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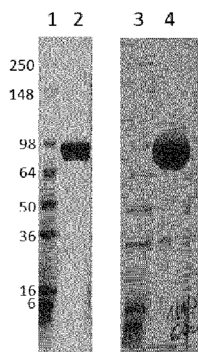
(54) **Title:** BISPECIFIC ANTIBODY TARGETING IL-1R1 AND NLPR3

(57) **Abstract:** The present disclosure concerns modulators of the NLRP3 inflammasome pathway,in particular an NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3, for use in the treatment or prophylaxis of inflammatory eye diseases such as glaucoma.

Figure 1:



Bispecific Antibody Targeting IL-1R1 and NLPR3

Technical Field

The present invention relates to modulators of the NLRP3 inflammasome pathway, particularly antibodies and fragments thereof as well as aptamer molecules (small RNA/DNA molecules that can form secondary and tertiary structures capable of specifically binding proteins or other cellular targets), each of which have binding specificity for members of the NLRP3 inflammasome. In particular, the invention extends to use of such antibodies and aptamers, and their fragments, for the treatment and prevention of inflammatory diseases mediated by NLRP3 inflammasome signalling and activation, particularly inflammatory eye diseases such as glaucoma.

Background Art

Inflammasomes are a group of protein complexes that recognize a large variety of inflammation inducing stimuli that include pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). Different inflammasome complexes are known; among these, NLRP3 is the most studied inflammasome due to the large variety of signals that activate it, including LPS, bacterial toxins, dust, stress signals such as ATP, crystallized and particulate materials, cholesterol crystals, oxidised LDL, amyloid beta, prion protein fibrils and fibrillar alpha synuclein, shear stress, pressure.

The NLRP3 (nucleotide-binding oligomerization domain (NACHT)), leucine rich repeat (LRR) domain, and pyrin domain-containing protein 3 inflammasome is implicated in a number of infectious diseases and a plethora of degenerative inflammatory type diseases including Atherosclerosis, Diabetes, Inflammatory eye disease, other eye diseases such as dry eye syndrome, Glaucoma, Age related macular degeneration, Depression, Alzheimer's Disease, Parkinson's Disease, Inflammatory Bowel Diseases, Arthritic conditions such as Rheumatoid Arthritis, Ageing, Dermatological conditions and Cancer.

The main role of the NLRP3 protein is to sense danger signals or foreign material, and relay the signal to caspase 1 in turn activating the secretion of the pro-inflammatory cytokine IL-1β, which then initiates inflammation in an attempt to protect the body. IL-1β is the most studied of all cytokines because of its central role in the inflammatory process. Although it is useful for the body to activate IL-1β, in many diseases this inflammation can get out of control and be responsible for the pathogenesis of the disease. Most therapeutic strategies to date have concentrated on developing therapies against IL-1β to dampen the inflammation, but as we propose here, there are number of advantages of targeting the upstream controllers of this cytokine, namely the NLRP3 inflammasome.

The mechanism of activation is not yet fully understood, but the processing of IL-1β via the inflammasome has been demonstrated to involve two pathways. First, the NFκB pathway is activated by a DAMP or PAMP via Toll-like receptors (TLRs) and or CD36 receptors. This leads to the transcription and expression of the pro form of IL-1β and NLRP3.

A second signal is also thought to be required whereby purinergic receptor stimulation by a DAMP such as ATP leads to increases in intracellular calcium and cell swelling that results in potassium efflux from the cell, lysosomal destabilisation, membrane permeabillisation, mitochondrial damage and subsequent generation of reactive oxygen species, leading to NLRP3 activation. Other work has demonstrated that oxidized LDL cholesterol can indeed itself act as the two signals required for NLRP3 activation. In all studies, potassium efflux appears to be the sole common denominator for NLRP3 activation.

The NLRP3 protein subsequently interacts with ASC (apoptosis-associated speck-like protein) through homotypic interactions of the pyrin domain. ASC then interacts with pro caspase 1 resulting in cleavage and activation of caspase 1, which in turn cleaves pro IL-1β to its active form. IL-1β is then cleaved to produce the biologically active and secreted form.

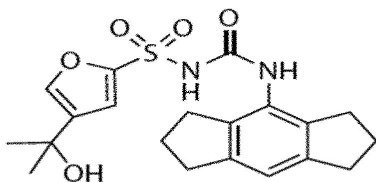
The current best treatments for inflammasome-related disorders target the main product of inflammasome activity, IL-1β. In the past 20 years, a number of anti- IL-1β therapies have been developed. However, there are several disadvantages of anti- IL-1β therapies. Host defence against opportunistic organisms as well as routine bacterial infections have become a major concern for all anti-cytokine agents because of the indolent and dangerous nature of these infections. Anti- IL-1β therapies have other side effects such as nausea, neutrophilia and adverse allergic responses.

Some advantages of an anti-NLRP3 therapy over the IL-1β therapies are as follows:

NLRP3 is a nod like receptor so dampening the recognition of the root cause of a disease, i.e. recognition of the foreign / danger material may be advantageous over dampening the response. This would mean that no IL-1β would be secreted via the NLRP3 pathway activated by disease specific stimuli, e.g. oxidized LDL, β amyloid or alpha synuclein or a particular pathogen. However, IL-1β could still be activated via other pathways in response to other non-disease-related stimuli as needed in extreme circumstances (such as large scale or opportunistic infections), since there are other pathways responsible for IL-1β activation.

The inflammasome has been associated with specialized forms of cell death, pyronecrosis (caspase1 independent) and pyroptosis, which may occur in cases of exacerbated inflammation. Therefore, an anti-NLRP3 therapy will also decrease such death pathways, which have been evidenced to be involved in the pathogenesis of certain diseases such as atherosclerosis. Pyroptosis is a risk factor for plaque disruption in this disease in response to oxidized LDL.

Several previously characterized small molecule inhibitors have more recently also been shown to affect NLRP3 inflammasome function. Glyburide, a sulfonylurea drug, is an example of such an inhibitor. MCC950 (illustrated below) is another example of a specific small molecule inhibitor of NLRP3 inflammasome:



However, there are several problems with currently available inhibitors. Indeed many of these currently available inhibitors of inflammasome function have either not been clinically successful, are nonspecific and importantly have very short half lives.

The development of humanized antibody type therapy could prove more advantageous than small molecule inhibitors for the NLRP3 inflammasome.

Some advantages of humanized antibodies over small molecule inhibitors are as follows:

- Non-recognition by the human immune system.
- A longer half-life in the circulation than non-human antibodies.
- Higher specificity than small-molecule inhibitors.
- Interact with challenging targets which have thus far eluded small molecule drugs. The best examples of this are protein-protein interactions which are characterised by large and often flat surfaces with few charged pockets.
- Chimeric and humanized mAbs, which have been the predominant mAbs entering clinical studies, have higher approval success rates (18% and 24%, respectively) than new chemical entities (NCEs) including small-molecule agents (5%), especially in the field of oncology.
- The commercial potential of biologics is very promising. The share of biologics in total sales of prescription and over-the-counter medicines grew from 12% in 2004 to 19% in 2011. More interestingly biologic products accounted for 17% of sales of the top 100 pharma products in 2004; 34% in 2011. The global biologics market is estimated to reach nearly $4bn by 2025.
- Biologics appear to be delivering a better overall economic return than small molecule drugs.
- Studies also show that the rate of attrition for biologics is less than that for small molecules. It has been reported that 24.4% of biologics that enter preclinical testing eventually reach the market compared with a success rate of only 7.1% for small molecule drugs.
- Biologics performed better than small molecules at all stages of development with an astonishing 116% rate of success at Phase 2.

NLRP3 (also known as NALP3 and cryopyrin) is a cytosolic protein; therefore, in order to target this protein, any therapy must gain entry to the cell. Humanized antibodies are quite large in size and entry to the cytosol may prove difficult. Small antibody fragment development also present a possibility to overcome such a challenge where an antibody fragment may be a Fab fragment, which is the antigen-binding fragment of an antibody, or a single-chain variable fragment, which is a fusion protein of the variable region of heavy and the light chain of an antibody connected by a peptide

linker. As discussed further below, the present inventor has devised additional strategies to ensure the therapeutic antibody or aptamer, and their fragments, can gain entry to the cell.

There are some reports in the field describing the targeting of the NLRP3 inflammasome or related molecules using various agents. For example, WO2013/007763A1 discloses an inhibitor capable of intracellular localisation and cytosolic binding to a member of the inflammasome group including NLRP3, for use in a method for the prevention/treatment of acne.

US20080008652A1 discloses methods and compositions for modulating immune responses and adjuvant activity, and in particular, via modulation of cryopyrin (NPRL3) signalling. Humanized antibodies that target cryopyrin modulating proteins, or cryopyrin signal pathway components, are mentioned, and methods of producing cryopyrin antibodies are disclosed.

WO2002026780A2 discloses antibodies that bind to PAAD-domain containing polypeptides, as well as methods of treating various pathologies, including inflammation, by administering an anti-PAAD antibody. Single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof are also mentioned.

WO2011109459A2 discloses a method of treating an inflammatory disease of skin/hair by providing a composition including at least one antibody that specifically binds to a component(s) of a mammalian inflammasome, such as ASC or NLRP1. Commercially available antibodies to ASC and NPRL1 are mentioned.

EP2350315B1 discloses methods and kits for the early diagnosis of atherosclerosis, involving the measurement of the expression levels of NLRP3, ASC and/or caspase-1. Expression levels may be measured by methods involving antibodies, including human antibodies, humanized antibodies, recombinant antibodies and antibody fragments, which in turn include Fab, Fab', F(ab)2, F(ab')2, Fv and scFv.

WO2013119673A1 discloses a method of evaluating a patient suspected of having a CNS injury comprising measuring the level of at least one inflammasome protein such as NLRP1 (NALP-1), ASC, and caspase-1. Commercially available antibodies to NPRL-1, ASC and caspase-1 are mentioned.

WO2007077042A1 discloses a method for the treatment of gout or pseudogout, comprising administering a NALP3 inflammasome inhibiting agent. The NALP3 inflammasome inhibiting agents are described as acting downstream of the NALP3 inflammasome and selected from among antibodies that inhibit the activity of IL-1.

WO2013138795A1 discloses a fusion protein comprising a Surf+ Penetrating Polypeptide and an antibody or antibody-mimic moiety (AAM moiety) that binds to an intracellular target, wherein the

fusion protein penetrates cells and binds to the intracellular target to inhibit binding between the target and another protein inside the cells.

The present invention provides novel and effective modulators of the NLRP3 inflammasome for the treatment and prevention of inflammatory diseases mediated by NLRP3 inflammasome signalling and activation, particularly inflammatory eye diseases such as glaucoma. Such modulators include a bi-antibody or aptamer, and their fragments, targeted to both of IL-1R1 and NLRP3. The bi-antibody first gains entry into the cell by binding to the IL-1R1 which triggers rapid internalisation and, once internalised, the bi-antibody then targets the intracellular protein NLRP3 inhibiting the assembly of the NLRP3 inflammasome, in turn preventing IL-1β secretion from the cells, and reducing the initiation/amplification of inflammation.

Summary of the Invention

Accordingly, in a first aspect of the present invention, there is provided an NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3 for use in the treatment or prophylaxis of an inflammatory eye disease.

Optionally, the inflammatory eye disease is glaucoma.

Optionally, the modulator is also capable of binding to the PYD domain of NLRP3.

Optionally, the modulator is selected from the group comprising: a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, a fusion protein, or an aptamer molecule, a combination thereof, and fragments of each thereof.

The modulator may be a bi-antibody capable of binding to both of : IL-1R1 and NLRP3. Optionally, the modulator is a recombinant humanized bi-antibody capable of binding to both of: IL-1R1 and NLRP3.

Optionally, the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody capable of binding IL-1R1 and one or more of the binding regions of a second antibody capable of binding NLRP3. Optionally, the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody capable of binding IL-1R1 and one or more CDRs of a second antibody capable of binding NLRP3. Optionally, the first and/or second antibody is a monoclonal antibody.

Optionally, the modulator is selected from an antibody fragment capable of binding to both: IL-1R1 and NLRP3. Optionally, the antibody fragment is selected from one or more of Fab, Fv, Fab', (Fab')2, scFv, bis-scFv, minibody, Fab2, and Fab3.

Optionally, the modulator is selected from a recombinant humanized antibody or antibody fragment capable of binding to both of: IL-1R1 and NLRP3.

Optionally, the modulator is an antibody or antibody fragment raised against one or more antigens selected from both of IL-1R1 and NLRP3. Optionally, the modulator is raised against one or more antigens selected from all or part of both of IL-1R1 and NLRP3. Optionally, the modulator is raised against one or more antigens selected from NLRP3, optionally conjugated to a carrier protein such as Keyhole Limpet Haemocyanin (KLH) (hereinafter, the NLRP3 immunogen), and IL-1R1, optionally recombinant IL-1R1.

Optionally, the extracellular domain of IL-1R1 (hereinafter, the IL-1R1 immunogen) comprises the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).
(* or ** denotes a stop codon throughout this specification).

Optionally, the NLRP3 immunogen comprises the sequence:
EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

Optionally, the NLRP3 immunogen comprises a carrier protein conjugated to the sequence EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), optionally conjugated to the N-terminal end of the sequence EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

A carrier protein, conjugated to a peptide, is known in the art to help the peptide generate a stronger immune response. Optionally, the carrier protein is KLH.

Optionally, the carrier protein is conjugated to the sequence EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30) via a linker, optionally the linker is Hydrazide-Ahx.

