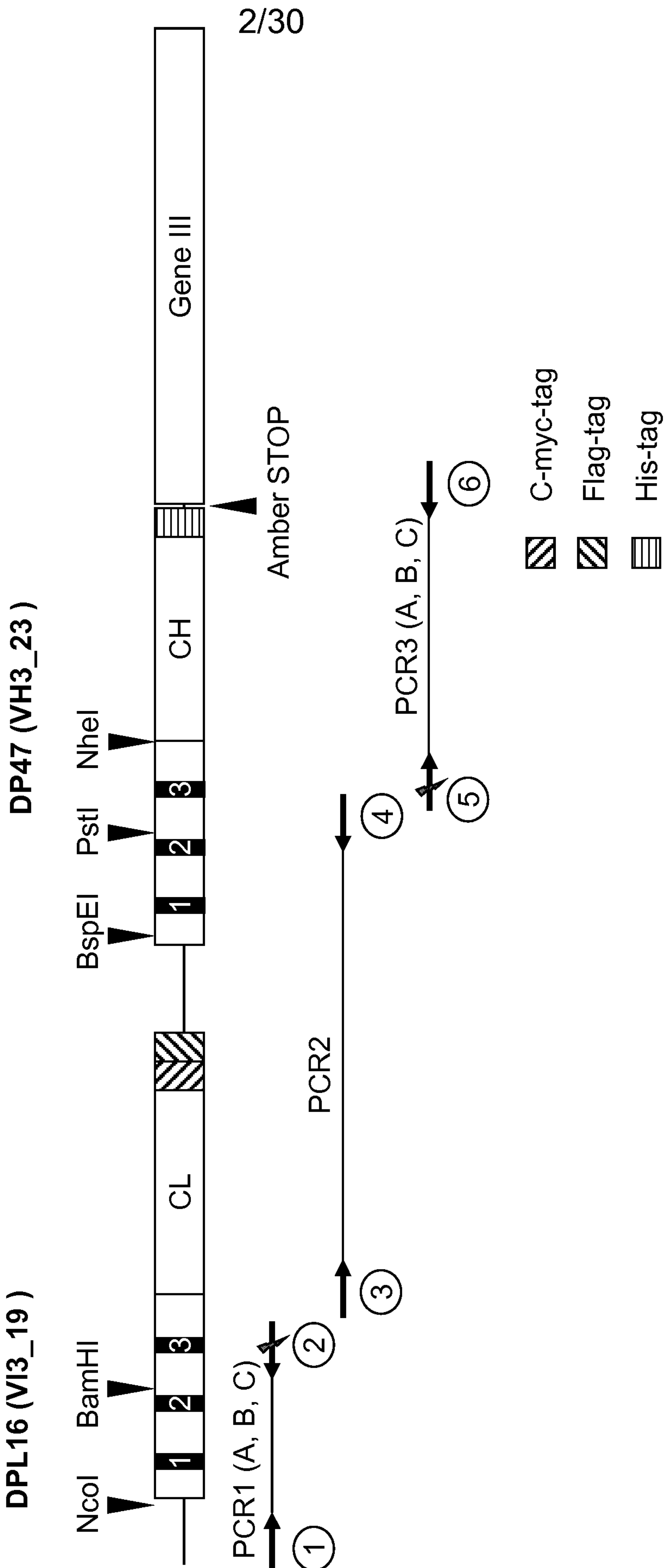


Figure 1

2/30

Figure 2



3/30

Figure 3

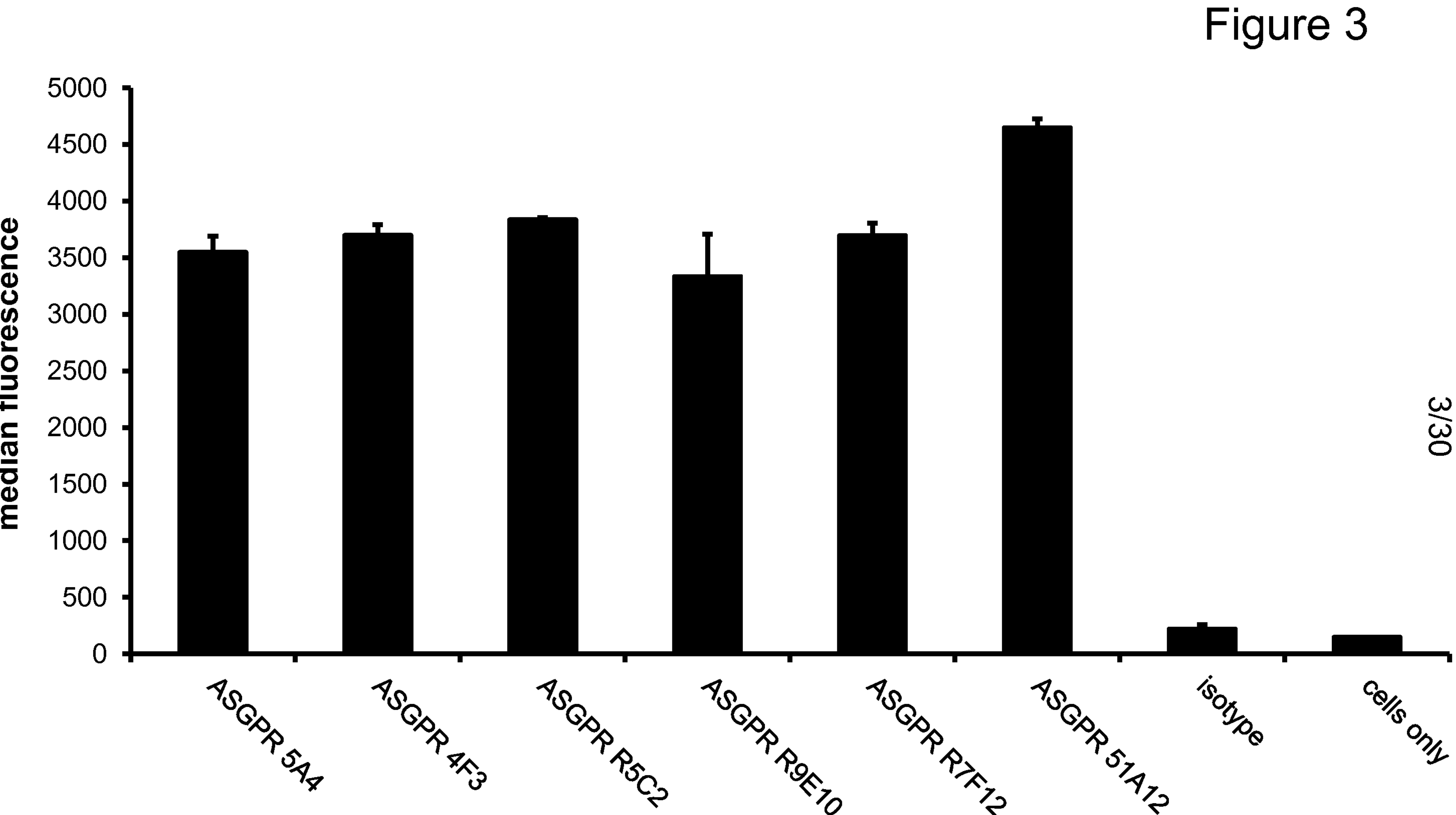
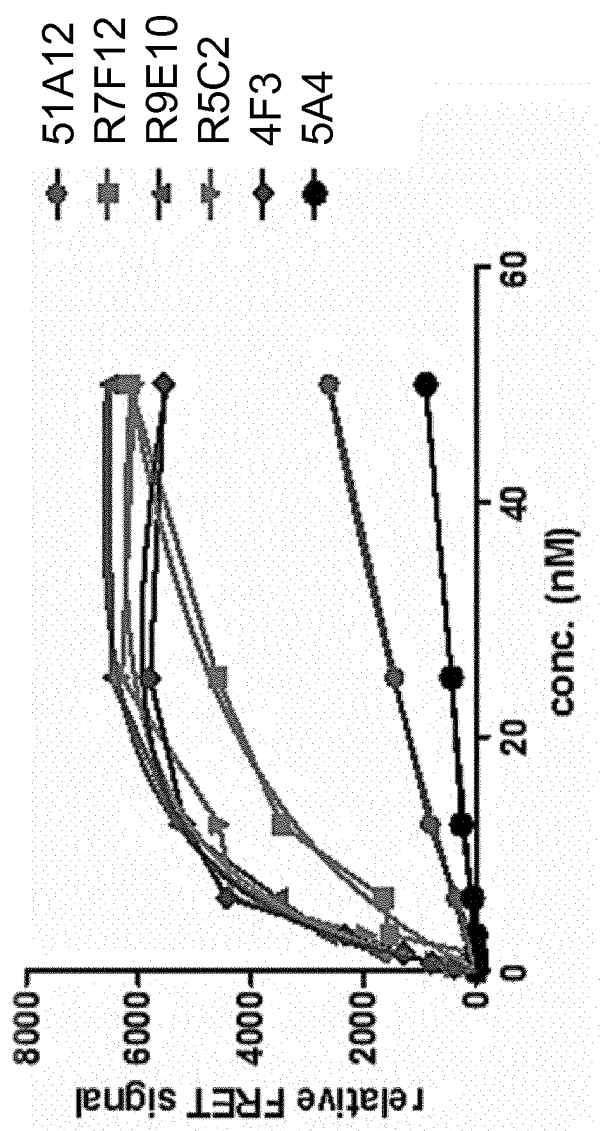


Figure 4


relative FRET signal
8000
6000
4000
2000
0
0
20
40
60
conc. (nM)
51A12
R7F12
R9E10
R5C2
4F3
5A4

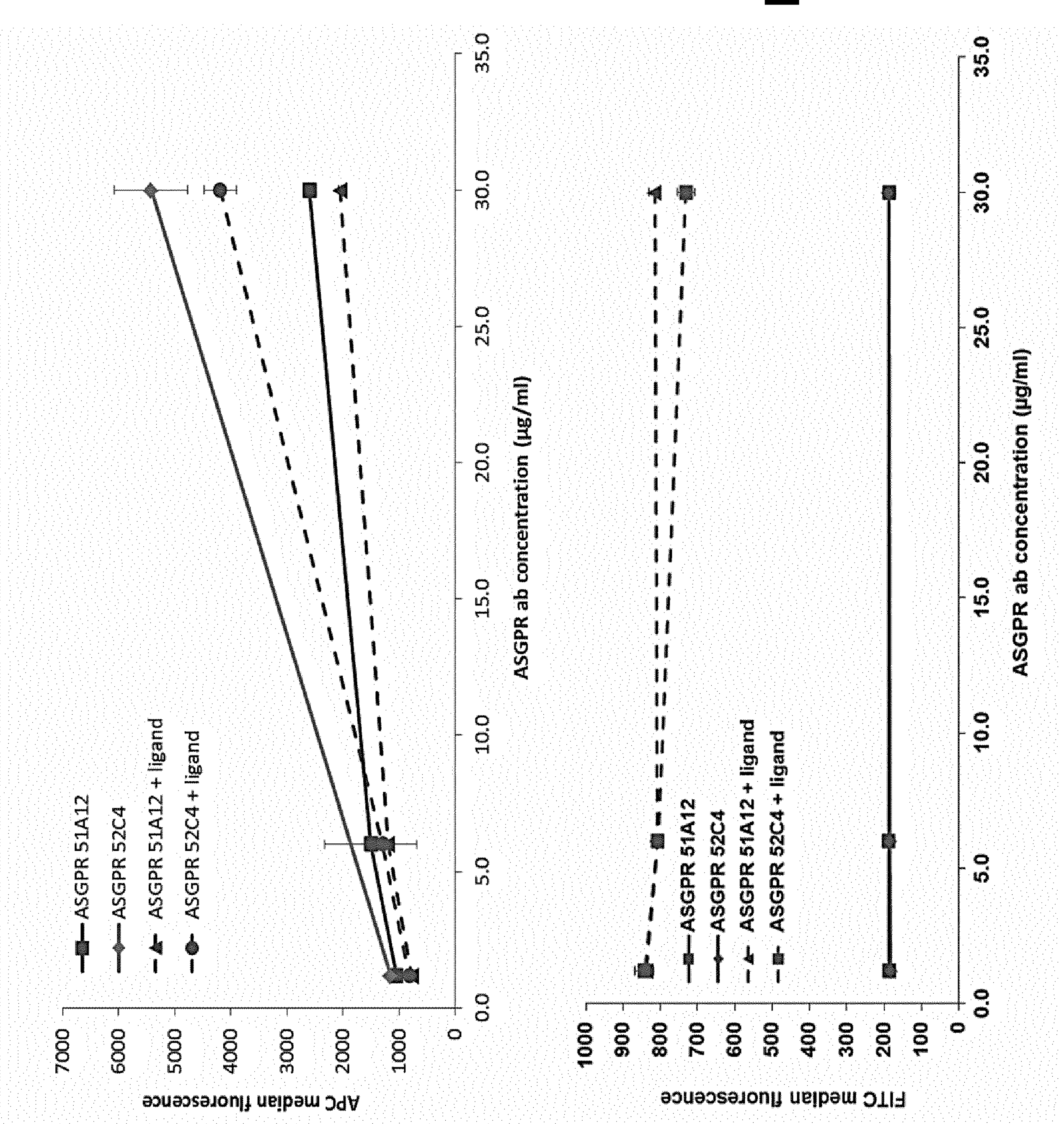
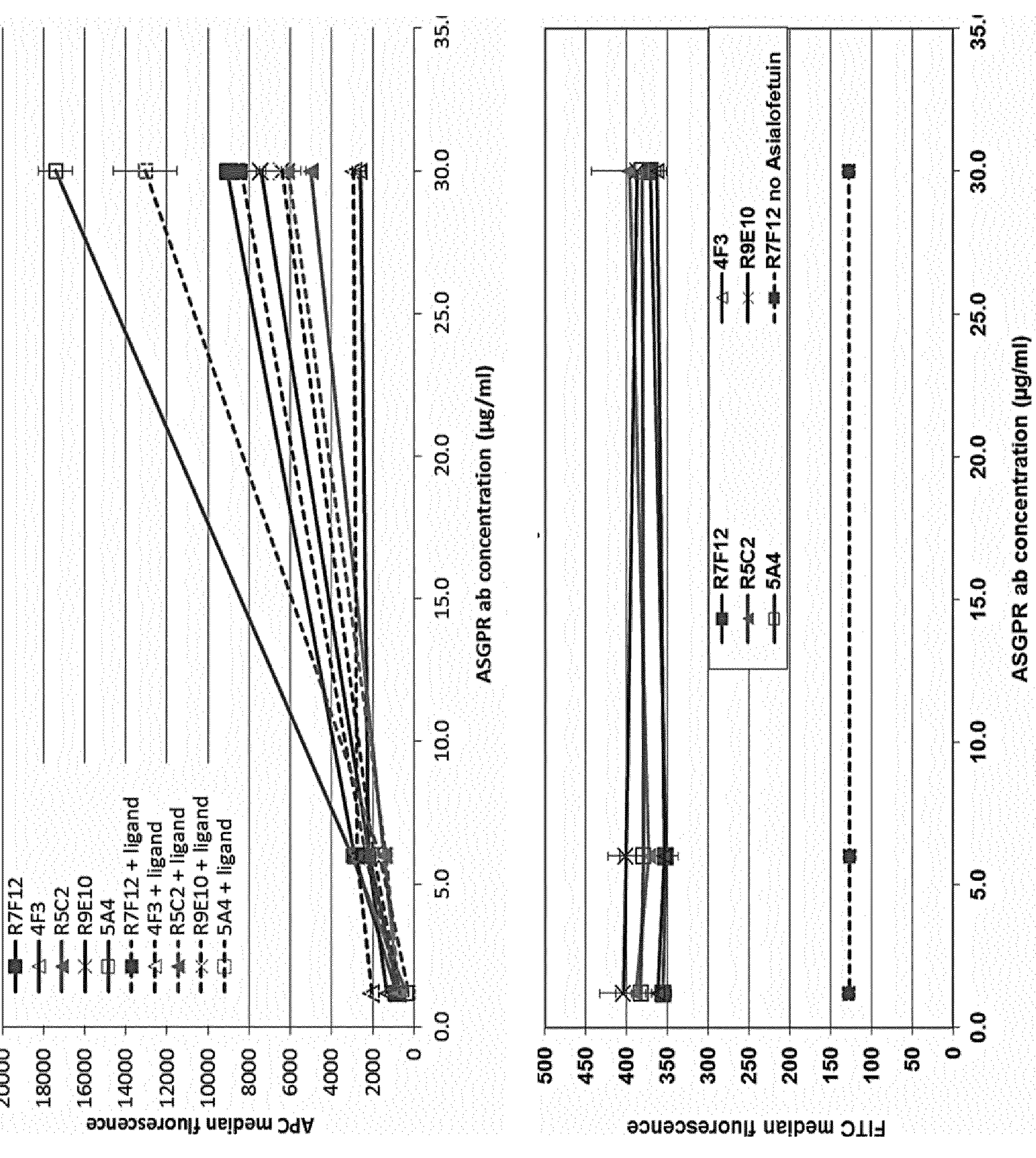


Figure 5

Figure 6



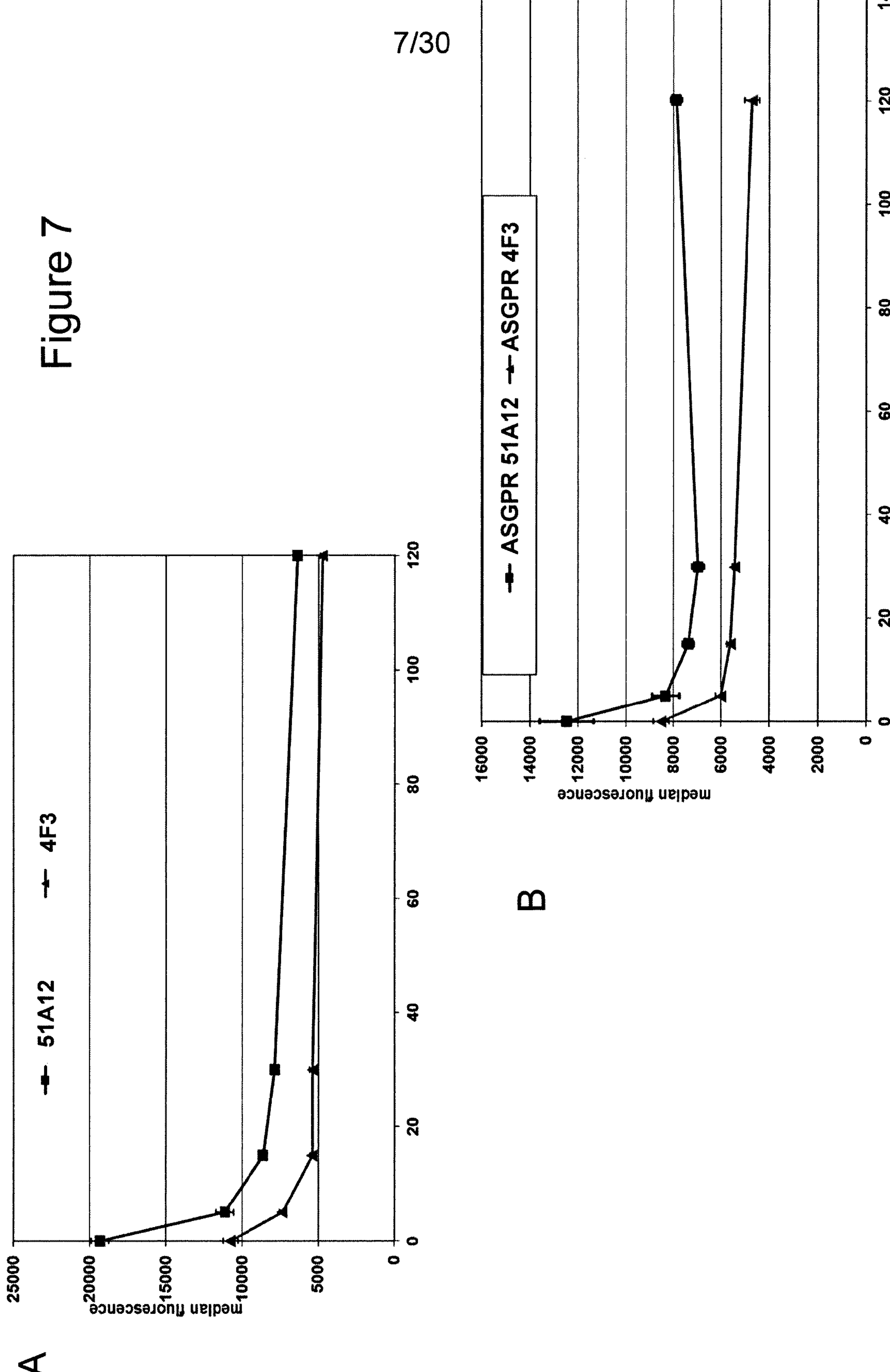


Figure 7
A
51A12
4F3
median fluorescence
25000
20000
15000
10000
5000
0
0
20
40
60
80
100
120
B
ASGPR 51A12
ASGPR 4F3
median fluorescence
16000
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120
140

Figure 7

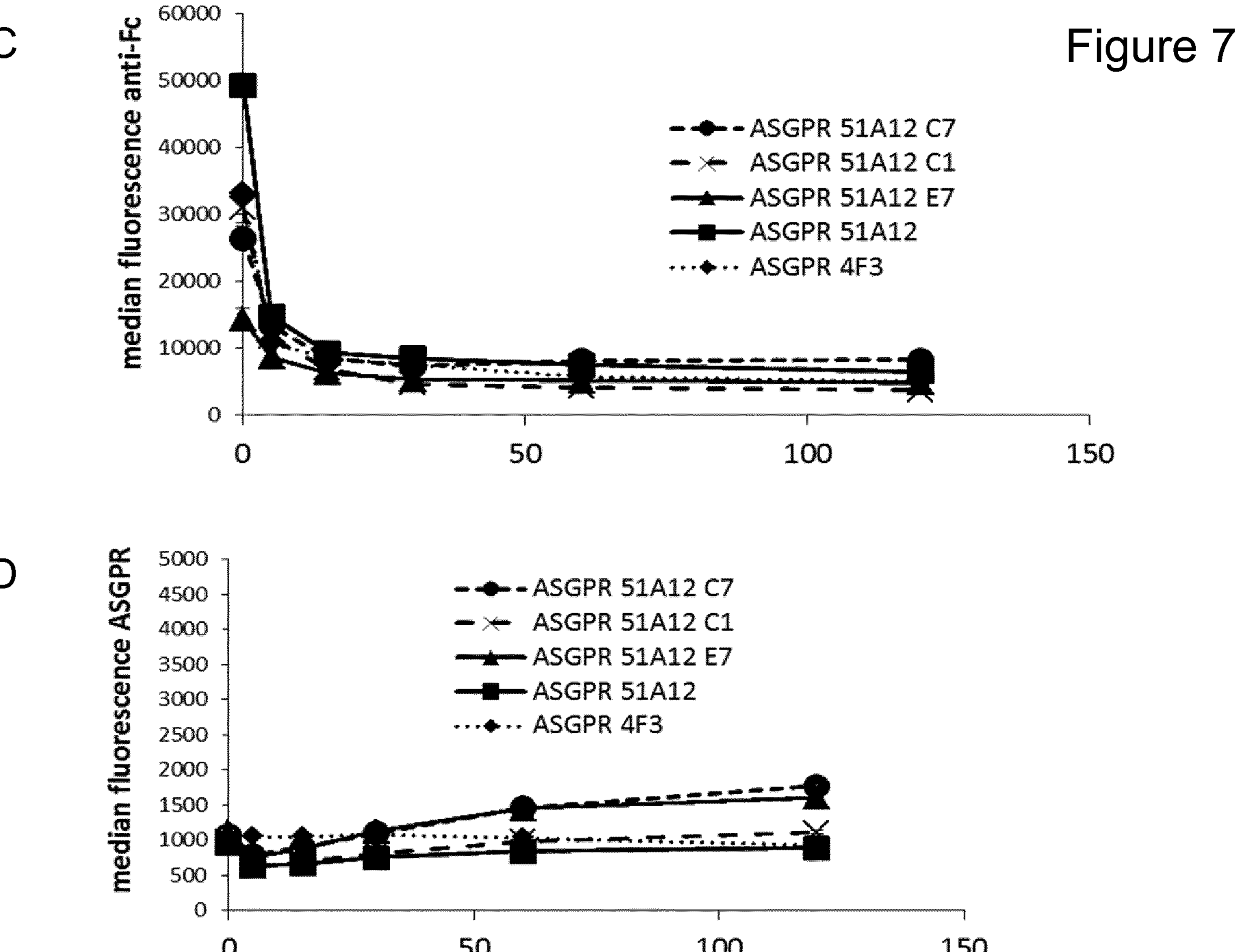


Figure 8

		89	90	91	92	93	94	95	95a	95b	95c	95d	96	97	98	99				
(A)	C	N	S	R	D	I	C	C	N	R	S	V	R	N	F	G	G	G	T	K
(B)	C	N	S	R	D	I	S	S	N	R	A	V	R	N	F	G	G	G	T	K
(C)				x	x	x	x	x	x	x	x	x	x							