Optionally, the NLRP3 immunogen is:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

As is understood in the art, a hydrazide is a class of organic compounds characterized by a nitrogen-nitrogen covalent bond with four substituents with at least one of them being an acyl group. Ahx denotes a 6-carbon linear aminohexanoic linker.

5 Optionally, the modulator is raisable, optionally raised, against one or more immunogens selected from NLRP3 immunogen and IL-1R1 immunogen, wherein the IL-1R1 immunogen comprises the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
10 ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
15 QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).
(* denotes a stop codon)
and the NLRP3 immunogen comprises the sequence:
20
KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

Optionally, the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:
25
MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
30 VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),
35 (* denotes a stop codon)

and one or more of the binding regions of a second antibody raised against NLRP3 immunogen comprising the sequence:

40 KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

Optionally, the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

5 MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
10 VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI SRSPGK* (SEQ ID NO: 1),
(* denotes a stop codon)

15

and one or more CDRs of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

20

Optionally, the first and/or second antibody is a monoclonal antibody.

Optionally, the consensus sequence of the heavy chain of the first antibody (to IL-1R1) is MGWVWNLPFLMAAAQSIQA**QIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGL**
25 **KWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVW** **GAGTTVTVSS**AKTTPPPVYPLA (SEQ ID NO: 7).

Optionally, the heavy chain CDRs of the first antibody comprise: **GYPFTTAG** (SEQ ID NO: 60)**;** **MNTQSEVP** (SEQ ID NO: 61)**;** and **AKSVYFNWRYFDV** (SEQ ID NO: 62).

30

Optionally, the consensus sequence of the light chain of the first antibody (to IL-1R1) is MRSPAQFLGLLLFWTSASRC**DIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRL** **IIKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELK**RADAAPT VSIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 12).

35

Optionally, the light chain CDRs of the first antibody comprise: **QSISDY** (SEQ ID NO: 63)**; YAS;** and **QHGHSFPLT** (SEQ ID NO: 64).

Optionally, the consensus sequence of the heavy chain of the second antibody (against NLRP3) is
40 MDFGLSWVFLVLVLKGVQC**EVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRL**

EWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSS FPYWGQGTLVTVSAAKTTPPSVYPLA (SEQ ID NO: 36).

Optionally, the heavy chain CDRs of the second antibody comprise: **GFTFSDYY** (SEQ ID NO: 65)**;** **ISDGGTYT** (SEQ ID NO: 66)**;** and **ARGWVSTMVKLLSSFPY** (SEQ ID NO: 67).

Optionally, the consensus sequence of the light chain of the second antibody (to NLRP3) is MAWISLLLSLLALSSGAIS**QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTG LIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVL**GQPKS SPSVTLFPPSTEELSL (SEQ ID NO: 43).

Optionally, the light chain CDRs of the second antibody comprise: **TGAVTTSNY** (SEQ ID NO: 68)**;** **GTN;** and **ALWYSNYWV** (SEQ ID NO: 69).

Optionally, the modulator is capable of binding simultaneously to IL-1R1 and NLRP3. Optionally, or additionally, the modulator is capable of binding sequentially to IL-1R1 and NLRP3.

Optionally, the light chain of a bi-specific antibody of the present invention has the amino acid sequence:
MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57).

Optionally, the heavy chain of a bi-specific antibody of the present invention has the amino acid sequence:
MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ PK** (SEQ ID NO: 59).

By “binding simultaneously” to both of IL-1R1 and NLRP3, it is meant that the modulator is capable of binding to each of IL-1R1 and / or NLRP3, whether said IL-1R1 and / or NLRP3 are formed as a complex, or whether they are not formed as a complex.

5 In a second aspect, the invention provides an NLRP3 inflammasome modulator as defined herein in relation to the first aspect of the invention for use in the treatment or prophylaxis of an inflammation-related disorder, optionally an inflammatory eye disease, such as glaucoma, as described in the first aspect of the invention, in which the NLRP3 inflammasome is known to play a key role in the disease pathogenesis.

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An advantage of the bispecific antibody as the modulator is that it can be used at lower, and thus less toxic, concentrations than single antibodies, therefore, reducing cytotoxicity potential. Being bi-specific allows for a more stable antibody with greater purity.

15 Being a biological has a longer half live thus confers a major advantage over small molecule inhibitors.

In a third aspect, the present invention provides a method for the treatment and/or prophylaxis of an inflammation-related disorder, optionally an inflammatory eye disease, such as glaucoma, the
20 method comprising the steps of:

providing a therapeutically effective amount of an NLRP3 inflammasome modulator as defined herein in relation to the first aspect of the invention which suppresses activation and/or signalling of the NLRP3 inflammasome, and
25 administering the therapeutically effective amount of said compound to a subject in need of such treatment.

In a fourth aspect, the present invention provides for use of an NLRP3 inflammasome modulator as defined herein in relation to the first aspect of the invention in the preparation of a medicament for the
30 treatment of an inflammation-related disorder, optionally an inflammatory eye disease, such as glaucoma.

In a fifth aspect, the present invention provides a method to reduce or prevent or treat at least one symptom of an inflammation-related disorder, optionally an inflammatory eye disease, such as
35 glaucoma, in a subject comprising selectively inhibiting and / or reducing activation of the inflammasome pathway by the use of an NLRP3 inflammasome modulator as defined herein in relation to the first aspect of the invention.

Optionally, the modulator is for use in the treatment or prevention of at least one symptom of an
40 inflammation-related disorder in a subject comprising selectively inhibiting and or reducing activation of the inflammasome pathway by the use of the modulator.

Optionally, the light chain of a bi-specific antibody has the amino acid sequence of SEQ ID NO: 57 and the heavy chain of a bi-specific antibody the amino acid sequence of SEQ ID NO: 59 and may be referred to herein as InflaMab or Inflamab.

Optionally, InflaMab may have disease modifying effects in systemic conditions such as but not limited to Atherosclerosis, whereby it prevents/inhibits inflammation therefore preventing plaque build up and/or plaque rupture thus reducing risk of myocardial infarction.

Optionally, InflaMab may have disease modifying effects in eye diseases such as but not limited to Glaucoma, whereby it prevents/inhibits inflammation, reduces intraocular pressure and / or prevents loss of retinal ganglion cells and axons, protecting the optic nerve and preserving visual acuity, and/or preventing blindness.

Optionally, InflaMab may have disease modifying effects in neurological conditions such as but not limited to Alzheimer’s Disease, whereby it prevents/inhibits inflammation, reduces/inhibits amyloid plaque load, and/or prevents of cognitive dysfunction.

The modulator as defined herein may have utility in individuals with multi-morbidities or co-morbidities associated with inflammation.

Optionally, the modulator as defined in relation to of any of the aforementioned aspects of the invention, denoted as Inflamab, is a 210 kiloDalton (kDa) bispecific mouse antibody composed of two pairs of light chain and two pairs of heavy chains with scFv domains fused to the N-terminal, complexed together via disulphide bonds.

As used herein, an “inflammation-related disorder” includes, but is not limited to, Atherosclerosis, inflammatory eye conditions such as Age-Related Macular degeneration, Dry Eye Syndrome, Glaucoma, Sjogren’s syndrome, Diabetes, Inflammatory eye disease, Depression, Alzheimer’s Disease, Parkinson’s Disease, Inflammatory Bowel Disease, Rheumatoid Arthritis, Ageing, Dermatological conditions and Cancer.

Optionally, the subject is a mammal, such as a human.

The term “antibody” should be construed as covering any binding member or substance having a binding domain with the required specificity. The antibody of the invention may be a monoclonal antibody, or a fragment, functional equivalent or homologue thereof. The term includes any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included.

Fragments of a whole antibody can perform the function of antigen binding. Examples of such binding fragments are; a Fab fragment comprising of the VL, VH, CL and CH1 antibody domains; an Fv fragment consisting of the VL and VH domains of a single antibody; a F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments; a single chain Fv molecule (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site; or a bi- or tri-specific antibody, which may be multivalent or multispecific fragments constructed by gene fusion.

A fragment of an antibody or of a polypeptide for use in the present invention, generally means a stretch of amino acid residues of at least 5 to 7 contiguous amino acids, often at least about 7 to 9 contiguous amino acids, typically at least about 9 to 13 contiguous amino acids, more preferably at least about 20 to 30 or more contiguous amino acids and most preferably at least about 30 to 40 or more consecutive amino acids.

The term "antibody" includes antibodies which have been "humanized". Methods for making humanised antibodies are known in the art.

Aptamers are peptide molecules that bind to specific target molecules. Aptamers are in the realm between a small molecule and a biologic. They exhibit significant advantages relative to antibody therapeutics in terms of size, synthetic accessibility and modification.

Modulators as described herein may be used in assays, such as ELISAs, to detect NLRP3 from human blood or tissue samples. Thus, in a further aspect, the present invention provides a kit comprising one or more modulators of the first aspect of the invention. Optionally, the kit further comprises instructions for use of said kit. Optionally, the kit is for detecting NLRP3 in human cells, in blood or tissue samples.

In the drawings:

Figure 1: 4-20% denaturing, reducing and non-reducing, SDS-PAGE analysis of IL-1R1 FC. Molecular weight marker shown in kiloDaltons.
Figure 2: UUC IL-1R *1st Bleed.*
Figure 3: UUC IL-1R *2nd Bleed.*
Figure 4: *Post Fusion Screening Results.*
Figure 5: *1st Protoclones 24 well.*
Figure 6: *LD1 Screening Results.*
Figure 7: *24 Well-Plate Screening Results.*
Figure 8: *Final Selected Hybridomas from F237 5D1-1A8.*
Figure 9: *Final Selected Hybridomas from F237 5D1-1A8 final 24w Screening.*

Figure 10: IL-1R1 Internalisation in THP1 cells- immunofluorescence imaging. Fluorescence microscopic images taken from THP1 macrophages treated with LPS and ATP to induce the expression of the IL-1R1.

Figure 11: IL-1R1 Internalisation in THP1 cells- flow cytometry.

5 **Figure 12:** PCR using several combinations of Ig variable domain primers.

Figure 13: Graphical representation of the CDR loops. Ref: Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003) PMID: 12477501).

Figure 14: Graphical representation of the CDR loops (Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003) PMID: 12477501).

10 **Figure 15**: Structure of NLRP3 inflammasome. Bergsbaken, T.; Fink, S. L.; Cookson, B. T. (2009). "Pyroptosis: Host cell death and inflammation". *Nature Reviews Microbiology*. **7** (2): 99–109. doi:10.1038/nrmicro2070. PMC 2910423 . PMID 19148178. and Dagenais, M.; Skeldon, A.; Saleh, M. (2011). "The inflammasome: In memory of Dr. Jurg Tschopp". *Cell Death and Differentiation*. 19 (1): 5–12. doi:10.1038/cdd.2011.159. PMC 3252823 . PMID 22075986.

15 http://jonlieffmd.com/blog/cellular-intelligence-blog/inflammasomes-are-large-complex-signaling-platforms

Figure 16: Sequence alignment using CLUSTAL 0 (1.2.4) of the consensus sequences of C-term domains of human and mouse NALP (NLRP) proteins.

Figure 17: Novafold predicted structure of Peptide FUS_746_001 (Yellow) aligned to NLRP3 PDB:
20 3QF2 showing secondary structural features using Protean 3D, version 14.0.1

Figure 18: Immunized mice expressed high levels of the NLRP3 mAb.

Figure 19: UUC NLRP3 *1st Bleed.*

Figure 20: UUC NLRP3 *2nd Bleed.*

Figure 21: *Post Fusion Screening Results.*

25 **Figure 22**: *1st Protoclones 24 well.*

Figure 23: *LD1 Screening Results.*

Figure 24: *24 Well-Plate Screening Results.*

Figure 25: *Final Selected Hybridomas from F226.*

Figure 26: Dot Blot analysis.

30 **Figure 27**: Western Blot Analysis.

Figure 28: PCR using several combinations of Ig variable domain primers.

Figure 29: Graphical representation of the CDR loops (Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003) PMID: 12477501).

Figure 30: Graphical representation of the CDR loops (Lefranc, M.-P. et al., Dev. Comp. Immunol.,
35 27, 55-77 (2003) PMID: 12477501).

Figure 31: Diagram illustrating the bispecific design and the plasmid map of InflaMab.

Figure 32: 4-20% SDS-PAGE analysis of InflaMab. Molecular weight marker shown in kiloDaltons.

Figure 33: Inflamab prevents IL-1β release. (Note, “Ulster Ab” is synonymous with “Inflamab” and “Bi-specific Ab”.)

40 **Figure 34:** Inflamab prevents caspase-1 activation in THP1 cells.

Figure 35: Internalization of Inflamab.

Figure 36: Constitutive expression of NLRP3 in the mouse and human ONH.

Figure 37: Constitutive NLRP3 expression in the astrocytes of the human ONH.

Figure 38: Assembly of the NLRP3 inflammasome in the ONH coincides with the induction of inflammatory mediators at 7 days post microbead injection.

Figure 39: Early induction of inflammatory mediators and accumulation of Iba1+ cells in the ONH is abrogated in inflammasome deficient (ASC KO) mice.

Figure 40: ASC and NLRP3 are required for IOP-induced axon degeneration and death of RGCs in microbead-induced mouse model of glaucoma.

Figure 41: NLRP3 small molecule inhibitor, MCC950, prevents death of RGCs in microbead model of glaucoma.

Figure 42: InflaMab prevents death of RGCs in microbead model of glaucoma.