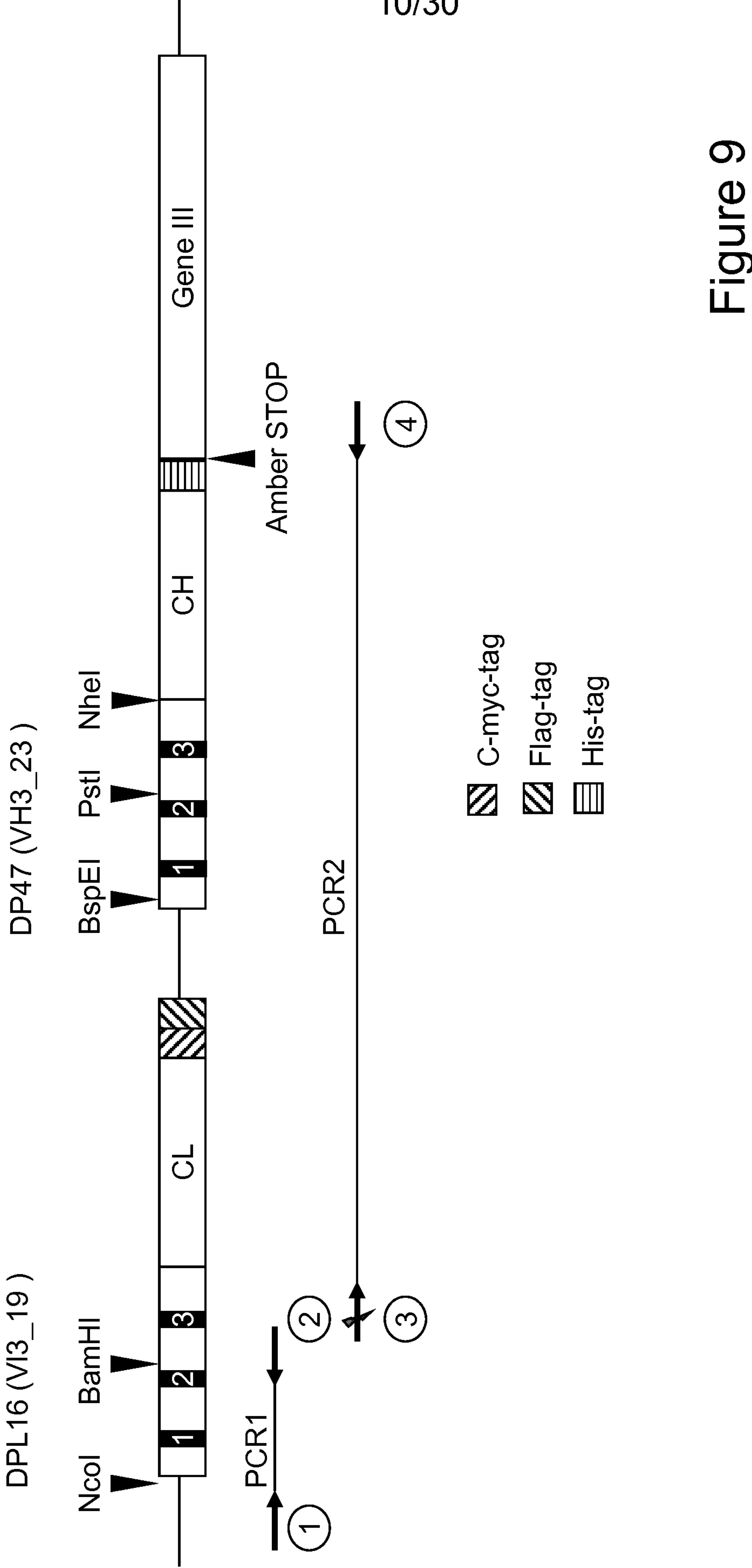


Figure 9

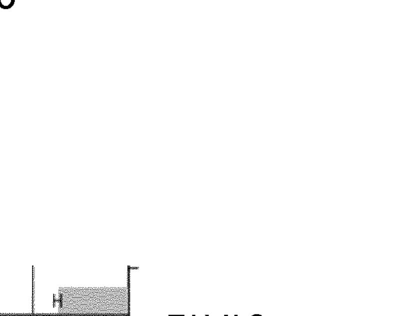
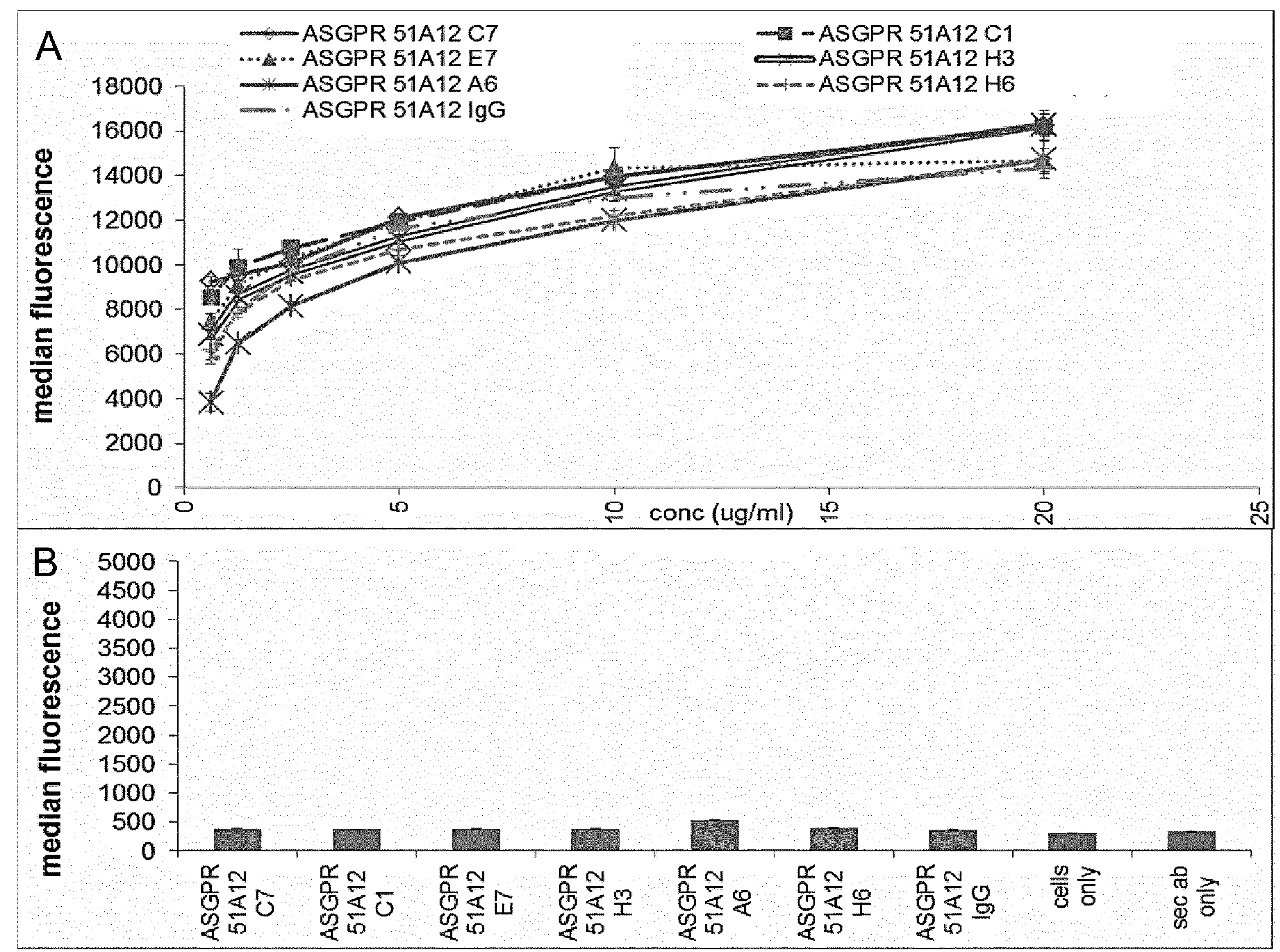
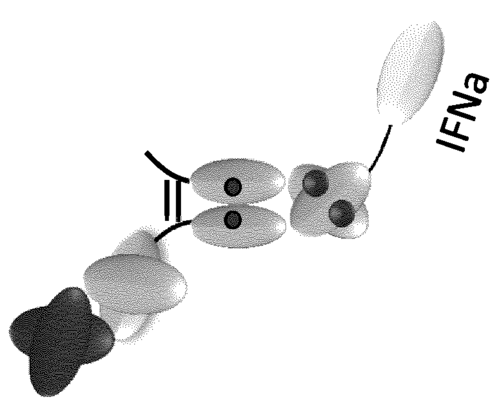


Figure 10

Figure 11



B



A

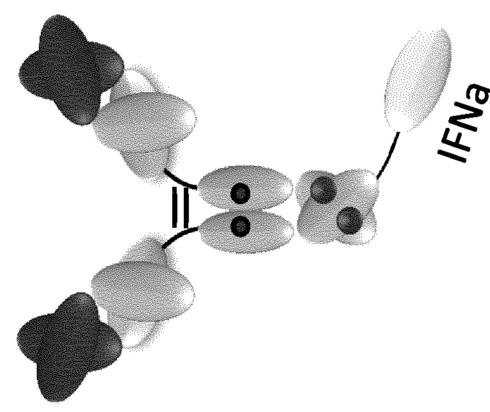


Figure 12

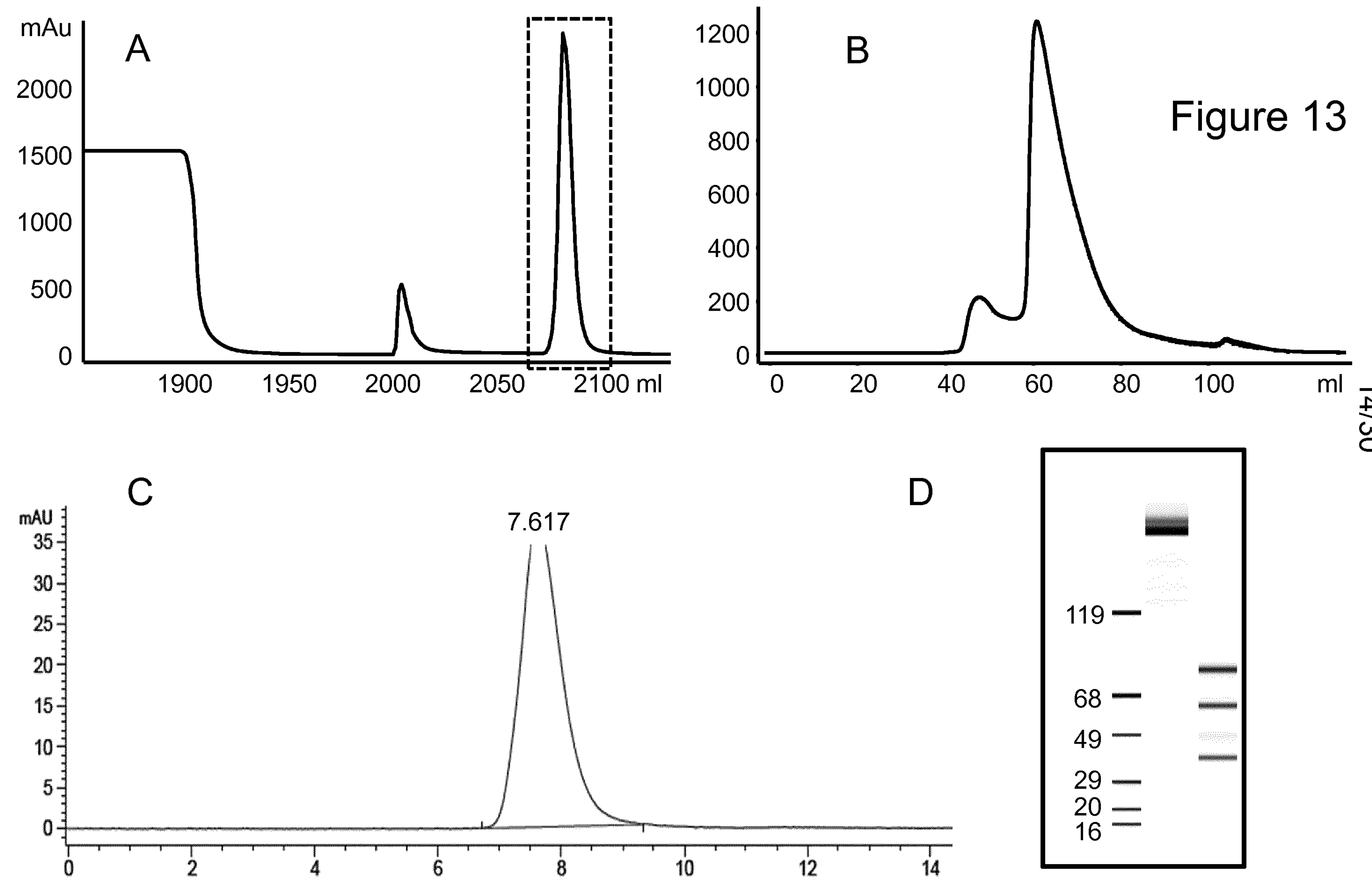
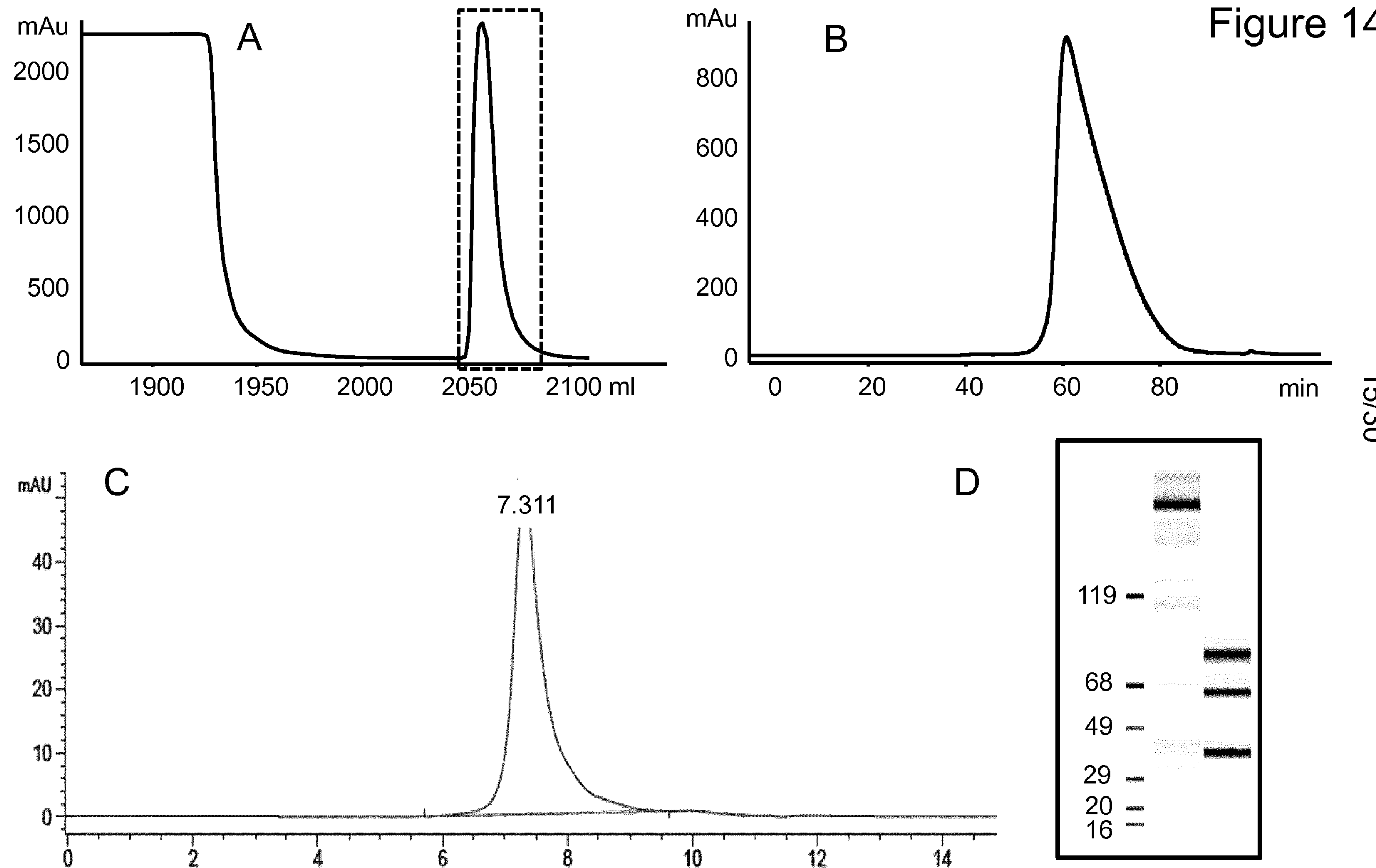