In a particular use or method of treatment, the modulator of the invention, e.g. the bi-specific antibody, acts according to steps which include:

1. Targeting the bispecific antibody to IL-1R1 to allow internalisation and entry of the antibody into the cell.
2. Targeting the antibody to NLRP3 in order to inhibit NLRP3 inflammasome assembly and subsequent IL-1β release from the cell, thus reducing inflammation.
3. Targeting the antibody to IL-1R1 triggers internalisation of the IL-1R1, thus making less IL-1R1 available for IL-1β binding resulting in further inhibiting the potentiation and amplification of inflammation.

Such a modulator of the first aspect of the invention provides a surprisingly additive inhibitory effect upon the inflammasome as a whole, not only the NLRP3 protein portion and thus will provide a more effective inhibitor of inflammasome-related diseases.

Examples

- Transient Expression of IL-1R1 FC fusion (Example 1)
- Generation of a monoclonal antibody against IL-1R1 (Example 2)
- IL-1R1 monoclonal antibody sequencing report (Example 3)
- NLRP3 peptide synthesis (Example 4)
- Generation of a monoclonal antibody against NLRP3 (Example 5)
- NLRP3 monoclonal sequencing report (Example 6)
- InflaMab design (Example 7)
- InflaMab transient expression (Example 8)
- InflaMab for Atherosclerosis/Coronary Artery Disease (Example 9)

<u>Example 1:</u> Transient Expression of IL-1R1 Fc fusion

IL-1R1 Fc is transiently expressed and purified in HEK293 cells. The purified protein is evaluated for size and purity by SDS PAGE and tested for endotoxin levels. Finally the protein is evaluated for activity by ELISA.

A mammalian expression vector encoding interleukin-1 receptor (IL-1R1) Fc fusion protein was transfected into HEK293 cells. The expressed Fc fusion protein was subsequently purified from cell culture supernatant using standard chromatography techniques. The concentration and purity were determined for the purified product.

Transient Transfection of HEK293 cells and purification of protein

DNA coding for the amino acid sequence of IL-1R1 Fc (**see Example 1A**) was synthesised and cloned into a mammalian transient expression plasmid pD2610-v1 (DNA2.0). IL-1R1 Fc was expressed using a HEK293 cell based transient expression system and the resulting antibody containing cell culture supernatants was clarified by centrifugation and filtration. Two lots of IL-1R1 Fc were purified (using AKTA chromatography equipment) from cell culture supernatants via protein A affinity chromatography. Purified protein was dialysed/buffer exchanged into phosphate buffered saline solution. The purity of the recombinant protein was determined to be >95%, as judged by Sodium Dodecyl Sulphate Polyacrylamide gels (**Figure 1)**. Protein concentration was determined by measuring absorbance (1.0 mg/ml = A280 of 1.37). Details of the purified product are summarized in **Table 1**.

Figure 1 shows 4-20% denaturing, reducing and non-reducing, SDS-PAGE analysis of IL-1R1 FC. Molecular weight marker shown in kiloDaltons. Lanes are as follows:

Lane Number	Sample	Lot	Amount (µg)	Conditions
1	*See Blue plus 2 (Thermo Fisher)*	-	-	Reducing
2	IL-1R1 FC	1	-	Reducing
3	Blank	-	-	NA
4	IL-1R1 FC	2	-	Reducing

Table 1: Purification summary: IL-1R1 Fc

Sample	Lot	Concentration (mg/ml)	Volume (ml)	Total (mg)	Purity	Endotoxin (EU/mg)
IL-1R 1Fc	1	0.64	1.6	1.02	>95%	ND
IL-1R1 Fc	2	0.95	1.4	1.33	>95%	ND

Abbreviations are as follows; ND, not determined.

<u>Example 1A:</u> IL-1R1 Fc amino acid sequence

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1)

Example 2: **Generation of a monoclonal antibody against IL-1R1**

The aim of this project is to generate a monoclonal antibody against IL-1R1. A population of 5 mice were immunised and screened for positive immune responses. After selecting a suitable candidate for fusion, splenocytes were fused with partner cells to produce a population of hybridomas. This population underwent a series of limiting dilutions and screening assays to produce fully monoclonal cell lines.

Cell Line Nomenclature

The product name “F237 5D1-1A8-2A5” refers to one of the 10 chosen monoclonal hybridoma cell lines. The name is comprised of components describing the production pathway at each stage. Each hybridoma selected from the post-fusion screening and each limiting dilution was given a number corresponding to the plate number and well location on that plate for which the hybridoma was chosen (i.e. 5D1-1A8-2A5). This nomenclature traces the derivation of each individual hybridoma allowing for clear differentiation in the screening process.

Abbreviations

Ab	Antibody
DMSO	Dimethyl Sulfoxide
FCS	Fetal Calf Serum
ELISA	Enzyme-Linked Immunosorbent Assay
RT	Room Temperature
OD	Optical Density
PBST	Phosphate-buffered saline + 1% Tween 20
PBS	Phosphate-buffered saline
RPM	Revolutions per minute
NP, LP, RP, LRP, 2LP	Mouse Identification: No Punch, Left Punch, Right Punch Left/Right Punch, 2 Left Punches
HAT	Hypoxanthine, Aminopterin, Thymidine supplement
HATR Media[1]	DMEM supplemented with 2% Roche (HFCS), 2% HAT, 1% Pen/Strep, 1% L-Glutamine
SFM	Serum Free Medium
PEG	Polyethylene Glycol
GAM-HRP	Goat Anti-Mouse-Horse Radish Peroxidase
HT	Hypoxanthine and Thymidine
LD1	First Limiting Dilution

LD2	Second Limiting Dilution

[1] This is the media that was used for all cultures following fusion and screening.

Materials

5 **Reagents and Media**

Reagent	Supplier	Catalogue No.
L-glutamine	Gibco	25030-024
HAT Supplement	Gibco	21060-017
HFCS	Roche	11363735001
DMEM Glutamax	Gibco	61965-059
Penicillin/Streptomycin	Gibco	15140-122
FCS	Gibco	16000.044
DMSO	Sigma	D2650
Trypan Blue	Sigma	T8154
PEG	Sigma	10783641001
Freund's Adjuvant Complete	Sigma	F5881
Freund's Adjuvant Incomplete	Sigma	F5506
Sodium Hydrogen Carbonate	VWR	27778.260
Sodium Carbonate	Sigma	S2127-500G
Powdered Milk	Marvel	Original Dried Skimmed
Tween 20 (10%)	Sigma	P1379-1L
GAM-HRP (Fc Specific)	Sigma	A2554
TMB	Biopanda	TMB-S-002
Mr. Frosty	Nalgene	55710-200

Disposables

Name	Supplier	Catalogue No.
25cm^2 static flasks	Corning	430639
75cm^2 static flasks	Corning	430641
96-well plate sterile	Corning	3595
96-well plate sterile TPP	Primer Scientific	92696T
Cryovials	Fisher Scientific	366656
Maxi Sorb 96- well plates	Nunc	442404

Equipment

10
- CO_2 Cell culture static incubators (SANYO)
- Plate reader Sunrise (Tecan)
- Centurion Scientific K40R Centrifuge
- Grant-Bio Multishaker PSU 20

15

Methods

Antigen Preparation

Once the immunogen (IL-1R1) was purified, these solutions were diluted to 200 µg/ml in sterile, EF-
20 PBS and aliquoted in volumes of 600 µl for immunisation and 150 µl for boosts and ELISA screening. These aliquots were labelled and stored at -20°C.

Immunisations

A population of 5 BalbC mice were immunised subcutaneously with 200 µl of a 1:1 emulsion of Freund's Adjuvant Complete (Sigma) and a 600 µl aliquot of IL-1R1 prepared herein. Two weeks after the 1st immunisation, the population was immunized with a 2nd injection at the same volumes and concentrations as the original injection only using Freund's Adjuvant Incomplete (Sigma) instead. One week after the 2nd immunisation, the mice were tagged by ear punches (NP, RP, LP, LRP, 2LP), and test bleeds were screened as described herein for preliminary results. Three weeks after the 2nd immunisation, the population was immunised a 3rd time using the same method as the 2nd injection. One week after the 3rd immunisation, test bleeds were screened, and the mouse with an ear tag of RP was then selected for fusion.

Test Bleed ELISAs

Tail bleeds were taken from the population of 5 BalbC mice and centrifuged at 8000 rpm for 10 min at RT (room temperature). The blood serum from each mouse was collected, loaded onto the plate the same day as screening, and stored at -20°C. This screening was performed twice for the selection of a suitable mouse for fusion.

The day prior to screening, a Maxi Sorp plate was coated by adding 100 µl/well of 50 mM sodium carbonate coating buffer (pH 9.5) containing the IL-1R1 at 1 µg/ml. A separate coating solution was prepared by diluting APO-A1 in the same coating buffer at 1 µg/ml. These solutions were loaded onto the plate in alternating rows so as to provide two wells to load each sample that demonstrates a positive and negative result. This plate was incubated overnight at 4°C in static conditions.

The following morning, coating buffer was removed, and 200µl/well of blocking solution (4.0% w/v semi skim milk powder, 1x PBS) was added and agitated at 150 rpm for 2 hr at RT. The plate was washed three times with PBS-T (0.1% v/v Tween 20). PBS was loaded into each well at 100 µl/well, and 1 µl of each test bleed serum was loaded into each positive and negative well. The plate was incubated at 150 rpm (Grant Shaker) for 2 hrs at room temperature. These samples were then removed and washed four times with PBS-T. 100 µl/wel,l GAM-HRP diluted 1:5000 (Sigma, UK) was added, and the plate was incubated for 1 hr with agitation at 150 rpm at RT. The secondary antibody was removed, and the plate was washed four times with PBS-T and once in PBS. 100 µl/well of TMB substrate solution was added and incubated at 37°C for 10 minutes. 50 µl 1M HCl was added per well and the plate immediately read at 450 nm on a Tecan Sunrise plate reader.

After the second test bleed ELISA screening, the mouse with an ear tag of RP was selected for fusion by expressing the most positive immune response.

Boost Injections

One week after the 3rd and final immunization, a boost injection was given to BalbC mouse RP by injecting 100 µl of aliquoted IL-1R1 at 200 µg/ml without any adjuvant.

Fusion F237

One week before fusion, SP2 cells were broken out from liquid nitrogen and were passaged in 10% FCS DMEM supplemented with 1% Pen/Strep, 1% L-glutamine until 3x12 ml T75 flasks were 75%-90% confluent on the day of fusion. On the day of the fusion, SP2 cells were dislodged by tapping the flask and were centrifuged at 1000 rpm for 5 min at 37°C. The cells were resuspended in 20 ml SFM DMEM, centrifuged again, and resuspended in 10 ml SFM DMEM. SP2 cells were stored in a Sterilin tube in SFM at 37°C, 6% CO_2 until needed.

After euthanasia, the spleen was aseptically removed from the mouse that showed the strongest immune response. Splenocytes were extracted by puncturing both ends of the spleen with a fine gauge needle and flushing 10-15 ml SFM DMEM. Splenocytes were transferred to a sterilin tube and washed twice with 20 ml serum free DMEM by centrifugation at 1300 rpm for 5 min at 37°C and gently removing the supernatant. The splenocytes were resuspended in 10 ml Serum free DMEM in a sterilin tube.

Using the SP2 cells stored at 37°C, the SP2 cells were added to the splenocytes. This SP2/splenocytes culture was centrifuged at 1300 rpm for 5 min at 37°C. After discarding the supernatant, 1 ml PEG was added to the SP2/splenocytes culture dropwise while stirring continuously over a period of 3 min. 1 ml SFM DMEM was added to the fusion mixture and stirred for 4 min. 10 ml SFM DMEM was added dropwise to the fresh culture and incubated for in 37°C water bath for 5 min. The cells were then centrifuged at 1000 rpm for 5 min at 37°C. The pellet was resuspended in 200 mL HATR media and was plated at 200 µl/well in 10x 96 well culture plates which were incubated 11 days at 37°C in 6% CO_2 prior to screening.

Post-Fusion Screening and Post-LD Screening

Eleven days after fusion, protoclones were screened by ELISA. 20x Maxi Sorp 96 well plates were coated as described herein using APO-A1 at 1 µg/ml as the negative control for specificity. The coating solution was removed and the plates were blocked as described herein. Samples were prepared by removing 160 µl of supernatant from each well of the ten fusion plates, limiting dilution plates, or 24-well plates and transferring to fresh 96 well culture plates containing 50 µl 1xPBS. After 2 hours of blocking, the blocking solution was removed, and the plates were washed 3x with PBS-T. The samples from each dilution plate were loaded onto the ELISA plates at 100 µl/well by adding 1 row from each dilution plate per 2 rows on the ELISA plates to account for specificity of the coating antigens. Two wells per ELISA were incubated with 100 µl 1xPBS as a negative control. These samples were incubated at 150 rpm for 2 hours at room temperature.

Limiting Dilutions

Once the hybridoma populations were expanded in 24-well plates and growing well, a secondary screen was performed to select the most specific and highest producing populations for rounds of limiting dilutions.

Both limiting dilutions were performed for 1-3 protoclones each by seeding 2-4x 96-well plates at 1 cell/ well in 200 µl culture/well. The plates were prepared by counting each culture in the 24-well plate and were diluted 10x as an intermediate dilution, then were diluted to 200 cells in 40 ml. The culture was plated at 200 µl/well and left to incubate at 37°C, 6% CO_2 for 7-10 days until the wells were 80%-90% confluent. Each well for both limiting dilutions were screened by ELISA as described herein.

Final Clone Selection

Following the second limiting dilution, 10 clones were selected for expansion in a 24 well plate. Each clone was left to grow in 37°C, 6% CO_2 for 6 days until each well became 80%-90% confluent. When the clones were well established in the 24-well plates, each clone at 1 ml/well was transferred to a T25 flask containing 5 ml fresh 10% HATR DMEM for cryopreservation.

Cryopreservation of Monoclonal Cell Lines

Once the clones were well established (80%-90% confluency) in T25 flasks, each 5 ml culture was centrifuged at 1000 rpm for 5 min at 37°C and was resuspended in 1 ml of fresh 10% DMEM HATR media. Each 1 ml culture was transferred to a cryovial containing 300 µl of a 1:1 ratio of FCS to DMSO. The vials were sealed and placed in a Mr. Frosty and transferred to the -70°C freezer for short-term storage.

Cell Preparation for Sequencing

Anti-IL-1R1 produced from clone F237 5D1-1A8-2A5 was selected for sequencing. Once the culture was confluent in the T25 flask, the supernatant was discarded. The cells were dislodged by cell scraping into 2 ml fresh media and were centrifuged at 7,600 rpm for 5 min at RT. The supernatant was then discarded and the pellet was flash frozen in liquid nitrogen and placed in -70°C until ready for mRNA extraction.

Immunisation and Screening of Test Bleeds

A colony of mice were immunised with an IL-1R1 immunogen (produced in house in CHO cells) and regular test bleeds were taken over an 11 week period. Test bleeds were screened for IL-1R1 mAb expression levels using ELISA and internalisation capability using the pHrodo fluorescent assay (Thermo Fisher Scientific, UK https://www.thermofisher.com/order/catalog/product/P35369 and https://www.sigmaaldrich.com/catalog/product/sigma/m4280?lang=en®ion=GB).

Results

Test Bleed 1

One week after the 2nd immunisation, a tail bleed was taken from each of the 5 mice and screened against IL-1R and APO-A1 for determination of a suitable animal for fusion and a relative specificity of the polyclonal antibody produced – see Figure 2.

Test Bleed 2

After screening sera from tail bleeds, the mouse with an ear tag of RP was selected for the fusion of its splenocytes to fusion partner SP2 culture as it demonstrated the best immune response – see Figure 3.

Post-Fusion Screening

Once the wells in each plate had reached 70%-80% confluency, the plates were screened by ELISA against IL-1R1 and APO-A1. The hybridoma population producing the highest responses were selected for expansion in a 24-well plate – see Figure 4.

1st 24-Well Plate Screening

Clones were selected from the post-fusion screening and were arrayed into a 24 well plate for expansion followed by a secondary screening that determines suitable protoclones for the first round of limiting dilutions – see Figure 5.

Limiting Dilution 1 Screening

Once the 1st limiting dilution plates were confluent, the limiting dilution was screened by ELISA against IL-1R1 and APO-A1. Eleven hybridoma populations were selected from F237 2H12, F237 5D1, and F237 7E6 that demonstrated the highest and most specific response – see Figure 6.

2nd 24-Well Plate Screening

When the clones became confluent in the 24-well plate, each clone was screened by ELISA against IL-1R1 and APO-A1. F237-5D1-1A8 was selected for the 2nd round of limiting dilution over 4x 96 well plates – see Figure 7.

Limiting Dilution 2 Screening

Once the wells in each plate had reached 70%-80% confluency, the plates were screened by ELISA against IL-1R1 and APO-A1. The hybridoma population producing the highest response and highest specificity were selected for expansion in a 24-well plate and cryopreservation – see Figure 8.

IL-1R1 Internalisation in THP1 cells was immunofluorescence imaged

Fluorescence microscopic images taken from THP1 macrophages treated with LPS and ATP to induce the expression of the IL-1R1 – see Figure 10. The cells were incubated with mouse serum from several different mice, containing the test antibody against the IL-1R1, which was conjugated to a pHrodo™ dye (that will only fluoresce within a cell). Strong IL-1R1 immunoreactivity was observed in the nucleus and cytoplasm of the THP1 cells. IL-1R1 and DAPI staining at X40 magnification. No staining was observed in the secondary antibody only treated control cells. Images are from four different wells used in two different experiments. The best mouse was selected to take forward to the fusion hybridoma and cloning stages.

THP1 macrophages (see Figure 11) treated with LPS and ATP to induce the expression of the IL-1R1. The cells were incubated with mouse serum from several mice containing the test monoclonal antibody against the IL-1R1, which was conjugated to a pHrodo dye (that will only fluoresce within a cell) and analysed with flow cytometry. More fluorescence was seen in the IL-1R1 antibody treated cells (i) as compared to the control secondary antibody only treated cells (ii). Using this data and that from Figure 3, the best mouse was chosen to take forward to the fusion hybridoma and cloning stages.

Conclusions

The aim of the project was to produce a range of antibodies against IL-1R1. Once the mice were immunised and screened, RP was selected for fusion. 10 monoclonal hybridoma cell lines were produced from two rounds of limiting dilutions. Each population was selected by highest production and highest specificity for IL-1R1. These final cell lines have been frozen down, and the antibody expressed by this cell line will be sequenced.

Example 3: IL-1R1 monoclonal antibody sequencing

mRNA was extracted from the hybridoma cell pellets. Total RNA was extracted from the pellets using a conventional RNA extraction protocol. Cell pellets were homogenised using RNA STAT-60 reagent. Upon addition of chloroform, the homogenate separated into an aqueous phase and an organic phase, and total RNA was isolated in the aqueous phase. Isopropanol was used to precipitate the RNA, followed by ethanol washes and solubilisation in water.

RT-PCR

cDNA was created from the RNA by reverse-transcription with an oligo(dT) primer. PCR reactions are set up using variable domain primers to amplify both the VH and VL regions of the monoclonal antibody DNA giving the following bands – see Figure 12.

The VH and VL products were cloned into the Invitrogen sequencing vector pCR2.1 and transformed into TOP10 cells and screened by PCR for positive transformants. Selected colonies were picked and analyzed by DNA sequencing on an ABI3130xl Genetic Analyzer, the result may be seen below.

Sequencing Results

Heavy Chain

V_H Amino Acid Sequence Alignment:

```
                   1                                                50
      VH1.1   (1)  MEWSCVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH1.4   (1)  MECSCVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISRKASGYPFTT
      VH1.3   (1)  MGWSWVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH2.1   (1)  MGWVWNLLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH2.5   (1)  MGWVWTLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH2.3   (1)  MGWVWNLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH1.2   (1)  MDWVWTLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
      VH2.4   (1)  MDWLWNLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
  Consensus   (1)  MGWVWNLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTT
                   51                                              100
      VH1.1  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH1.4  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH1.3  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH2.1  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH2.5  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH2.3  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH1.2  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
      VH2.4  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
  Consensus  (51)  AGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYL
                   101                                             150
      VH1.1 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
      VH1.4 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPSVFPL
      VH1.3 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
      VH2.1 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
      VH2.5 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
      VH2.3 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
      VH1.2 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPSVYPL
      VH2.4 (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
  Consensus (101)  QINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSSAKTTPPPVYPL
                   151
      VH1.1 (151)  A
      VH1.4 (151)  A
      VH1.3 (151)  A
      VH2.1 (151)  V
      VH2.5 (151)  V
      VH2.3 (151)  A
      VH1.2 (151)  A
      VH2.4 (151)  A
  Consensus (151)  A
```

VH1.1 (SEQ ID NO: 2)

VH1.4 (SEQ ID NO: 3)

5 VH1.3 (SEQ ID NO: 4)

VH2.1 (SEQ ID NO: 5)

VH2.5 (SEQ ID NO: 6)

VH2.3 (SEQ ID NO: 7)

VH1.2 (SEQ ID NO: 8)

10 VH2.4 (SEQ ID NO: 9)

Consensus (SEQ ID NO: 7)

Key to amino acid shading:

Black non-similar residues

Blue on cyan	consensus residue derived from a block of residues at a given position
Black on green	residues similar in structure to consensus residue or each other when no consensus found
Red on yellow	consensus residue derived from a completely conserved residue at a given position
Green	residue weakly similar to consensus residue at given position

V_H Consensus Amino Acid Sequence:

MGWVWNLPFLMAAAQSIQA**QIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKWIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGTTVTVSS**AKTTPPPVYPLA (SEQ ID NO: 7)

The variable domain is highlighted in **BOLD.**
The Complementarity Determining Regions (CDRs) are underlined as determined by the IMGT numbering system (Lefranc, M.-P. et al., Nucleic Acids Research, 27, 209-212 (1999)) – see Figure 13.

Key to amino acid shading, in Figure 13:
Blue shaded circles are hydrophobic (non-polar) residues in frameworks 1-3 at sites that are hydrophobic in the majority of antibodies.
Yellow shaded circles are proline residues.
Squares are key residues at the start and end of the CDR.
Red amino acids in the framework are structurally conserved amino acids.

Light Chain

V_L Amino Acid Sequence Alignment:

```
                    1                                                50
     VK1.1     (1) MRAPAQFLGLLLLWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
     VK1.5     (1) MRAPAQLLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
     VK1.3     (1) MRSPAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
     VK1.4     (1) MRSPAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
     VK2.1     (1) MVSSAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
     VK2.6     (1) MVSTAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
 Consensus     (1) MRSPAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSIS
                    51                                              100
     VK1.1    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
     VK1.5    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLNINSVEP
     VK1.3    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
     VK1.4    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
     VK2.1    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
     VK2.6    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
 Consensus    (51) DYLSWYQQRSHESPRLIIKYASQSISGIPSRFSGSGSGSDFTLSINSVEP
                    101                                             150
     VK1.1   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
     VK1.5   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
     VK1.3   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
     VK1.4   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
     VK2.1   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
     VK2.6   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
 Consensus   (101) EDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTVSIFPPSSEQLTSGGA
                    151      162
     VK1.1   (151) SVVCFLNNFYPK
     VK1.5   (151) SVVCFLNNFYPK
     VK1.3   (151) SVVCFLNNFYPK
     VK1.4   (151) SVVCFLNNFYPR
     VK2.1   (151) SVVCFLNNFYPK
     VK2.6   (151) SVVCFLNNFYPR
 Consensus   (151) SVVCFLNNFYPK
```

VK1.1 (SEQ ID NO: 10)

VK1.5 (SEQ ID NO: 11)

5 VK1.3 (SEQ ID NO: 12)

VK1.4 (SEQ ID NO: 13)

VK2.1 (SEQ ID NO: 14)

VK2.6 (SEQ ID NO: 15)

Consensus (SEQ ID NO: 12)

10 Key to amino acid shading:

Black non-similar residues

Blue on cyan consensus residue derived from a block of residues at a given position

Black on green residues similar in structure to consensus residue or each other
15 when no consensus found

Red on yellow consensus residue derived from a completely conserved residue at a given position

Green residue weakly similar to consensus residue at given position

20 V_L Consensus Amino Acid Sequence:

MRSPAQFLGLLLFWTSASRC**DIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRL**
IIKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPT
VSIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 12)

The variable domain is highlighted in **BOLD.**

The Complementarity Determining Regions (CDRs) are underlined as determined by the IMGT numbering system (Lefranc, M.-P. et al., Nucleic Acids Research, 27, 209-212 (1999)) – see Figure 14.

Key to amino acid shading, in Figure 14:
Blue shaded circles are hydrophobic (non-polar) residues in frameworks 1-3 at sites that are hydrophobic in the majority of antibodies.
Yellow shaded circles are proline residues.
Squares are key residues at the start and end of the CDR.
Red amino acids in the framework are structurally conserved amino acids.

VH Sequencing results:

VH1.1 DNA Sequence:
ATGGAATGGAGCTGTGTCATGCTCTTTCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
CCTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTTTATCCAC
TGGCC(SEQ ID NO: 16)

VH1.1 Amino Acid Sequence:
MEWSCVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
GTTVTVSSAKTTPPPVYPLA (SEQ ID NO: 2)

VH1.3 DNA Sequence:
ATGGGATGGAGCTGGGTCATGCTCTTTCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
CTTCTGGGTATCCCTTCACAACTGCTGGACTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT

GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTTTATCCCT
TGGCC (SEQ ID NO: 17)

VH1.3 Amino Acid Sequence:
5 MGWSWVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
GTTVTVSSAKTTPPPVYPLA (SEQ ID NO: 4)
VH1.4 DNA Sequence:
ATGGAATGCAGCTGTGTAATGCTCTTTCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
10 AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCCGCAAGG
CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
15 GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCATCCGTCTTCCCCC
TGGCA (SEQ ID NO: 18)

VH1.4 Amino Acid Sequence:
MECSCVMLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISRKASGYPFTTAGLQWVQKMSGKGLK
20 WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
GTTVTVSSAKTTPPSVFPLA (SEQ ID NO: 3)

VH2.1 DNA Sequence:
ATGGGTTGGGTGTGGAACTTGCTATTCCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
25 AGCTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
30 GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTCTATCCAC
TGGTC (SEQ ID NO: 19)

VH2.1 Amino Acid Sequence:
MGWVWNLLFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
35 WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
GTTVTVSSAKTTPPPVYPLV (SEQ ID NO: 5)

VH1.2 DNA Sequence:
ATGGATTGGGTGTGGACCTTGCCATTCCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
40 AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT

GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCATCTGTCTATCCAC
5 TGGCC (SEQ ID NO: 20)

VH1.2 Amino Acid Sequence:
MDWVWTLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
10 GTTVTVSSAKTTPPSVYPLA (SEQ ID NO: 8)

VH2.3 DNA Sequence:
ATGGGTTGGGTGTGGAACTTGCCATTCCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
15 CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTACCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGCACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTCTATCCAT
20 TGGCC (SEQ ID NO: 21)