Figure 13
A
mAu
2000
1500
1000
500
0
1900
1950
2000
2050
2100 ml
B
1200
1000
800
600
400
200
0
0
20
40
60
80
100
ml
C
mAU
35
30
25
20
15
10
5
0
7.617
0
2
4
6
8
10
12
14
D
119
68
49
29
20
16

Figure 14



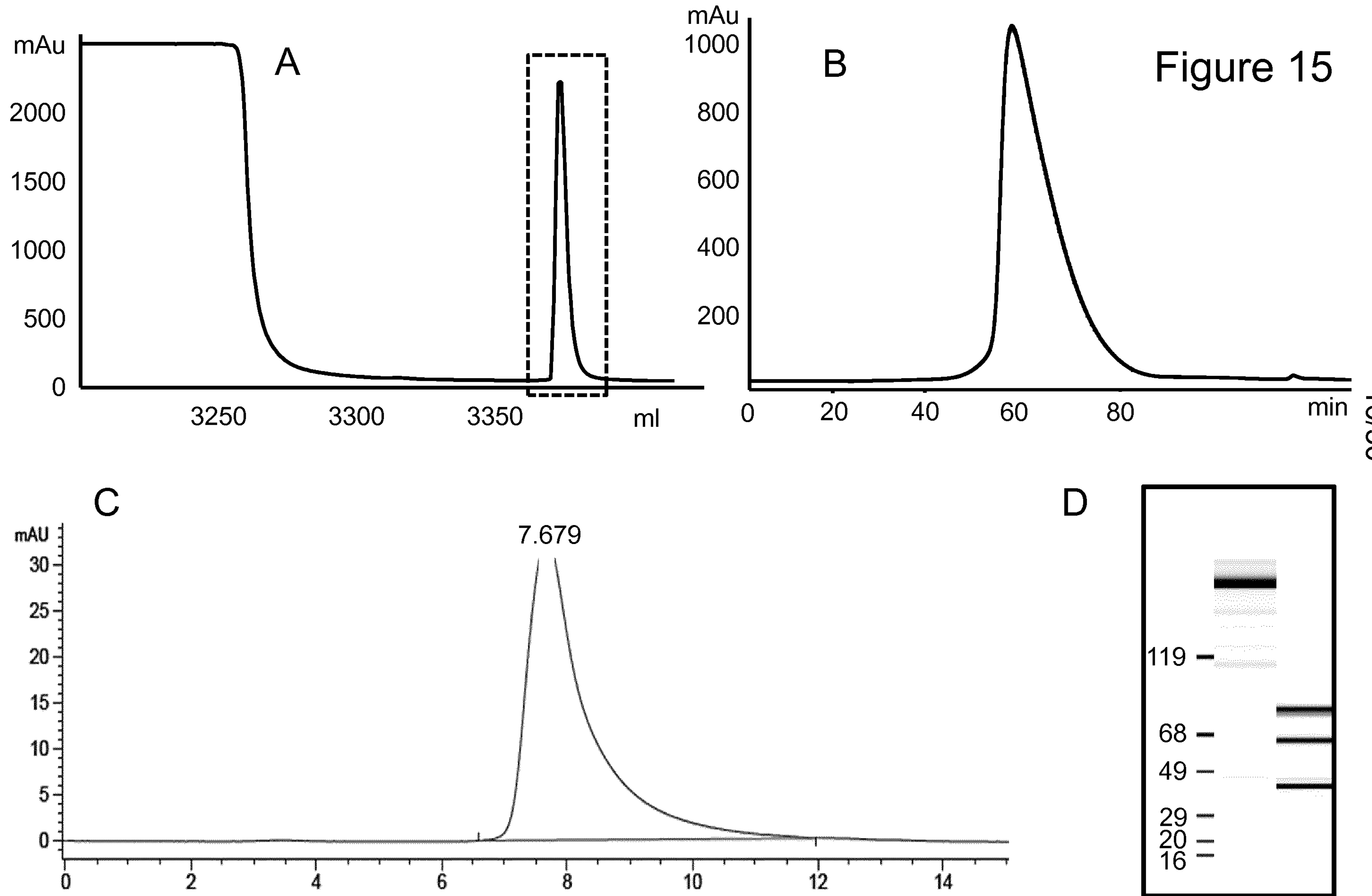
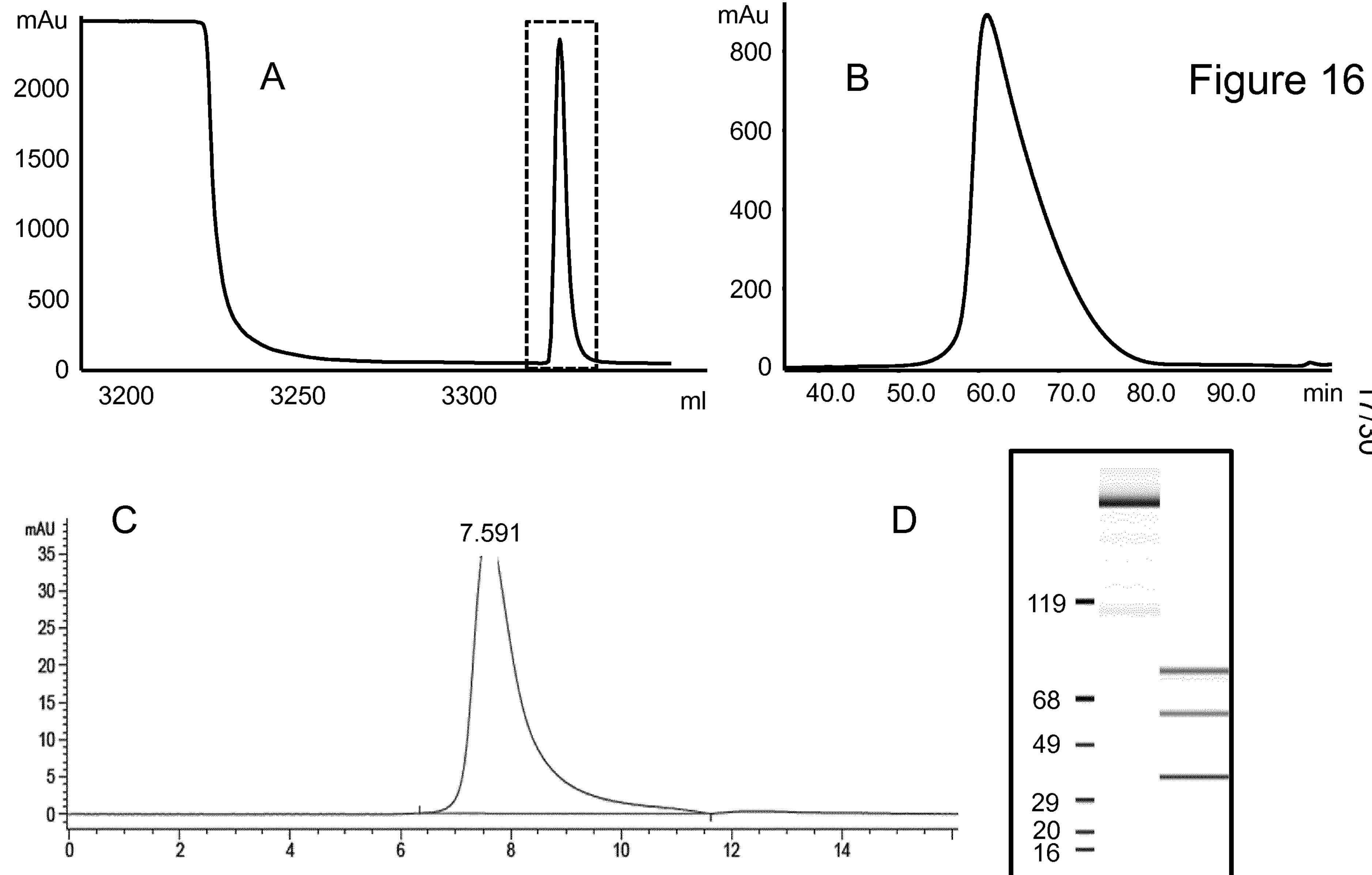


Figure 15
A
mAu
2000
1500
1000
500
0
3250
3300
3350
ml
B
mAu
1000
800
600
400
200
0
20
40
60
80
min
C
mAU
30
25
20
15
10
5
0
7.679
0
2
4
6
8
10
12
14
D
119
68
49
29
20
16