VH2.3 Amino Acid Sequence:
MGWVWNLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
25 GTTVTVSSAKTTPPPVYPLA (SEQ ID NO: 7)

VH2.4 DNA Sequence:
ATGGATTGGCTGTGGAACTTGCCATTCCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
30 CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCAACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTCTATCCAC
35 TGGCC (SEQ ID NO: 22)

VH2.4 Amino Acid Sequence:
MDWLWNLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
40 GTTVTVSSAKTTPPPVYPLA (SEQ ID NO: 9)

VH2.5 DNA Sequence:
ATGGGTTGGGTGTGGACCTTGCCATTCCTCATGGCAGCAGCTCAAAGTATCCAAGCACAGATCC
AGTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAGGATCTCCTGCAAGG
CTTCTGGGTATCCCTTCACAACTGCTGGATTGCAGTGGGTACAGAAGATGTCAGGAAAGGGTTT
5 GAAATGGATTGGCTGGATGAACACCCAGTCTGAAGTGCCAAAATATGCAGAAGAGTTCAAGGGA
CGGATTGCCTTCTCTTTGGAAACCGCTGCCAGTACTGCATATTTACAGATAAACAACCTCAAAAC
TGAGGACACGGCGACGTATTTCTGTGCGAAATCGGTCTATTTTAACTGGAGATATTTCGATGTCT
GGGGTGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACACCCCCACCCGTCTATCCCC
TGGTC (SEQ ID NO: 23)

10

VH2.5 Amino Acid Sequence:
MGWVWTLPFLMAAAQSIQAQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLK
WIGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGA
GTTVTVSSAKTTPPPVYPLV (SEQ ID NO: 6)

15

VL Sequencing Results:

VK1.1 DNA Sequence:
ATGAGGGCCCCTGCTCAGTTTCTTGGGCTTTTGCTTCTCTGGACTTCAGCCTCCAGATGTGACA
20 TTGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTG
CAGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCA
AGGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTG
GATCAGGGTCAGACTTCACTCTCAGTATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTA
CTGTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACG
25 GGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGT
GCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGA (SEQ ID NO: 24)

VK1.1 Amino Acid Sequence:
MRAPAQFLGLLLLWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLI
30 IKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 10)

VK1.3 DNA Sequence:
ATGAGGTCCCCTGCTCAGTTCCTTGGGCTTTTGCTTTTCTGGACTTCAGCCTCCAGATGTGACAT
35 TGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTGC
AGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCAA
GGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTGG
ATCAGGGTCAGACTTCACTCTCAGTATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTACT
GTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACGGG
40 CTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGC
CTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAA (SEQ ID NO: 25)

VK1.3 Amino Acid Sequence:
MRSPAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLI
IKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
5 SIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 12)

VK1.4 DNA Sequence:
ATGAGGTCCCCAGCTCAGTTTCTGGGGCTTTTGCTTTTCTGGACTTCAGCCTCCAGATGTGACA
TTGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTG
10 CAGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCA
AGGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTG
GATCAGGGTCAGACTTCACTCTCAGTATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTA
CTGTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACG
GGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGT
15 GCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAGAGA (SEQ ID NO: 26)

VK1.4 Amino Acid Sequence:
MRSPAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLI
IKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
20 SIFPPSSEQLTSGGASVVCFLNNFYPR (SEQ ID NO: 13)

VK1.5 DNA Sequence:
ATGAGGGCCCCTGCTCAGCTCCTGGGGCTTTTGCTTTTCTGGACTTCAGCCTCCAGATGTGACA
TTGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTG
25 CAGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCA
AGGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTG
GATCAGGGTCAGACTTCACTCTCAATATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTAC
TGTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACGG
GCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTG
30 CCTCAGTCGTGTGCTTCTTGAACAACTTCTATCCCAAAGA (SEQ ID NO: 27)

VK1.5 Amino Acid Sequence:
MRAPAQLLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLI
IKYASQSISGIPSRFSGSGSGSDFTLNINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
35 SIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 11)

VK2.1 DNA Sequence:
ATGGTATCCTCAGCTCAGTTCCTTGGACTTTTGCTTTTCTGGACTTCAGCCTCCAGATGTGACAT
TGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTGC
40 AGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCAA
GGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTGG

ATCAGGGTCAGACTTCACTCTCAGTATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTACT
GTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACGGG
CTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGC
CTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAA (SEQ ID NO: 28)

5

VK2.1 Amino Acid Sequence:
MVSSAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLI
IKYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPK (SEQ ID NO: 14)

10

VK2.6 DNA Sequence:
ATGGTGTCCACAGCTCAGTTCCTTGGACTTTTGCTTTTCTGGACTTCAGCCTCCAGATGTGACAT
TGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTGC
AGGGCCAGCCAGAGTATTAGCGACTACTTATCCTGGTATCAACAAAGATCTCATGAGTCTCCAA
15 GGCTTATCATCAAATATGCTTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTGG
ATCAGGGTCAGACTTCACTCTCAGTATCAACAGTGTGGAACCTGAAGATGTTGGAGTGTATTACT
GTCAACATGGTCACAGCTTTCCGCTCACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACGGG
CTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGC
CTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAGAGA (SEQ ID NO: 29)

20

VK2.6 Amino Acid Sequence:
MVSTAQFLGLLLFWTSASRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPR (SEQ ID NO: 15)

25

<u>Example 4</u> - NLRP3 antigen synthesis

Design of a peptide (antigen) to NLRP3 that will generate an antibody response capable of inhibiting formation of the NLRP3 inflammasome.

30

The NLRP-3 inflammasome is a heterogenous protein complex that forms in mammalian cells in response to inflammatory stimulus, the ability to regulate and attenuate its formation could have important therapeutic potential for a range of inflammatory disorders. A peptide will be designed, derived from the NALP3 protein sequence which should generate antibodies capable of blocking the
35 binding of NALP3 to the other protein components in the NLRP3 inflammasome complex.

NLRP3 activation occurs by the self-assembly of NLRP protein with ASC, which is a hetero-complex of CARD, PYD and Caspase-1 domains. NLRP3 and ASC interact through their respective PYD domains, which contain a large proportion of highly conserved charged amino acid residues which
40 interact to form electrostatic interactions, which stabilize the complex – see Figure 15.

Figure 16 shows sequence alignment using CLUSTAL 0 (1.2.4) of the consensus sequences of C-terminal domains of human and mouse NALP (NLRP) proteins. Red indicates a residue recognized as essential for NLRP/ASC interaction by site-directed mutagenesis (Vajjhala et al, 2012).

5 Peptide selection was concentrated on the sequence region from 1-61 which has been studied extensively and is involved in the interaction with ASC (Vajjhala et al, 2012). The region has also been well modelled by crystallography, with a number of PDB structures available for this domain. PDB model 3QF2, which consists of the PYR domain of NLRP3 was selected as the most useful PDB structural reference. Initial peptide candidate sequences were selected on the basis of 10 accessibility and visibility as potential epitopes, and also degree of similarity between mouse and human sequence, whilst maintaining difference with other NLRP variants. These initial 3 peptides were modelled into 3D structures using NovaFold.

NovaFold Analysis

15 NovaFold is a 3D protein modelling software that uses the I-TASSER algorithm, a combination of template based threading (from PDB) and *ab initio* methods to predict the folding of a protein or peptide. It is used in this context to predict the presence of secondary structural features within a peptide which are known to be exhibited by the sequence *in situ* within the parent protein. This can help optimise the selection of a peptide sequence which best reflects the folding and proximity based 20 relationships within the parent protein, helping to maximize the potential of the immunogenic protein resulting in an antibody with full activity towards the corresponding epitope in the full length protein.

Modelling of Peptides and Alignment

Four distinct sequences were modelled using NovaFold, and the resulting highest scoring models 25 were assessed and then aligned to the parent NLRP3 structure as represented by PDB:3QF2 .

Peptide ID	**Location in Consensus sequence**	**Sequence**	**Secondary Structure**
FUS_746_001	30-53	EDYPPQKGCIPLPRGQTEKA DHVD (SEQ ID NO: 30)	α-helix
FUS_746_002	35-53	QKGCIPLPRGQTEKADHVD (SEQ ID NO: 31)	none
FUS_746_003	35-49	QKGCIPLPRGQTEKA (SEQ ID NO: 32)	none

Table 2: Comparison of alignment and structural features of peptide candidates modelled using Novafold 12.0 and aligned to NLRP3 structure PDB: 3QF2 using Protean 3D, version 14.0.1.

30 The modelling and comparison indicates that peptide FUS_746_001 is the preferred candidate for use as a peptide immunogen. In addition to demonstrating the greatest alignment with the model of the parent protein, it also demonstrates high similarity in prediction of secondary structure and is an accessible epitopic target.

35 Peptide FUS_746_001 Alignment using a Novafold predicted structure is shown in Figure 17.

Conclusion

The modelling of the software should always be taken as advisory, rather than definitive and interpreted on this basis, particularly if strong secondary structural features are not known to be found within the parent molecule. With this in mind, however, the modelling suggests that peptide FUS_746_001, sequence EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30) would be a best candidate for selection as the immunogen for this project on the basis of alignment to the parent protein, and predicted antigenicity. The peptide also shows only a few points of difference between the mouse and human sequence, which supports the production of an antibody response in mice that may allow for cross reactivity between these species, which is also a desirable feature, whilst minimising cross reactivity to other NLRP types. Note: It is recommended to add an N-terminal Cys residue for cross-linking to KLH.

References

Zhang, Y., 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics,* 23 Jan.9(40).
Vajjhala, P. R., Mirams, R. E., and Hill, J. M. (2012). Multiple binding sites on the pyrin domain of ASC protein allow self-association and interaction with NLRP3 protein. *J. Biol. Chem.* 287, 41732–41743

NLRP3 antigen synthesis

The NLRP3 peptide was synthesised by bioSynthesis Inc, Texas, conjugated to KLH using maleimide coupling through an additional C-terminal cysteine residue.

ELISA screening results of 1st bleed from mice immunised with NLRP3 immunogen – see Figure 18.

<u>Example 5</u> - Generation of a monoclonal antibody against NLRP3

A population of 5 mice were immunised and screened for positive immune responses. After selecting a suitable candidate for fusion, splenocytes were fused with partner cells to produce a population of hybridomas. This population underwent a series of limiting dilutions and screening assays to produce fully monoclonal cell lines.

Cell Line Nomenclature

The product name “F226 7A7-1E1-2D5” refers to one of the 10 chosen monoclonal hybridoma cell lines. The name is comprised of components describing the production pathway at each stage. Each hybridoma selected from the post-fusion screening and each limiting dilution was given a number corresponding to the plate number and well location on that plate for which the hybridoma was chosen (i.e. 7A7-1E1-2D5). This nomenclature traces the derivation of each individual hybridoma allowing for clear differentiation in the screening process.

Abbreviations

Ab	Antibody
DMSO	Dimethyl Sulfoxide
FCS	Fetal Calf Serum
ELISA	Enzyme-Linked Immunosorbent Assay
RT	Room Temperature
OD	Optical Density
PBST	Phosphate-buffered saline + 1% Tween 20
PBS	Phosphate-buffered saline
RPM	Revolutions per minute
NP, LP, RP, LRP, 2LP	Mouse Identification: No Punch, Left Punch, Right Punch Left/Right Punch, 2 Left Punches
HAT	Hypoxanthine, Aminopterin, Thymidine supplement
HATR Media[2]	DMEM supplemented with 2% Roche (HFCS), 2% HAT, 1% Pen/Strep, 1% L-Glutamine
SFM	Serum Free Medium
PEG	Polyethylene Glycol
GAM-HRP	Goat Anti-Mouse-Horse Radish Peroxidase
HT	Hypoxanthine and Thymidine
LD1	First Limiting Dilution
LD2	Second Limiting Dilution

[1] This is the media that was used for all cultures following fusion and screening.

Materials

5 **Reagents and Media**

Reagent	**Supplier**	**Catalogue No.**
L-glutamine	Gibco	25030-024
HAT Supplement	Gibco	21060-017
HFCS	Roche	11363735001
DMEM Glutamax	Gibco	61965-059
Penicillin/Streptomycin	Gibco	15140-122
FCS	Gibco	16000.044
DMSO	Sigma	D2650
Trypan Blue	Sigma	T8154
PEG	Sigma	10783641001
Freund's Adjuvant Complete	Sigma	F5881
Freund's Adjuvant Incomplete	Sigma	F5506
Sodium Hydrogen Carbonate	VWR	27778.260
Sodium Carbonate	Sigma	S2127-500G
Powdered Milk	Marvel	Original Dried Skimmed
Tween 20 (10%)	Sigma	P1379-1L
GAM-HRP (Fc Specific)	Sigma	A2554
TMB	Biopanda	TMB-S-002
Mr. Frosty	Nalgene	55710-200

Disposables

Name	**Supplier**	**Catalogue No.**
25cm^2 static flasks	Corning	430639
75cm^2 static flasks	Corning	430641
96-well plate sterile	Corning	3595
96-well plate sterile TPP	Primer Scientific	92696T
Cryovials	Fisher Scientific	366656
Maxi Sorb 96- well plates	Nunc	442404

Equipment

- CO_2 Cell culture static incubators (SANYO)
- Plate reader Sunrise (Tecan)
- Centurion Scientific K40R Centrifuge
- 5 Grant-Bio Multishaker PSU 20

Methods

Antigen Preparation

Once the immunogen; NLRP3 peptide-KLH conjugate (bioSynthesis Inc, Texas) was received, these solutions were diluted to 400 µg/ml in sterile, EF-PBS and aliquoted in volumes of 600 µl for immunisation and 150 µl for boosts and ELISA screening. These aliquots were labelled and stored at -20°C.

Immunisations

A population of 5 BalbC mice were immunised subcutaneously with 200 µl of a 1:1 emulsion of Freund's Adjuvant Complete (Sigma) and a 600 µl aliquot of NLRP3 peptide-KLH conjugate prepared herein. Two weeks after the 1st immunisation, the population was immunized with a 2nd injection at the same volumes and concentrations as the original injection only using Freund's Adjuvant Incomplete (Sigma) instead. One week after the 2nd immunisation, the mice were tagged by ear punches (NP, RP, LP, LRP, 2LP), and test bleeds were screened as described herein for preliminary results. Three weeks after the 2nd immunisation, the population was immunised a 3rd time using the same method as the 2nd injection. One week after the 3rd immunisation test bleeds were screened, and RP was then selected for fusion.

Test Bleed ELISAs

Tail bleeds were taken from the population of 5 BalbC mice and centrifuged at 8000 rpm for 10 min at RT. The blood serum from each mouse was collected, loaded onto the plate the same day as screening, and stored at -20°C. This screening was performed twice for the selection of a suitable mouse for fusion.

The day prior to screening, a Maxi Sorb plate was coated by adding 100 µl/well of 50 mM sodium carbonate coating buffer (pH 9.5) containing the free NLRP3 peptide at 1 µg/ml. A separate coating solution was prepared by diluting APO-A1 in the same coating buffer at 1 µg/ml. These solutions were loaded onto the plate in alternating rows so as to provide two wells to load each sample that demonstrates a positive and negative result. This plate was incubated overnight at 4°C in static conditions.

The following morning coating buffer was removed, and 200µl/well of blocking solution (4.0% w/v semi skim milk powder, 1x PBS) was added and agitated at 150 rpm for 2 hr at RT. The plate was

washed three times with PBS-T (0.1% v/v Tween 20). PBS was loaded into each well at 100 µl/well, and 1 µl of each test bleed serum was loaded into each positive and negative well. The plate was incubated at 150 rpm (Grant Shaker) for 2 hrs at room temperature. These samples were then removed and washed four times with PBS-T. 100 µl/well GAM-HRP diluted 1:5000 (Sigma, UK) was added, and the plate was incubated for 1 hr with agitation at 150 rpm at RT. The secondary antibody was removed, and the plate was washed four times with PBS-T and once in PBS. 100 µl/well of TMB substrate solution was added and incubated at 37°C for 10 minutes. 50 µl 1M HCl was added per well and the plate immediately read at 450 nm on a Tecan Sunrise plate reader.

After the second test bleed ELISA screening, RP was selected for fusion by expressing the most positive immune response.

Boost Injections

One week after the 3rd and final immunization, a boost injection was given to BalbC mouse RP by injecting 100 µl of aliquoted IL-1R at 200 µg/ml without any adjuvant.

Fusion F226

One week before fusion, SP2 cells were broken out from liquid nitrogen and were passaged in 10% FCS DMEM supplemented with 1% Pen/Strep, 1% L-glutamine until 3x12 ml T75 flasks were 75%-90% confluent on the day of fusion. On the day of the fusion, SP2 cells were dislodged by tapping the flask and were centrifuged at 1000 rpm for 5 min at 37°C. The cells were resuspended in 20 ml SFM DMEM, centrifuged again, and resuspended in 10 ml SFM DMEM. SP2 cells were stored in a Sterilin tube in SFM at 37°C, 6% CO_2 until needed.

After euthanasia, the spleen was aseptically removed from the mouse that showed the strongest immune response. Splenocytes were extracted by puncturing both ends of the spleen with a fine gauge needle and flushing 10-15 ml SFM DMEM. Splenocytes were transferred to a sterilin tube and washed twice with 20 ml serum free DMEM by centrifugation at 1300 rpm for 5 min at 37°C and gently removing the supernatant. The splenocytes were resuspended in 10 ml Serum free DMEM in a sterilin tube.

Using the SP2 cells stored at 37°C, the SP2 cells were added to the splenocytes. This SP2/splenocytes culture was centrifuged at 1300 rpm for 5 min at 37°C. After discarding the supernatant, 1 ml PEG was added to the SP2/splenocytes culture dropwise while stirring continuously over a period of 3 min. 1 ml SFM DMEM was added to the fusion mixture and stirred for 4 min. 10 ml SFM DMEM was added dropwise to the fresh culture and incubated for in 37°C water bath for 5 min. The cells were then centrifuged at 1000 rpm for 5 min at 37°C. The pellet was resuspended in 200 mL HATR media and was plated at 200 µl/well in 10x 96 well culture plates which were incubated 11 days at 37°C in 6% CO_2 prior to screening.

Post-Fusion Screening and Post-LD Screening

Eleven days after fusion, protoclones were screened by ELISA. 20x Maxi Sorb 96 well plates were coated as described in section 0 using APO-A1 at 1 µg/ml as the negative control for specificity. The coating solution was removed and the plates were blocked as described herein. Samples were prepared by removing 160 µl of supernatant from each well of the ten fusion plates, limiting dilution plates, or 24-well plates and transferring to fresh 96 well culture plates containing 50 µl 1xPBS. After 2 hours of blocking, the blocking solution was removed, and the plates were washed 3x with PBS-T. The samples from each dilution plate were loaded onto the ELISA plates at 100 µl/well by adding 1 row from each dilution plate per 2 rows on the ELISA plates to account for specificity of the coating antigens. Two wells per ELISA were incubated with 100 µl 1xPBS as a negative control. These samples were incubated at 150 rpm for 2 hours at room temperature.

Limiting Dilutions

Once the hybridoma populations were expanded in 24-well plates and growing well, a secondary screen was performed to select the most specific and highest producing populations for rounds of limiting dilutions.

Both limiting dilutions were performed for 1-3 protoclones each by seeding 2-4x 96-well plates at 1 cell/ well in 200 µl culture/well. The plates were prepared by counting each culture in the 24-well plate and were diluted 10x as an intermediate dilution, then were diluted to 200 cells in 40 ml. The culture was plated at 200 µl/well and left to incubate at 37°C, 6% CO_2 for 7-10 days until the wells were 80%-90% confluent. Each well for both limiting dilutions were screened by ELISA as described in section 0.

Final Clone Selection

Following the second limiting dilution, 10 clones were selected for expansion in a 24 well plate. Each clone was left to grow in 37°C, 6% CO_2 for 6 days until each well became 80%-90% confluent. When the clones were well established in the 24-well plates, each clone at 1 ml/well was transferred to a T25 flask containing 5 ml fresh 10% HATR DMEM for cryopreservation.

Cryopreservation of Monoclonal Cell Lines

Once the clones were well established (80%-90% confluency) in T25 flasks, each 5 ml culture was centrifuged at 1000 rpm for 5 min at 37°C and was resuspended in 1 ml of fresh 10% DMEM HATR media. Each 1 ml culture was transferred to a cryovial containing 300 µl of a 1:1 ratio of FCS to DMSO. The vials were sealed and placed in a Mr. Frosty and transferred to the -70°C freezer for short-term storage.

Cell Preparation for Sequencing

Anti-NLRP3 produced from clone F226 7A7-1E1-2D5 was selected for sequencing. Once the culture was confluent in the T25 flask, the supernatant was discarded. The cells were dislodged by cell scraping into 2 ml fresh media and were centrifuged at 7,600 rpm for 5 min at RT. The supernatant

was then discarded and the pellet was flash frozen in liquid nitrogen and placed in -70°C until ready for mRNA extraction.

Immunisation and Screening of Test Bleeds

A colony of mice were immunised with NLRP3 peptide-KLH conjugate (designed by bioinformatics and synthesised by bioSynthesis Inc, Texas) and regular test bleeds were taken over an 11 week period. Test bleeds were then screened against the antigen.

Upon identification of positive mice, a fusion was performed and supernatant from hybridoma clones were then validated. The specific antibodies then underwent limiting dilution and cloning to produce a stable hybridoma cell line against NLRP3.

The antibodies were screened using ELISA against the target protein – NLRP3 - and clones with a signal of at least 3 times the background were selected. Antibodies from 24 clones were selected and further in house testing was performed to pick the best 6 clones.

Results

Test Bleed 1

One week after the 2nd immunisation, a tail bleed was taken from each of the 5 mice and screened against unconjugated NLRP3 peptide and APO-A1 for determination of a suitable animal for fusion and a relative specificity of the polyclonal antibody produced – see Figure 19.

Test Bleed 2

After screening sera from tail bleeds, 2RP was selected for the fusion of its splenocytes to fusion partner SP2 culture as it demonstrated the best immune response – see Figure 20.

Post-Fusion Screening

Once the wells in each plate had reached 70%-80% confluency, the plates were screened by ELISA against NLRP3 peptide and APO-A1. The hybridoma population producing the highest responses were selected for expansion in a 24-well plate – see Figure 21.

1st 24-Well Plate Screening

Clones were selected from the post-fusion screening and were arrayed into a 24 well plate for expansion followed by a secondary screening that determines suitable protoclones for the first round of limiting dilutions. 3 clones were selected and limiting dilutions prepared – see Figure 22.

Limiting Dilution 1 Screening

Once the 1st limiting dilution plates were confluent, the limiting dilution was screened by ELISA against NLRP3 Peptide and APO-A1. 31 hybridoma populations were selected from F226 5B7 and 7A7 that demonstrated the highest and most specific response. No clones from 3D4 were suitable – see Figure 23.

2nd 24-Well Plate Screening

When the clones became confluent in the 24-well plate, each clone was screened by ELISA against NLRP3 peptide and APO-A1. F226 5B7-1E10, 5B7-1G2, 7A7-1C4 and 7A7-1E1 selected for the 2nd round of limiting dilution over 2x 96 well plates per clone – see Figure 24.

Limiting Dilution 2 Screening

Once the wells in each plate had reached 70%-80% confluency, the plates were screened by ELISA against NLRP3 peptide and APO-A1. The 24 hybridoma populations producing the highest response and highest specificity were selected for expansion in a 24-well plate and cryopreservation – see Figure 25.

Dot Blot analysis is shown in Figure 26. Dot blots were performed using protein lysates from THP-1 macrophages to test supernatant containing the anti NLRP3 monoclonal antibody collected from the best 24 clones from a fusion hybridoma cell line (A25=positive control commercial anti NLRP3 monoclonal antibody (R&D Systems), A26=negative control PBS). Clones 6, 11, 15, 16, 18 and 20 were selected and further tested by Western blotting.

Western Blot Analysis is shown in Figure 27. Western blots were performed using protein lysates from THP-1 macrophages to test supernatant containing anti-NLRP3 monoclonal antibody collected from the best 6 clones from a fusion hybridoma cell line untreated (lane 1) and stimulated with LPS and ATP (lane 2, (protein ladder lane 3)). Clone 18 was selected for sequencing and was used in the bispecific monoclonal antibody development.

Conclusions

The aim was to produce a range of antibodies against NLRP3 that were functional in preventing assembly of the NLRP3 inflammasome. Once the mice were immunised and screened, 2RP was selected for fusion. 24 monoclonal hybridoma cell lines were produced from two rounds of limiting dilutions. Each population was selected by highest production and highest specificity for NLRP3. The clone F226 7A7-1E1-2D5 was shown to be most active in preventing NLRP3 assembly in the *in vitro* assay. These final cell lines have been frozen down, and the antibody expressed by this 7A7-1E1-2D5 will be sequenced for the next stage in the production of the bi-specific, InflaMab.

<u>Example 6</u> – NLRP3 monoclonal sequencing

mRNA was extracted from the hybridoma cell pellets on 23/02/16. Total RNA was extracted from the pellets using Fusion Antibodies Ltd in-house RNA extraction protocol (see Example 3).

RT-PCR

cDNA was created from the RNA by reverse-transcription with an oligo(dT) primer. PCR reactions are set up using variable domain primers to amplify both the VH and VL regions of the monoclonal antibody DNA giving the following bands (see Figure 28):

The VH and VL products were cloned into the Invitrogen sequencing vector pCR2.1 and transformed into TOP10 cells and screened by PCR for positive transformants. Selected colonies were picked and analyzed by DNA sequencing on an ABI3130xl Genetic Analyzer, the result may be seen below.

Sequencing Results

Heavy Chain

V_H Amino Acid Sequence Alignment:

```
                 1                                                50
    VH1.1    (1) MNFGLSLVFLVLVLKGAQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
    VH3.7    (1) --------FLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
    VH3.4    (1) MDFGLSRVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
    VH3.1    (1) MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
    VH3.5    (1) MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
    VH3.8    (1) MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
Consensus    (1) MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSD
                 51                                              100
    VH1.1   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
    VH3.7   (43) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
    VH3.4   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
    VH3.1   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
    VH3.5   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
    VH3.8   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
Consensus   (51) YYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYL
                 101                                             150
    VH1.1  (101) QMNSLK--------------------------------------------
    VH3.7   (93) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
    VH3.4  (101) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
    VH3.1  (101) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
    VH3.5  (101) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
    VH3.8  (101) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
Consensus  (101) QMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSAAKTTPPS
                 151
    VH1.1  (107) -----
    VH3.7  (143) VYPLA
    VH3.4  (151) VYPLA
    VH3.1  (151) VYPLA
    VH3.5  (151) VYPLA
    VH3.8  (151) VYPLA
Consensus  (151) VYPLA
```

VH1.1 (SEQ ID NO: 33)

VH3.7 (SEQ ID NO: 34)

VH3.4 (SEQ ID NO: 35)

VH3.1 (SEQ ID NO: 36)

VH3.5 (SEQ ID NO: 36)

VH3.8 (SEQ ID NO: 36)

Consensus (SEQ ID NO: 36)

Key to amino acid shading:

Black	non-similar residues
Blue on cyan	consensus residue derived from a block of residues at a given position
Black on green	residues similar in structure to consensus residue or each other when no consensus found
Red on yellow	consensus residue derived from a completely conserved residue at a given position
Green	residue weakly similar to consensus residue at given position

V_H Consensus Amino Acid Sequence:

MDFGLSWVFLVLVLKGVQC**EVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVTVSA**AKTTPPSVYPLA (SEQ ID NO: 35)

The variable domain is highlighted in **BOLD.**

The Complementarity Determining Regions (CDRs) are underlined as determined by the IMGT numbering system (Lefranc, M.-P. et al., Nucleic Acids Research, 27, 209-212 (1999)) – see Figure 29.