Figure 16



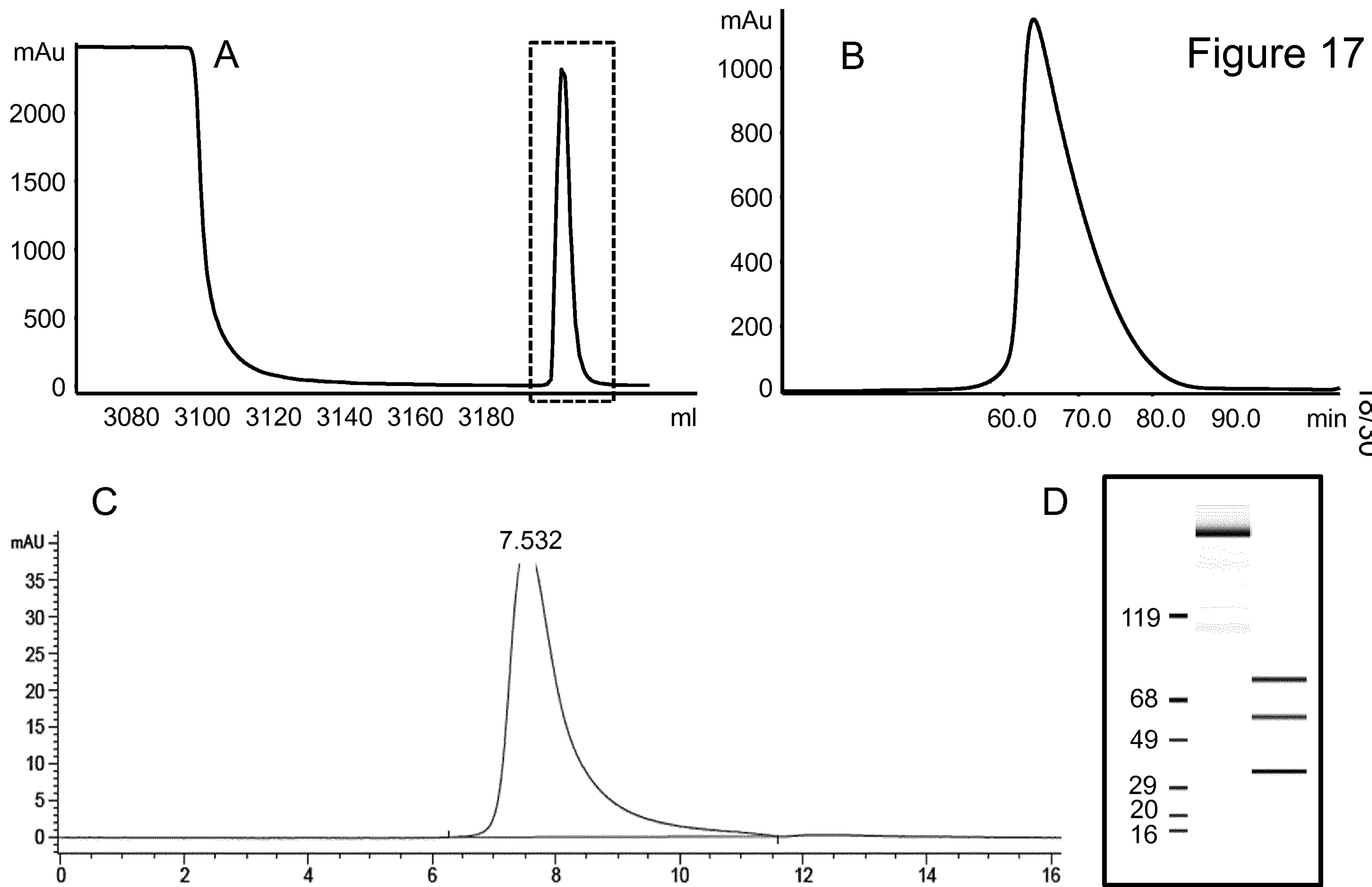


Figure 17
A
mAu
2000
1500
1000
500
0
3080
3100
3120
3140
3160
3180
ml
B
mAu
1000
800
600
400
200
0
60.0
70.0
80.0
90.0
min
C
mAU
7.532
D
119
68
49
29
20
16

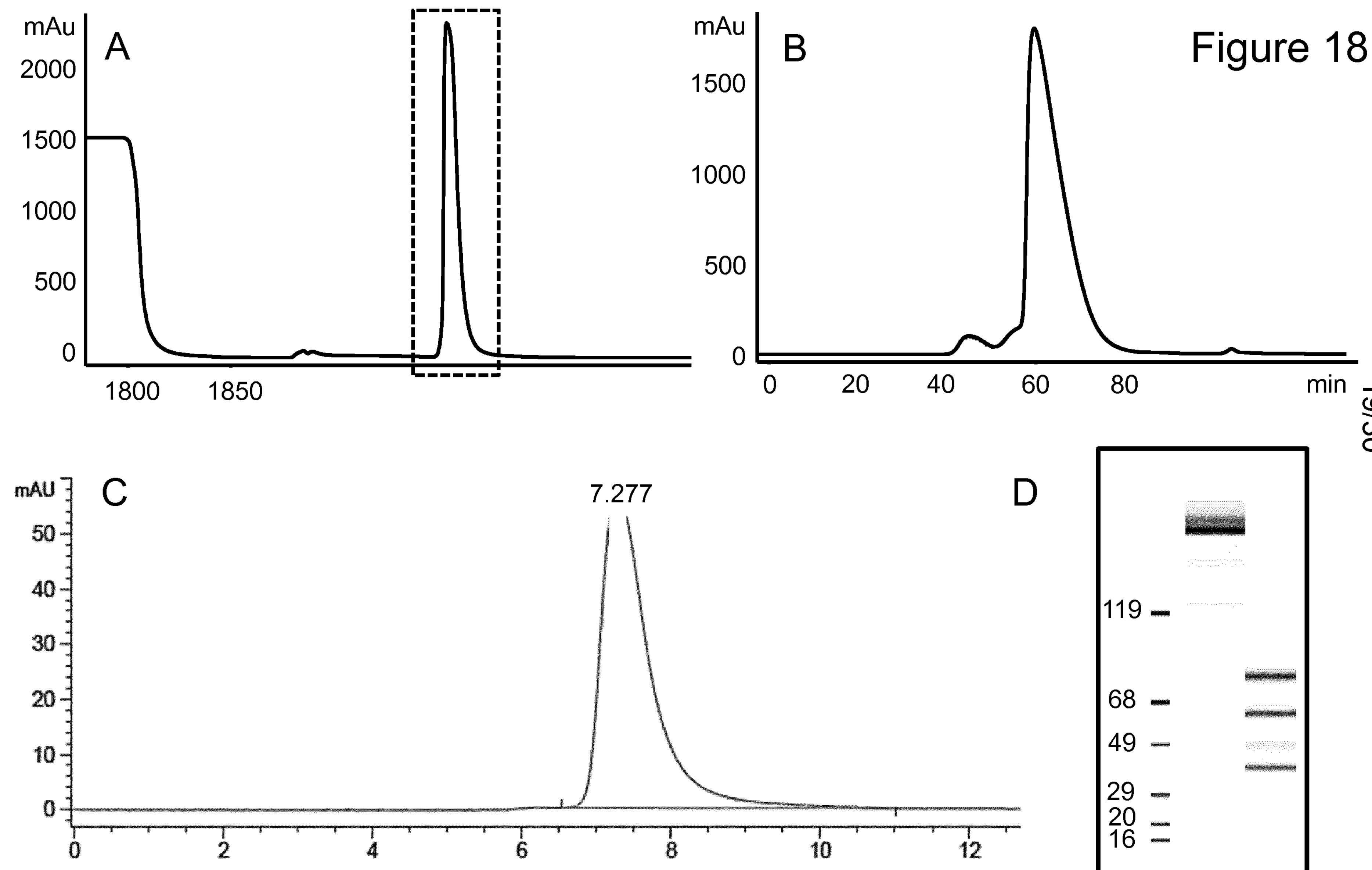


Figure 18
A
mAu
2000
1500
1000
500
0
1800
1850
B
mAu
1500
1000
500
0
0
20
40
60
80
min
C
mAU
50
40
30
20
10
0
0
2
4
6
8
10
12
7.277
D
119
68
49
29
20
16

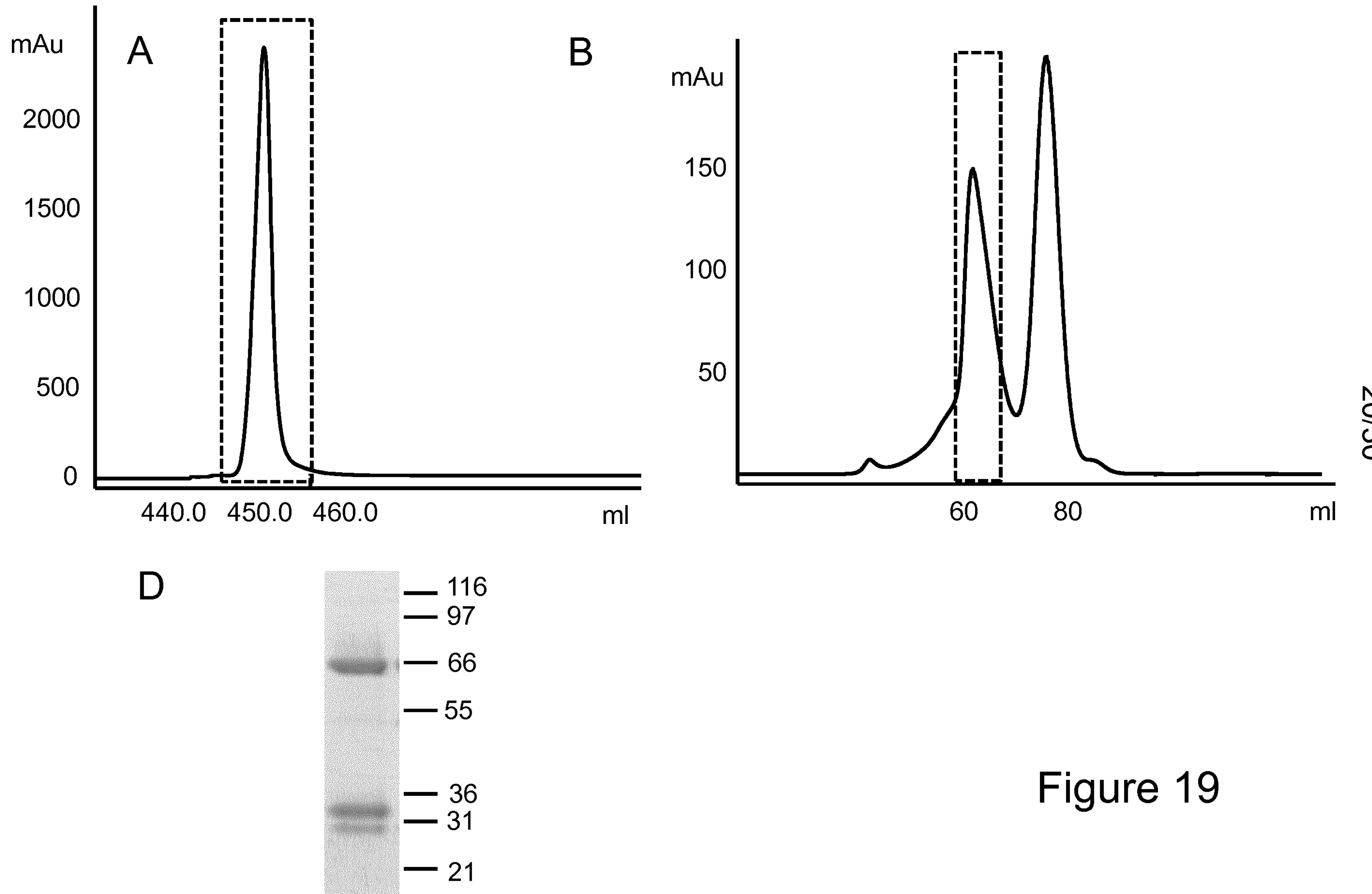


Figure 19

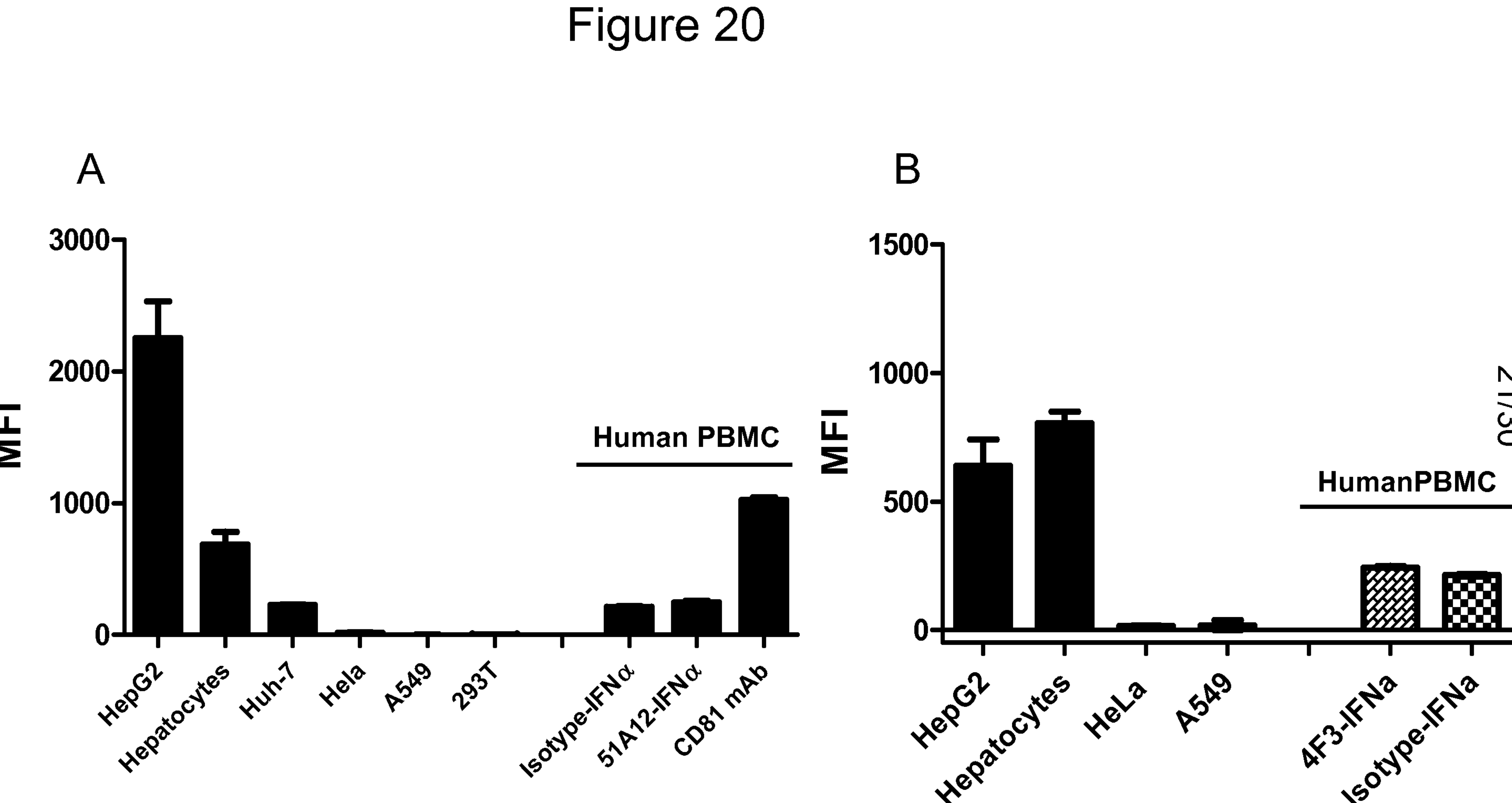


Figure 20
A
MFI
3000
2000
1000
0
HepG2
Hepatocytes
Huh-7
Hela
A549
293T
Isotype-IFNα
51A12-IFNα
CD81 mAb
Human PBMC
B
MFI
1500
1000
500
0
HepG2
Hepatocytes
HeLa
A549
4F3-IFNa
Isotype-IFNa
HumanPBMC