Key to amino acid shading, in Figure 29:
Blue shaded circles are hydrophobic (non-polar) residues in frameworks 1-3 at sites that are hydrophobic in the majority of antibodies.
Yellow shaded circles are proline residues.
Squares are key residues at the start and end of the CDR.
Red amino acids in the framework are structurally conserved amino acids.

Light Chain

V_L Amino Acid Sequence Alignment:

```
                      1                                                50
     VL1.1      (1)  MAWISLIFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
     VL1.6      (1)  MAWISLIFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
     VL1.2      (1)  MAWTSLLLSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
     VL1.7      (1)  MAWTSLLFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
     VL1.4      (1)  MAWIPLLFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
     VL1.5      (1)  MAWISLLLSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
 Consensus      (1)  MAWISLLFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTT
                     51                                               100
     VL1.1     (51)  SNYANWVQEKPDHLFTGLVGGTNNRAPGVPARFSGSLIGDKAALTITGAQ
     VL1.6     (51)  SNYANWVQEKPDHLFTGLIGGTSNRAPGVPARFSGSLIGDKAALTITGAQ
     VL1.2     (51)  SNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQ
     VL1.7     (51)  SNYANWVQEKPDHLFTGLIGGTNNRAPGAPARFSGSLIGDKAALTITGAQ
     VL1.4     (51)  SNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTIIGAQ
     VL1.5     (51)  SNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQ
 Consensus     (51)  SNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQ
                     101                                            149
     VL1.1    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLFPPSTEELSL
     VL1.6    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLYPPSTKELSL
     VL1.2    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLCPPSSEKLSL
     VL1.7    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLCPPSTEKLSL
     VL1.4    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLFPPSLEKLSL
     VL1.5    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLFPPSTEELSL
 Consensus    (101)  TEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPSVTLFPPSTEKLSL
```

VL1.1 (SEQ ID NO: 37)

VL1.6 (SEQ ID NO: 38)

VL1.2 (SEQ ID NO: 39)

VL1.7 (SEQ ID NO: 40)

VL1.4 (SEQ ID NO: 41)

VL1.5 (SEQ ID NO: 42)

Consensus (SEQ ID NO: 43)

Key to amino acid shading:

Black	non-similar residues
Blue on cyan	consensus residue derived from a block of residues at a given position
Black on green	residues similar in structure to consensus residue or each other when no consensus found
Red on yellow	consensus residue derived from a completely conserved residue at a given position
Green	residue weakly similar to consensus residue at given position

V_L Consensus Amino Acid Sequence:

MAWISLLLSLLALSSGAIS**QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVL**GQPKSSPSVTLFPPSTEELSL (SEQ ID NO: 43)

The variable domain is highlighted in **BOLD.**

The Complementarity Determining Regions (CDRs) are underlined as determined by the IMGT numbering system (Lefranc, M.-P. et al., Nucleic Acids Research, 27, 209-212 (1999)) – see Figure 30.

Key to amino acid shading, in Figure 30:
Blue shaded circles are hydrophobic (non-polar) residues in frameworks 1-3 at sites that are hydrophobic in the majority of antibodies.
Yellow shaded circles are proline residues.
Squares are key residues at the start and end of the CDR.
Red amino acids in the framework are structurally conserved amino acids.

VH Sequencing results:

VH1.1 DNA Sequence:
ATGAACTTCGGGTTGAGCTTGGTTTTCCTTGTCCTTGTTTTAAAAGGTGCCCAGTGTGAAGTGCA
GCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGC
CTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCTG
GAGTGGGTCGCAACCATTAGTGATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGGGC
GATTCACCATCTCCAGAGACAATGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAG
(SEQ ID NO: 44)

VH1.1 Amino Acid Sequence:
MNFGLSLVFLVLVLKGAQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLE
WVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLK (SEQ ID NO: 33)

VH3.1 DNA Sequence:
ATGGACTTCGGGTTGAGCTGGGTTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGCA
GCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGC
CTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCTG
GAGTGGGTCGCAACCATTAGTGATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGGGC
GATTCACCATCTCCAGAGACAATGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAGTCT
GAGGACACAGCCATGTATTACTGTGCAAGAGGATGGGTTTCTACTATGGTTAAACTTCTTTCCTC
CTTTCCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCT
GTCTATCCACTGGCC (SEQ ID NO: 45)

VH3.1 Amino Acid Sequence:
MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLE
WVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFP
YWGQGTLVTVSAAKTTPPSVYPLA (SEQ ID NO: 36)

VH3.4 DNA Sequence:
ATGGACTTCGGGCTGAGCAGGGTTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGC
AGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAG
CCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCT
GGAGTGGGTCGCAACCATTAGTGATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGG
GCGATTCACCATCTCCAGAGACAATGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAG
TCTGAGGACACAGCCATGTATTACTGTGCAAGAGGATGGGTTTCTACTATGGTTAAACTTCTTTC
CTCCTTTCCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCA
TCTGTCTATCCACTGGCC (SEQ ID NO: 46)

VH3.4 Amino Acid Sequence:
MDFGLSRVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLE
WVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFP
YWGQGTLVTVSAAKTTPPSVYPLA (SEQ ID NO: 35)

VH3.5 DNA Sequence:
ATGGACTTCGGGCTGAGCTGGGTTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGC
AGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAG
CCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCT
GGAGTGGGTCGCAACCATTAGTGATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGG
GCGATTCACCATCTCCAGAGACAATGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAG
TCTGAGGACACAGCCATGTATTACTGTGCAAGAGGATGGGTTTCTACTATGGTTAAACTTCTTTC
CTCCTTTCCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCA
TCTGTCTATCCACTGGCC (SEQ ID NO: 47)

VH3.5 Amino Acid Sequence:
MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLE
WVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFP
YWGQGTLVTVSAAKTTPPSVYPLA (SEQ ID NO: 36)

VH3.7 DNA Sequence:
TTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGCAGCTGGTGGAGTCTGGGGGAGG
CTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTGAC
TATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCTGGAGTGGGTCGCAACCATTAGTG
ATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAA
TGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAGTCTGAGGACACAGCCATGTATTACT
GTGCAAGAGGATGGGTTTCTACTATGGTTAAACTTCTTTCCTCCTTTCCTTACTGGGGCCAAGGG
ACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCTGTCTATCCACTGGCC (SEQ ID
NO: 48)

VH3.7 Amino Acid Sequence:
FLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDGG
TYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTLVT
VSAAKTTPPSVYPLA (SEQ ID NO: 33)

VH3.8 DNA Sequence:
ATGGACTTCGGGCTGAGCTGGGTTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGC
AGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAG
CCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCGGAAAAGAGGCT
GGAGTGGGTCGCAACCATTAGTGATGGTGGTACTTACACCTACTATCCAGACAGTGTGAAGGG
GCGATTCACCATCTCCAGAGACAATGCCAAGAACAACCTTTACCTGCAAATGAACAGTCTGAAG
TCTGAGGACACAGCCATGTATTACTGTGCAAGAGGATGGGTTTCTACTATGGTTAAACTTCTTTC
CTCCTTTCCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCA
TCTGTCTATCCACTGGCC (SEQ ID NO: 49)

VH3.8 Amino Acid Sequence:
MDFGLSWVFLVLVLKGVQCEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLE
WVATISDGGTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFP
YWGQGTLVTVSAAKTTPPSVYPLA (SEQ ID NO: 36)

VL Sequencing Results:

VL1.1 DNA Sequence:
ATGGCCTGGATTTCTCTTATATTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTGT
TGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTCA
AGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTATT
CACTGGTCTAGTAGGTGGTACCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTCC
CTGATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATAT
TTCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTAG
GCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTTCCCACCCTCCACTGAAGAGCTAAGCTTGGG
(SEQ ID NO: 50)

VL1.1 Amino Acid Sequence:
MAWISLIFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGL
VGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSP
SVTLFPPSTEELSL (SEQ ID NO: 37)

VL1.2 DNA Sequence:
ATGGCCTGGACTTCACTCTTACTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTG
TTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTC
AAGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTAT

TCACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTC
CCTGATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATA
TTTCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTA
GGCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTGCCCGCCCTCCTCAGAGAAGCTAAGCTTG
GG (SEQ ID NO: 51)

VL1.2 Amino Acid Sequence:
MAWTSLLLSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTG
LIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSS
PSVTLCPPSSEKLSL (SEQ ID NO: 39)

VL1.4 DNA Sequence:
ATGGCCTGGATTCCTCTTTTATTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTGT
TGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTCA
AGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTATT
CACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTCC
CTGATTGGAGACAAGGCTGCCCTCACCATCATAGGGGCACAGACTGAGGATGAGGCAATATATT
TCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTAGG
CCAGCCCAAGTCTTCGCCATCAGTCACCCTGTTCCCGCCCTCCTTAGAAAAGCTTAGCTTGGG
(SEQ ID NO: 52)

VL1.4 Amino Acid Sequence:
MAWIPLLFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGL
IGGTNNRAPGVPARFSGSLIGDKAALTIIGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPS
VTLFPPSLEKLSL (SEQ ID NO: 41)

VL1.5 DNA Sequence:
ATGGCCTGGATTTCACTTTTACTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTG
TTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTC
AAGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTAT
TCACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTC
CCTGATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATA
TTTCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTA
GGCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTTTCCACCCTCCACAGAAGAGCTAAGCTTGG
G (SEQ ID NO: 53)

VL1.5 Amino Acid Sequence:
MAWISLLLSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGL
IGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSP
SVTLFPPSTEELSL (SEQ ID NO: 42)

VL1.6 DNA Sequence:
ATGGCCTGGATTTCACTTATCTTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTG
TTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTC
AAGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTAT
TCACTGGTCTAATAGGTGGTACCAGCAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTC
CCTGATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATA
TTTCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTA
GGCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTACCCGCCCTCTACAAAGGAGCTTAGCTTG
GG (SEQ ID NO: 54)

VL1.6 Amino Acid Sequence:
MAWISLIFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLI
GGTSNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSSPS
VTLYPPSTKELSL (SEQ ID NO: 38)

VL1.7 DNA Sequence:
ATGGCCTGGACTTCTCTCTTATTCTCTCTCCTGGCTCTCAGCTCAGGGGCCATTTCCCAGGCTG
TTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAACAGTCACACTCACTTGTCGCTC
AAGTACTGGGGCTGTTACAACTAGTAACTATGCCAACTGGGTCCAAGAAAAACCAGATCATTTAT
TCACTGGTCTAATAGGTGGTACCAACAACCGAGCTCCAGGTGCTCCTGCCAGATTCTCAGGCTC
CCTGATTGGAGACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATATA
TTTCTGTGCTCTATGGTACAGCAATTATTGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTA
GGCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTGCCCGCCCTCTACAGAAAAGCTAAGCTTG
GG (SEQ ID NO: 55)

VL1.7 Amino Acid Sequence:
MAWTSLLFSLLALSSGAISQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTG
LIGGTNNRAPGAPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQPKSS
PSVTLCPPSTEKLSL (SEQ ID NO: 40)

Example 7 - InflaMab Design - Development of a bi-specific antibody against both of IL-1R1 and NLRP3

The variable domain sequences of the monoclonal antibodies IL-1R1 and NLRP3 were sequenced.

The antibody was constructed using the IL-1R1 antibody with an IgG2a mouse constant domain sequence. A short linker was added to the C-terminal end of the heavy chain and the NLPR3 variable domains in an ScFv format with the linker $(GGGGS)_3$ was attached to create the bispecific. The DNA and amino acid sequences can be found below.

The constructs were cloned into ATUM vector pD2610-v5 and verified by sequencing. **Figure 31** illustrates the bispecific design and the plasmid map of InflaMab.