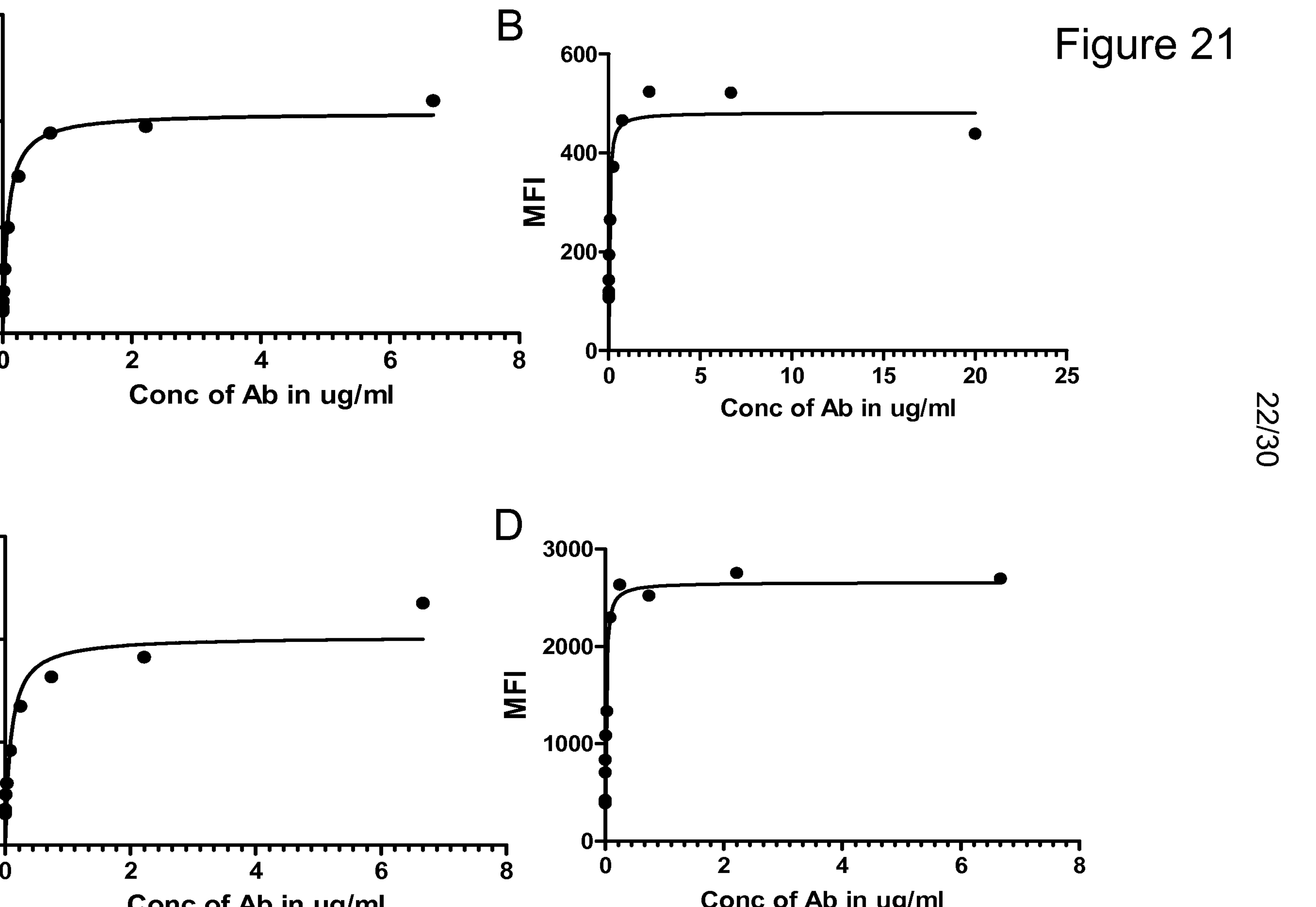
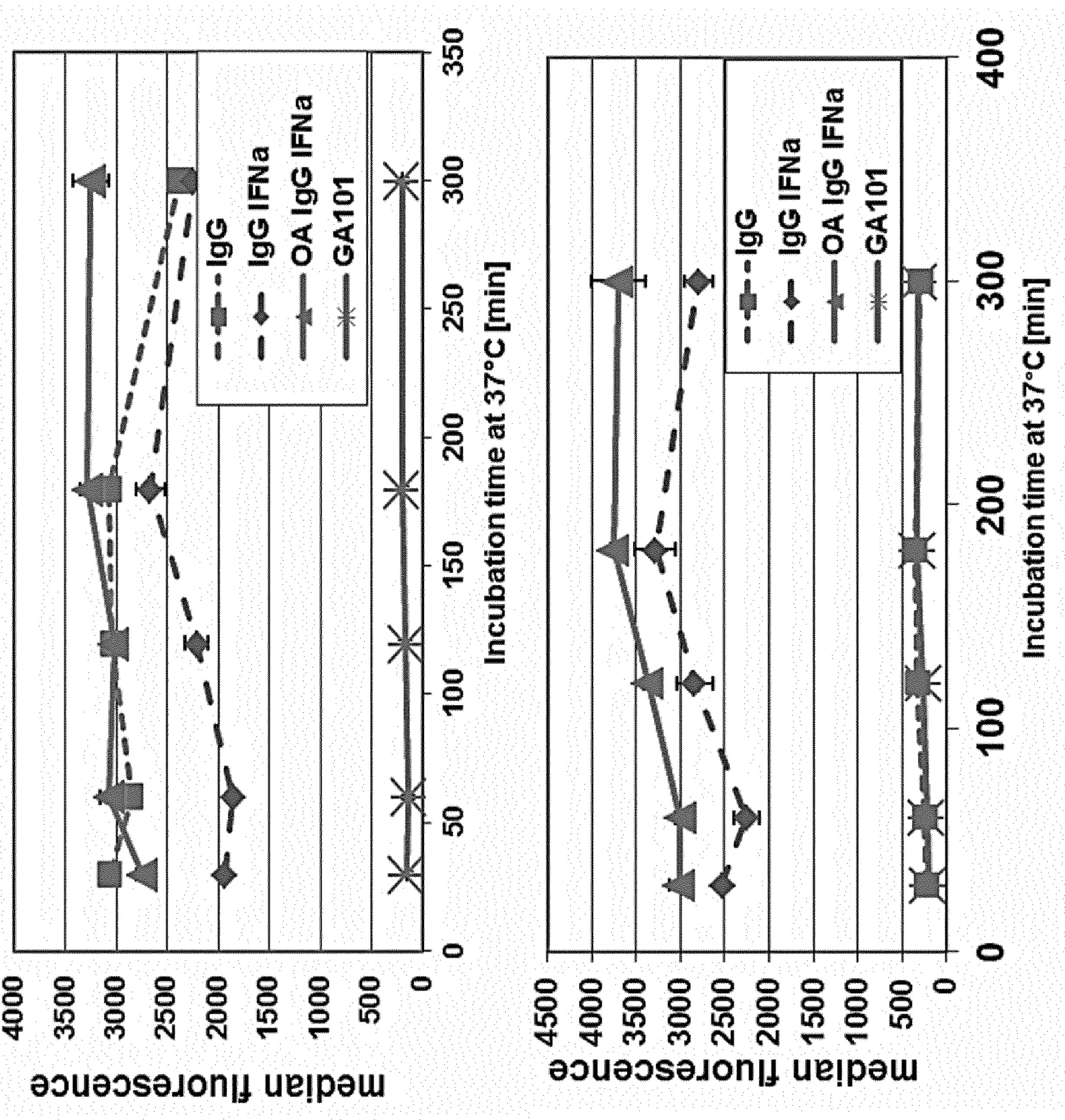


Figure 21
A
MFI
1500
1000
500
0
0
2
4
6
8
Conc of Ab in ug/ml
B
MFI
600
400
200
0
0
5
10
15
20
25
Conc of Ab in ug/ml
C
MFI
1500
1000
500
0
0
2
4
6
8
Conc of Ab in ug/ml
D
MFI
3000
2000
1000
0
0
2
4
6
8
Conc of Ab in ug/ml

Figure 22



A

B

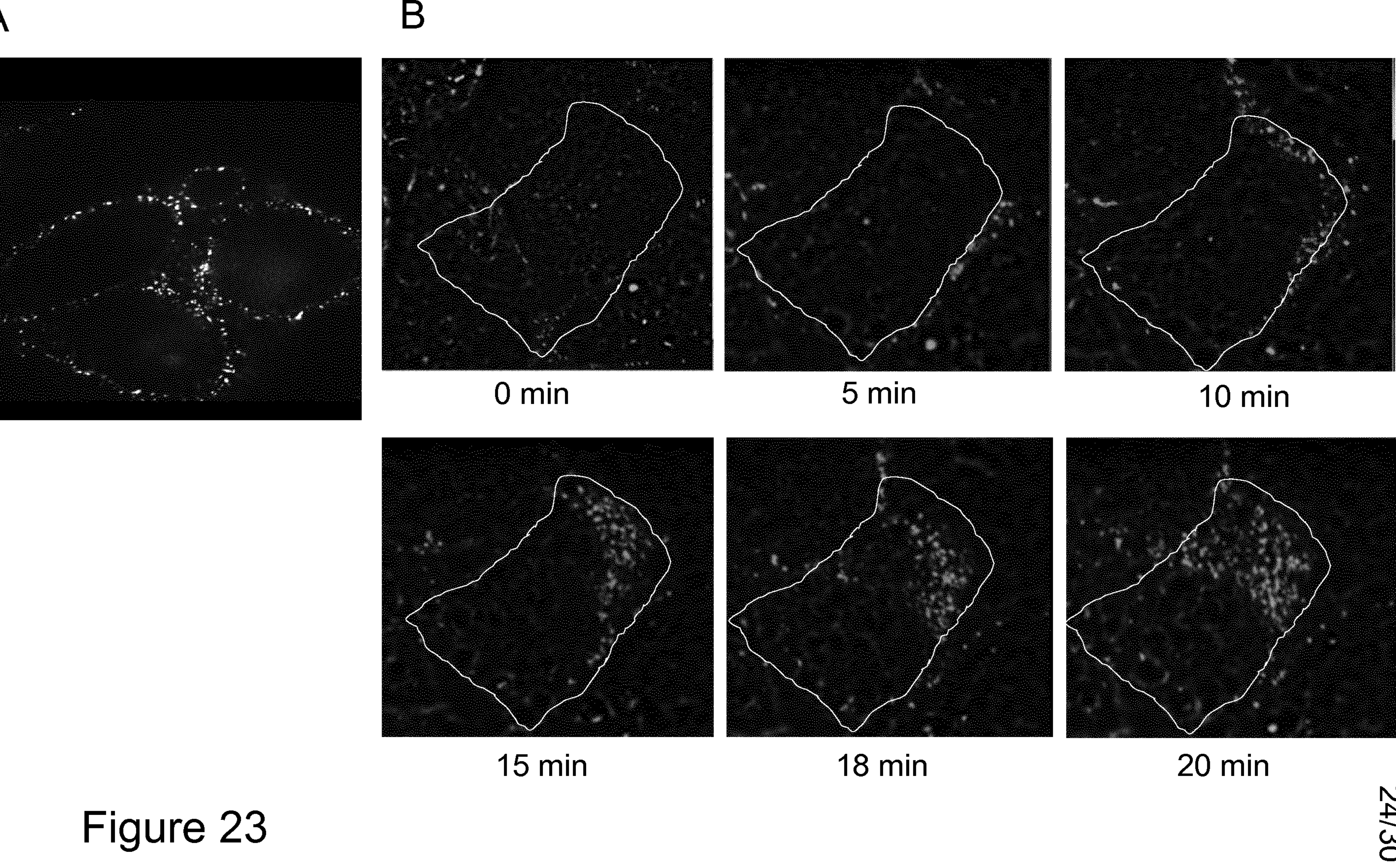


Figure 23

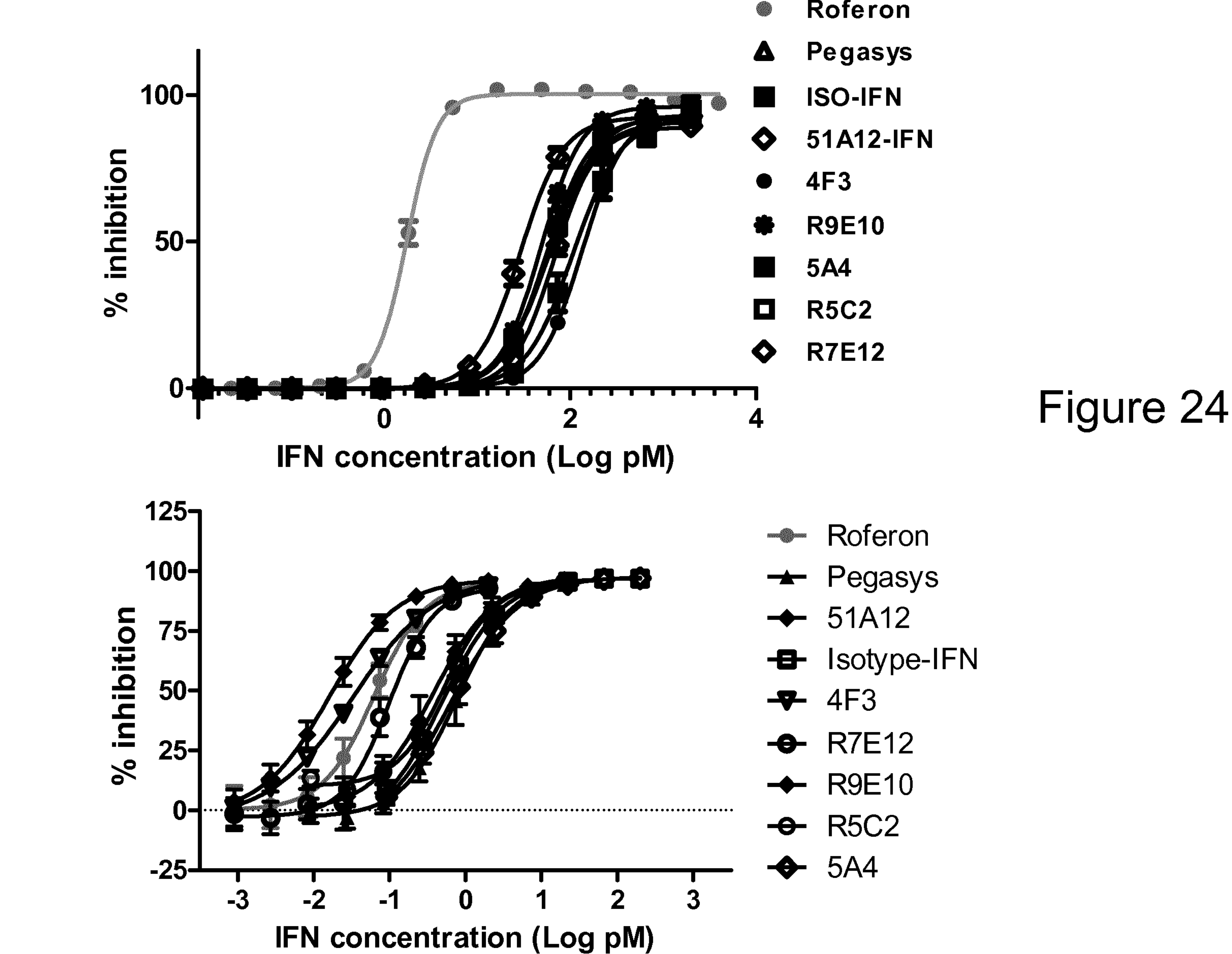


Figure 24

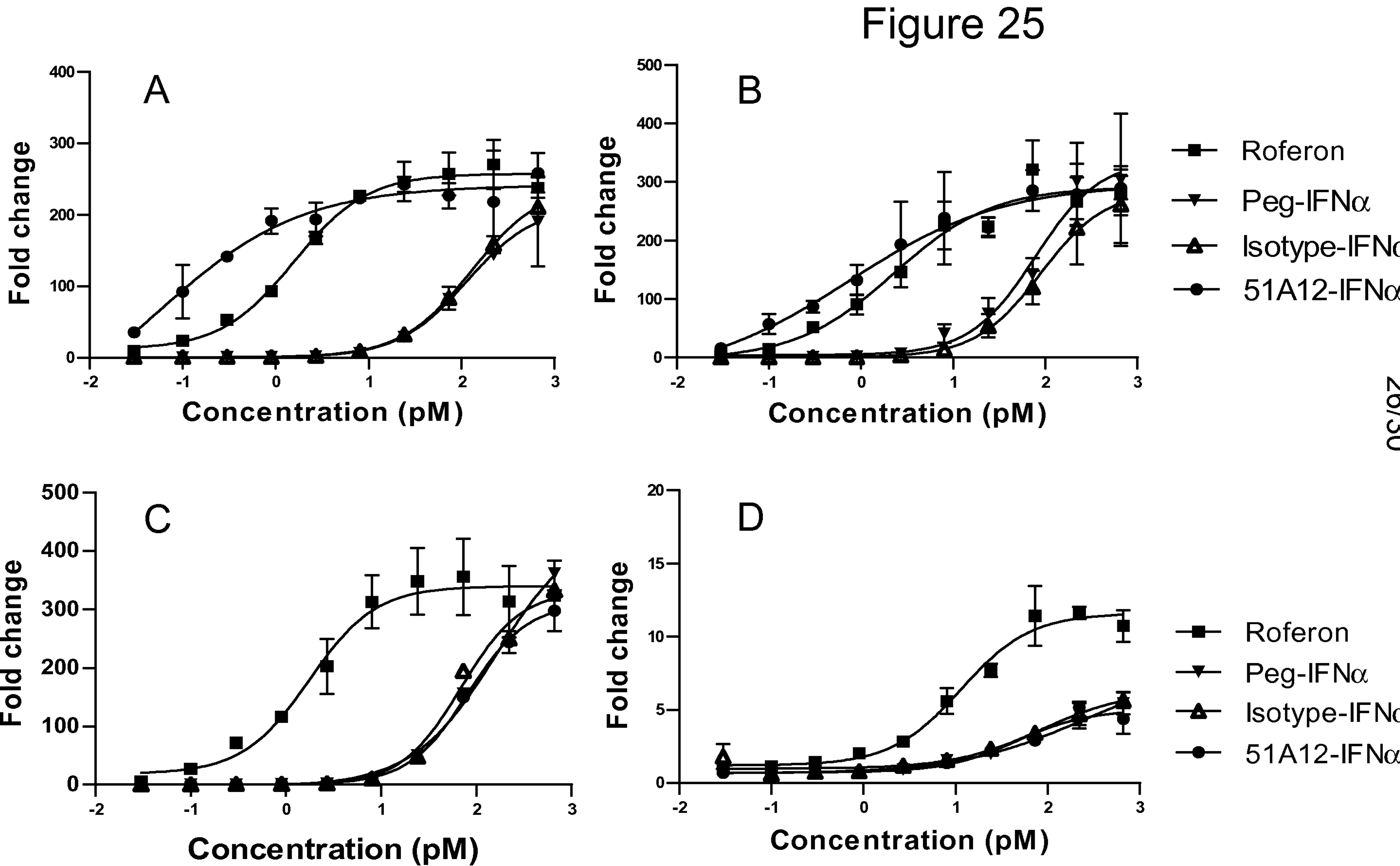


Figure 25
A
B
C
D
Fold change
Concentration (pM)
Roferon
Peg-IFNα
Isotype-IFNα
51A12-IFNα

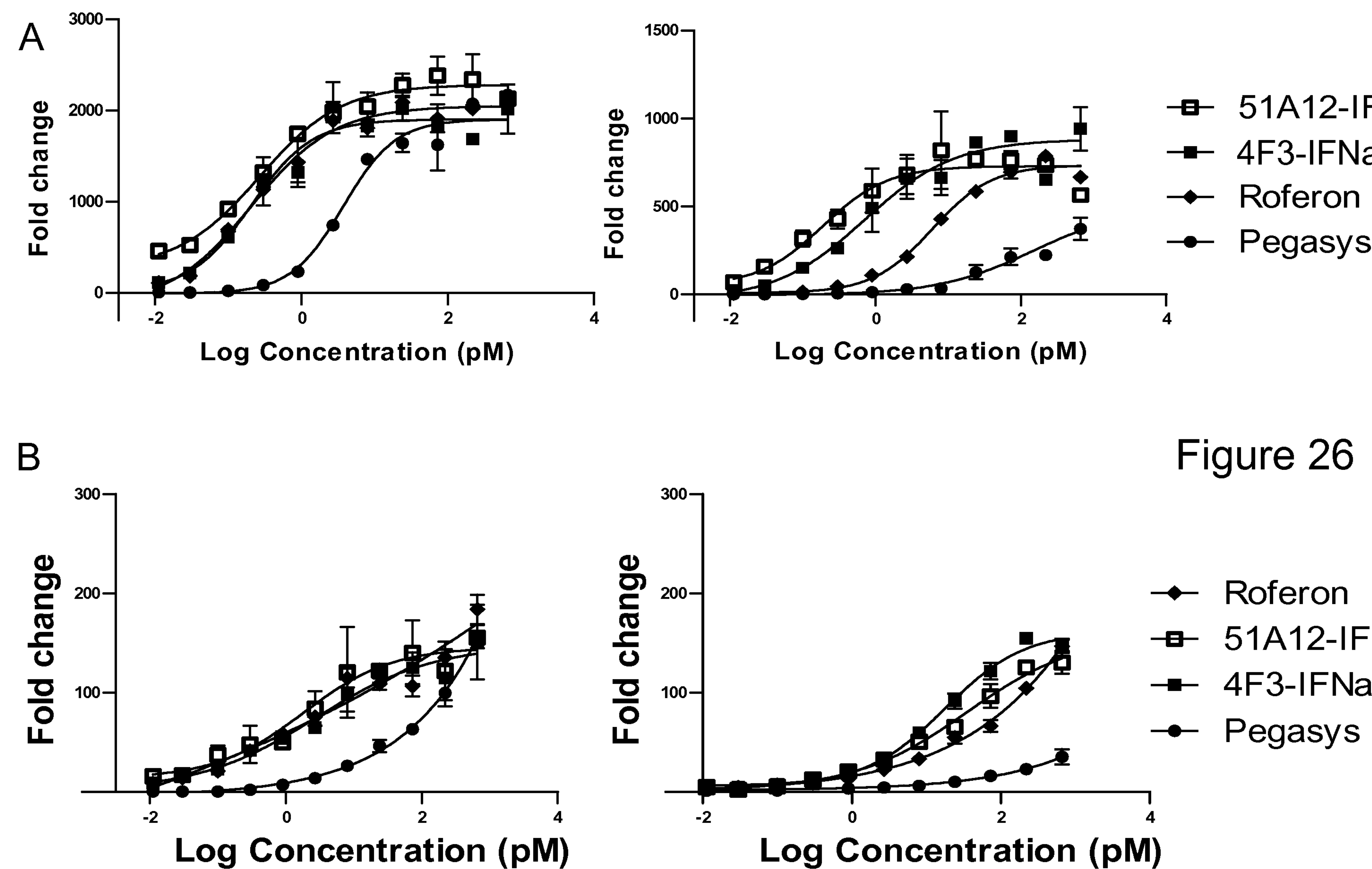


Figure 26

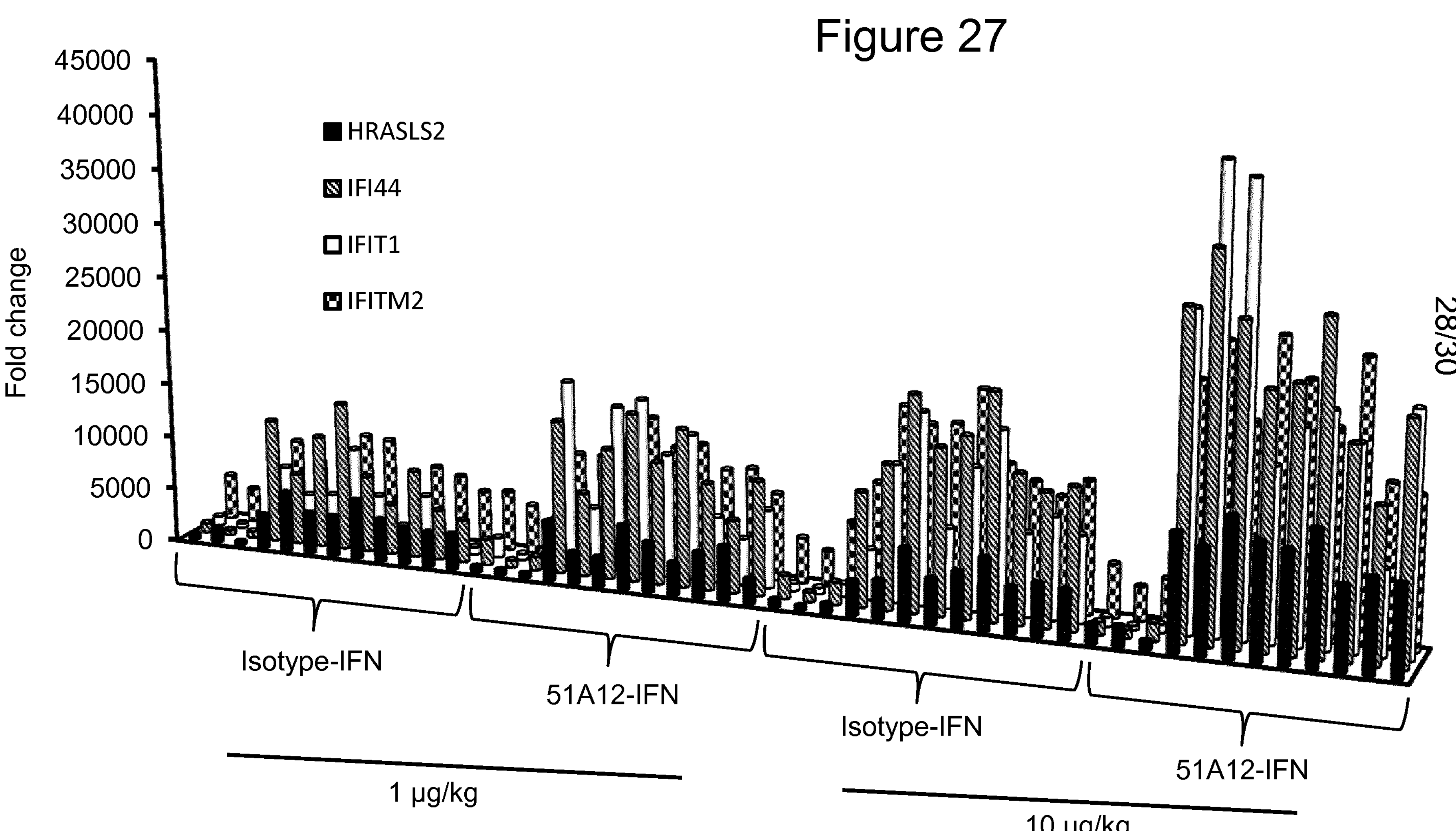
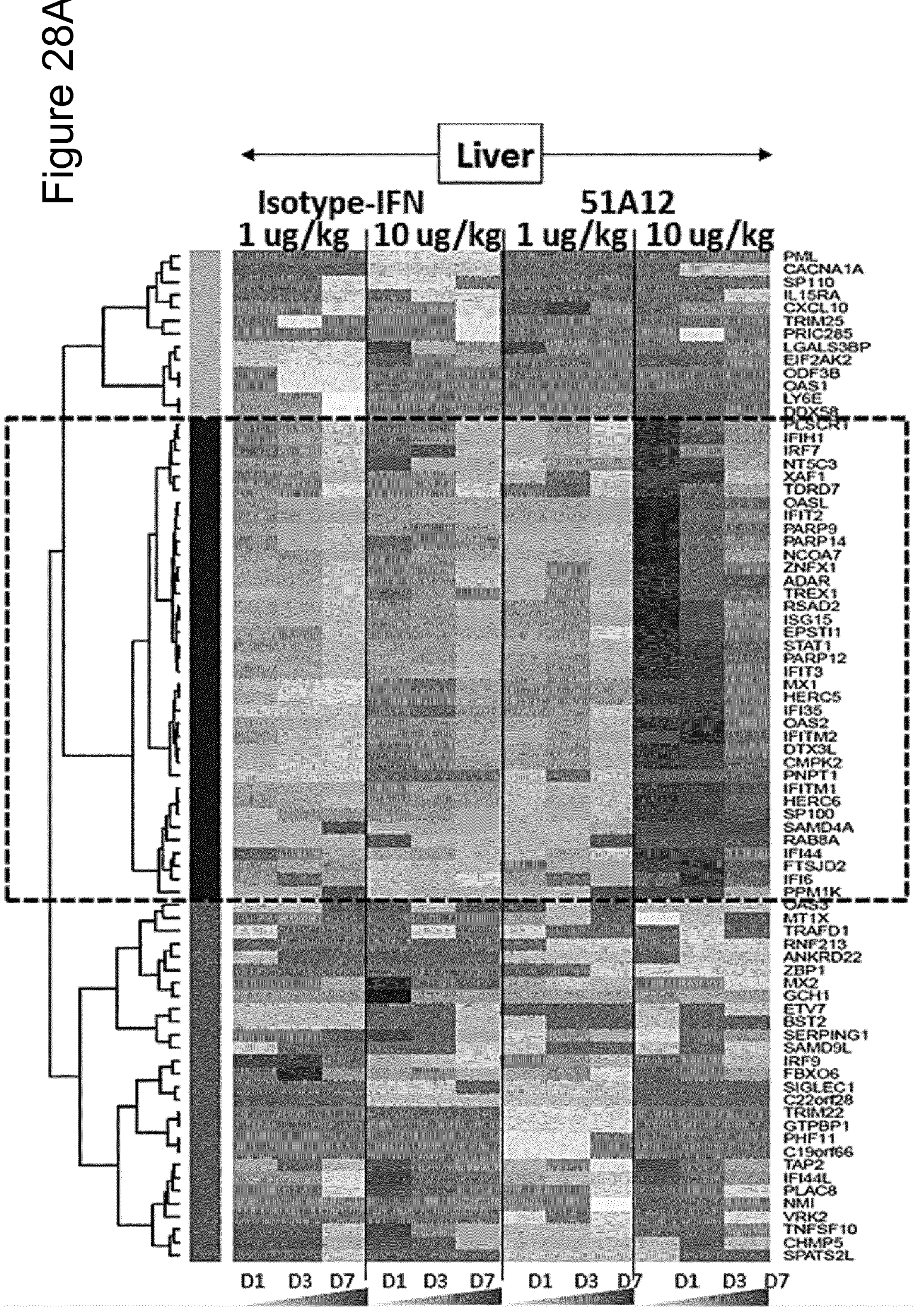


Figure 27
HRASLS2
IFI44
IFIT1
IFITM2
Fold change
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Isotype-IFN
51A12-IFN
Isotype-IFN
51A12-IFN
1 µg/kg
10 µg/kg

Figure 28A



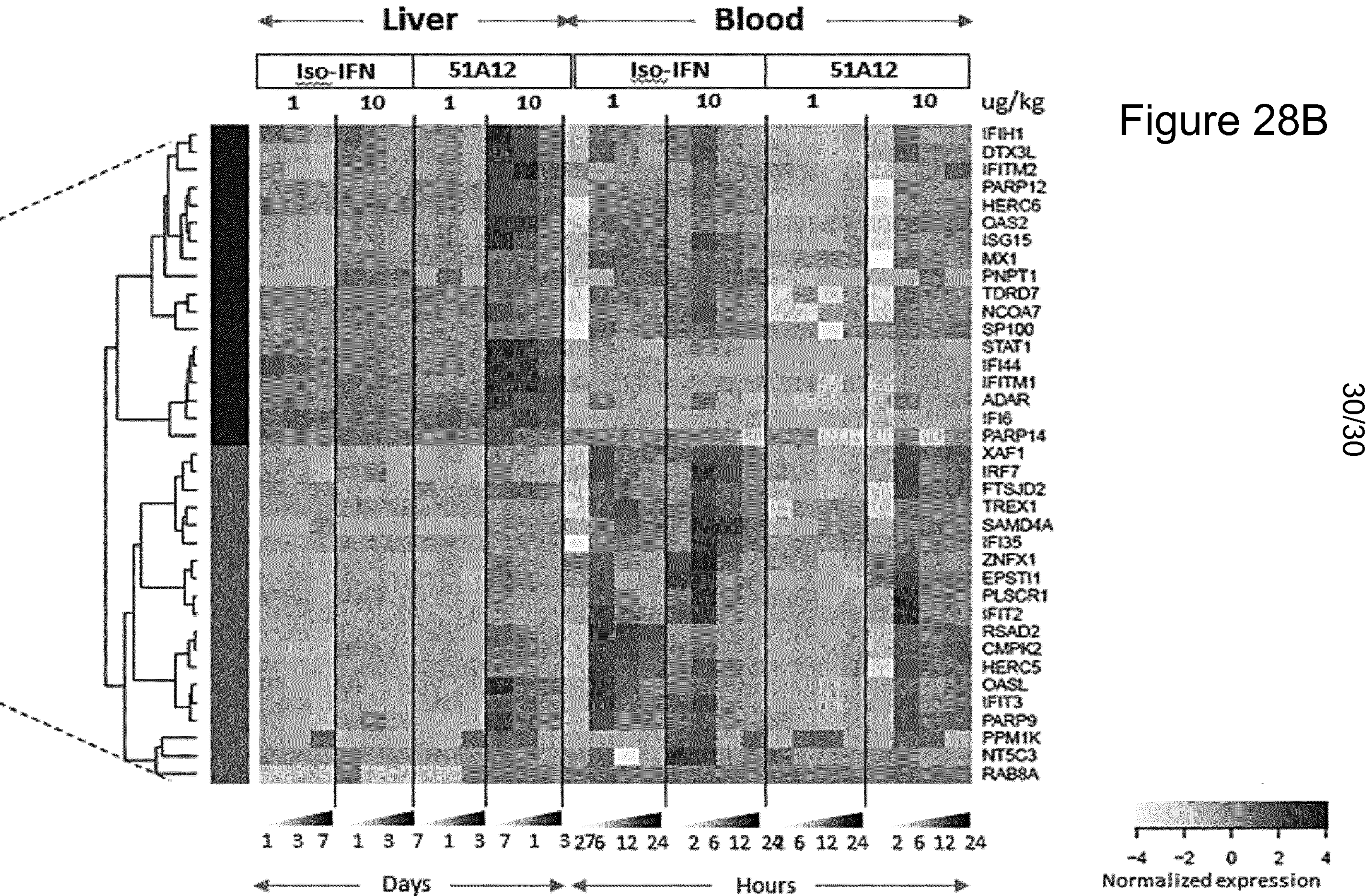


Figure 28B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/066432

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K38/19 A61P31/14 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIMIN CAO ET AL: "Characterization of a single-chain variable fragment (scFv) antibody directed against the human asialoglycoprotein receptor", BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY, ACADEMIC PRESS, US, vol. 44, no. 2, 1 May 2006 (2006-05-01), pages 65-72, XP002643344, ISSN: 0885-4513, DOI: 10.1042/BA20050081 [retrieved on 2010-12-23] page 65 page 71 -----	1-86

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 2013	31/10/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wagner, René

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIAORONG ZHAO ET AL: "Construction and characterization of an anti-asialoglycoprotein receptor single-chain variable-fragment-targeted melittin", BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY, vol. 58, no. 6, 1 November 2011 (2011-11-01), pages 405-411, XP055084712, ISSN: 0885-4513, DOI: 10.1002/bab.57 pages 407-708 -----	1-86
X	ALLA TRAHTENHERTS ET AL: "An Internalizing Antibody Specific for the Human Asialoglycoprotein Receptor", HYBRIDOMA, vol. 28, no. 4, 1 August 2009 (2009-08-01) , pages 225-233, XP055084704, ISSN: 1554-0014, DOI: 10.1089/hyb.2009.0019 figure 2 -----	1-86
X	WO 2011/086143 A2 (GLAXO GROUP LTD [GB]; DUNLEVY GRAINNE [GB]; HOLMES STEVEN [GB]; HONG Z) 21 July 2011 (2011-07-21) examples 4,6,7,12,13 -----	1-86
A	JUNG-HYUN PARK ET AL: "Detection of surface asialoglycoprotein receptor expression in hepatic and extra-hepatic cells using a novel monoclonal antibody", BIOTECHNOLOGY LETTERS, SPRINGER NETHERLANDS, DORDRECHT, vol. 28, no. 14, 24 June 2006 (2006-06-24) , pages 1061-1069, XP019391551, ISSN: 1573-6776, DOI: 10.1007/S10529-006-9191-7 the whole document -----	1-86

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/066432

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011086143 A2	21-07-2011	CA 2786660 A1	21-07-2011
		CN 102791293 A	21-11-2012
		EP 2523686 A2	21-11-2012
		JP 2013516967 A	16-05-2013
		US 2013078216 A1	28-03-2013
		WO 2011086143 A2	21-07-2011