Designed Bispecific Antibody Sequences

Light Chain DNA Sequence:
ATGGTCAGCTCTGCTCAATTTCTCGGACTCCTTCTTCTGTGCTTTCAAGGAACACGCTGCGATAT
TGTGATGACCCAGTCCCCCGCCACCCTGTCCGTGACTCCGGGCGACCGGGTGTCCCTGTCGTG
CCGGGCATCACAGAGCATCTCCGACTACCTGTCGTGGTACCAGCAGAGATCACACGAGAGCCC
TCGCCTGATCATCAAATACGCCAGCCAGTCAATCTCCGGCATCCCCTCGCGGTTCTCCGGGTCC
GGTTCCGGCTCCGACTTCACACTGTCCATTAACTCCGTGGAACCTGAGGACGTGGGAGTGTACT
ACTGTCAACACGGCCATTCGTTCCCGCTGACTTTCGGGTCGGGAACCAAGCTGGAATTGAAGA
GGGCGGACGCGGCCCCTACCGTGTCAATTTTCCCACCGAGCTCCGAACAGCTCACCAGCGGC
GGTGCCTCGGTCGTGTGCTTCCTCAACAACTTCTATCCAAAAGACATTAACGTCAAGTGGAAGA
TCGATGGATCGGAGAGACAGAACGGAGTGCTGAACAGCTGGACTGATCAGGACTCCAAGGATT
CGACCTACTCCATGAGCTCCACTCTGACCCTGACCAAGGACGAATACGAGCGGCACAATTCCTA
CACTTGCGAAGCCACCCACAAGACCTCAACGTCCCCCATCGTGAAGTCCTTCAACCGCAACGA
GTGTTGATAA (SEQ ID NO: 56)

Light Chain Amino Acid Sequence:
MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57)

Heavy Chain DNA Sequence:
ATGGGCTGGACCCTCGTGTTCCTGTTCCTGCTGAGCGTGACGGCGGGCGTGCACTCCCAAATC
CAGCTTGTGCAGTCCGGACCCGAGCTCAGGAAGCCGGGCGAAACTGTGCGCATCAGCTGCAA
GGCTTCAGGGTACCCTTTCACCACCGCCGGGCTGCAATGGGTGCAGAAGATGTCCGGGAAGG
GTCTGAAGTGGATCGGATGGATGAACACCCAGTCCGAAGTGCCTAAATACGCCGAAGAATTCAA
GGGCCGCATTGCGTTCAGCCTGGAGACAGCCGCCTCGACCGCGTACCTTCAGATCAACAATCT
CAAGACTGAGGACACTGCCACCTACTTCTGTGCCAAGAGCGTGTACTTCAACTGGAGATACTTC
GACGTGTGGGGCGCCGGAACCACCGTGACCGTGTCCAGCGCCAAGACTACCGCCCCGAGCGT
GTACCCTCTGGCGCCAGTGTGCGGCGACACGACTGGCAGCTCGGTGACCTTGGGCTGCCTCG
TGAAGGGTTACTTCCCCGAGCCCGTGACTCTGACTTGGAACTCGGGCTCACTGTCGTCCGGAG
TGCATACCTTCCCGGCTGTGCTGCAAAGCGACCTCTATACCTTGTCATCGTCCGTGACTGTGAC
CTCCTCCACCTGGCCGTCCCAGAGCATCACCTGTAATGTCGCCCACCCTGCTTCATCGACTAAG
GTCGACAAGAAGATCGAGCCCAGAGGACCTACCATCAAGCCCTGCCCGCCCTGCAAATGCCCG
GCCCCAAACTTGCTGGGAGGGCCTTCCGTGTTCATCTTCCCTCCGAAAATCAAGGACGTGCTGA
TGATCTCCCTGAGCCCAATTGTCACTTGCGTGGTGGTGGATGTGTCCGAAGATGACCCAGATGT
GCAGATTTCATGGTTCGTGAACAACGTCGAAGTCCATACCGCACAGACCCAGACCCACCGCGA

GGATTACAACTCGACGCTGCGCGTCGTCAGCGCCCTGCCGATTCAGCACCAGGATTGGATGAG
CGGAAAGGAATTCAAGTGCAAAGTCAACAACAAGGACCTTCCGGCGCCGATCGAACGGACCAT
CTCGAAGCCTAAGGGATCAGTGCGGGCGCCTCAGGTCTACGTGCTCCCGCCTCCGGAAGAGG
AAATGACCAAGAAACAAGTCACCCTGACTTGCATGGTCACCGACTTCATGCCTGAGGACATCTA
TGTGGAGTGGACTAACAACGGAAAGACTGAACTGAACTACAAAAACACCGAACCAGTGCTGGAC
TCTGACGGCTCCTACTTCATGTACTCGAAGCTGCGGGTGGAGAAGAAAAACTGGGTGGAACGA
AACTCCTACTCGTGTTCCGTGGTGCACGAGGGTCTGCACAACCACCATACCACCAAGTCCTTCT
CCCGGACCCCCGGAAAGGGATCCGCCGGGGGATCCGGAGGGGACTCCGAAGTGCAACTGGT
GGAGTCGGGTGGCGGACTCGTGAAGCCCGGGGGGTCATTGAAGCTTTCCTGTGCTGCCTCCG
GTTTCACTTTCTCCGACTATTACATGTACTGGGTCAGACAGACCCCGGAGAAGCGGCTCGAATG
GGTGGCCACCATTTCGGACGGTGGAACCTACACTTACTACCCTGACTCCGTCAAGGGCCGGTT
TACTATCTCCCGCGACAACGCGAAGAACAATCTGTACCTCCAAATGAACTCCCTGAAGTCCGAG
GACACCGCCATGTACTATTGCGCAAGGGGATGGGTCAGCACTATGGTCAAGCTGCTGTCATCCT
TCCCTTACTGGGGACAGGGAACCCTTGTGACTGTGTCAGCCGGTGGCGGGGGGTCGGGCGGC
GGCGGTTCCGGTGGAGGGGGATCCCAGGCCGTCGTGACCCAAGAGTCGGCTCTGACTACTTC
ACCCGGAGAAACCGTGACCCTGACATGCCGCTCCTCCACTGGCGCAGTGACCACGAGCAATTA
CGCCAACTGGGTGCAGGAAAAGCCCGATCACCTGTTCACTGGACTCATTGGGGGAACCAACAA
CCGGGCGCCGGGCGTGCCCGCTCGGTTTAGCGGCTCCCTGATTGGAGACAAGGCCGCCCTGA
CTATCACCGGAGCCCAGACCGAAGATGAAGCCATCTACTTTTGCGCACTCTGGTACTCTAACTA
CTGGGTGTTTGGCGGCGGAACCAAGCTGACTGTGCTCGGACAGCCGAAGTGATAAAA (SEQ ID NO: 58)

Heavy Chain Amino Acid Sequence:
MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW
IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT
TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI
KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM
SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG
KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG
GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL
VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH
LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ
PK** (SEQ ID NO: 59)

<u>Example 8</u> - InflaMab (Bispecific against IL-1R1 and NLRP3) transient expression

The aim was to carry out transient transfections of InflaMab vector DNA in ExpiCHO cells. Following culture, expressed InflaMab was purified from the culture supernatant and QC analysis carried out on the purified protein.

InflaMab is a 210 kiloDalton (kDa) bispecific mouse antibody composed of two pairs of light chain and two pairs of heavy chains with ScFv domains fused to the N-terminal, complexed together via disulphide bonds. A mammalian expression vector encoding InflaMab was transfected into ExpiCHO cells. The expressed antibody was subsequently purified from clarified culture supernatant via protein A affinity chromatography. The concentration of purified antibody was measured using a NanoDrop Lite, Thermofisher and purity was evaluated using SDS-PAGE.

Sequence

DNA coding for the amino acid sequences of InflaMab was synthesised and cloned into the mammalian transient expression plasmid pD2610-v5 (Atum).

Plasmid InflaMab:

>InflaMab Light chain (Theoretical MW = 26.7 kDa)
MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 57)

>InflaMab Heavy Chain (Theoretical MW = 79.3 kDa)
MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW
IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT
TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI
KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM
SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG
KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG
GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL
VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH
LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ
PK (SEQ ID NO: 59)

Transient transfection of CHO cells

Suspension adapted ExpiCHO cells (Thermo Fisher, UK) were routinely cultured at 1.0–3.0 x10^5 cells/ml every 2-3 days in 500ml vented Erlenmeyer flasks (Corning, Netherlands) agitated at 135 rpm at 37°C 8% CO_2. Plasmid DNA for transfection was isolated using a Purelink Hipure plasmid filter maxiprep kit (Thermo Fisher, UK) as per the manufacturer instructions. DNA was quantified using a Nano Drop lite spectrophometer as per the manufacturer instructions.

Twenty-four hours prior to transfection, ExpiCHO cells were seeded at a concentration of 4.0 x 10^6 cells/ml in ExpiCHO expression medium and grown overnight at 135 rpm, 37°C 8% CO_2. On the day of transfection, 250ml ExpiCHO cells were diluted to a final density of 6.0 X 10^6 cells/ml in ExpiCHO expression medium. 1.0µg/ml of plasmid DNA and 0.32% (v/v) Expifectamine CHO reagent (Thermo Fisher) were diluted separately in 4% (v/v) OptiPro SFM (Thermo Fisher). The Expifectamine CHO/Optipro complex was added to the Plasmid DNA/Optipro complex dropwise. The transfection mixture was immediately added to the ExpiCHO cells. Transfected cells were incubated overnight at 135rpm, 37°C, 8% CO_2.

Twenty hours post transfection, cultures were supplemented with 0.6% (v/v) Expi CHO enhancer (Thermo Fisher, UK) and 24% ExpiCHO feed (Thermo Fisher, UK). The viability of the cells were closely monitored and cultures were harvested on day 8 by centrifugation at 4000 rpm for 40 minutes at room temperature.

Purification of InflaMab

Purifications were performed using AKTA (GE Healthcare) chromatography equipment. Prior to use, all AKTA equipment was thoroughly sanitized using 1M NaOH. Following centrifugation, filtered (0.22 µm) cell culture supernatant was applied to an AKTA system fitted with a 1 ml HiTrap Protein A column (equilibrated with wash buffer). Following loading, the column was washed with 20 column volumes of wash buffer. Bound antibody was step eluted with 10 column volumes of elution buffer. All eluted fractions were neutralised with Tris pH 9.0 buffer. Eluted fractions corresponding to elution peak were selected for overnight dialysis into PBS. The purity of the antibody was >95%, as judged by SDS-polyacrylamide midi gels.

SDS-PAGE analysis – see Figure 32

Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS PAGE) was carried out on purified antibody using standard methods.

Molecular weight marker shown in kilodaltons. Lanes, in Figure 32, are as follows:

Lane Number	**Sample**	**Batch**	**Amount (µg)**	**Conditions**
1	*PageRuler Plus (Thermo Fisher)*	NA	NA	Reducing
2	InflaMab	1	5	Reducing
3	Blank	NA	NA	Reducing
4	InflaMab	1	5	Non-reducing

InflaMab is ≥95% pure as judged by analysis of SDS-polyacrylamide gels. Under reducing conditions, both heavy and the light chains of the antibody are visible and are observed at the expected molecular weight of approximately 80 and 27kDa, respectively. Under non-reducing conditions, a single major band and several minor bands are observed. The additional bands (impurities) are likely the result of non-glycosylated IgG and IgG degradation products (e.g. a single

[partial] light chain, a combination of two heavy and one light chain, two heavy chains, two heavy and one light chain).

Evaluation of purified InflaMab

Purified InflaMab was quantified using a Nanodrop Lite spectrophotometer and the extinction coefficient 330,685 M^{-1} cm^{-1} (or 1.0 mg/ml = A280 of 1.7 [assuming a MW = 184,276 Da]), as per the manufacturer instructions. A total of 17.5 mg of InflaMab was purified from 0.3 litres of transfected cell culture supernatant.

Sample	Batch	Vol. of culture Super. (L)	Concentration (mg/ml)	Volume (ml)	Total (mg)	Yield (mg/L)	Endotoxin (EU/mg)
InflaMab	1	0.3	3.15	5.57	17.55	58.49	ND

Table 3: Concentration and yield of Antibody InflaMab from a 250ml transfection.

Summary: InflaMab

Material: Purified Antibody

Origin: Produced in a Chinese Hamster (Cricetulus griseus) Ovary cell line (no hamster or animal component added)

Results	
Purity:	≥95% pure (as determined by SDS-polyacrylamide gels [**Fig.1**])
Endotoxin (EU/mg):	Not determined
Concentration (mg/ml):	3.15 (as determined by measurement of absorbance at 280nm)
Mycoplasma:	Not determined
Package contents and storage recommendations	
Volume (ml):	5.57
Total (mg):	17.55
Container:	2 ml tube x 3
Volume per container:	2.0 ml x 2; 1.57 ml x 1
Net weight:	Not determined
Formulation:	Provided as a 0.2 µm sterile-filtered solution in PBS.
Shipped:	Ice packs (+4°C)
Storage:	4°C refrigerated

Non-hazardous, non-infectious. For research use only.

Inflamab prevents IL-1β release – see Figure 33a and b. THP1 cells, for Figure 33a and b, were cultured in 96-well plates at 100,000 cells/200µl complete media. PMA (100µg/ml for 72 hours) was used to differentiate THP-1 cells into macrophages. Following 24 hours of rest, differentiated THP1 cells were stimulated with LPS (1µg/ml) for 3 hours, treated with MCC950 (1µM) or the IL-1R1/NLRP3 Ab in a dose dependent fashion from 0.0025ng/ml to 2.5ng/ml for figure 33a or with IL-1R1/NLRP3 Ab (1nM) for figure 33b or with IgG control antibody for 30 minutes, followed by ATP (5mM) for 1 hour. IL-1β release was measured in the supernatant by ELISA.

Inflamab prevents caspase-1 activation in THP1 cells – see Figure 34. THP1 cells, for Figure 34, were cultured in 96-well plates at 100,000cells/200ul complete media. PMA (100ug/ml for 72 hours) was used to differentiated THP-1 cells into macrophages. Following 24 hours of rest, differentiated THP1 cells were stimulated with LPS (1ug/ml) for 3 hours, treated with the IL-1R1/NLRP3 Ab (1ug/ml) for 30 minutes, followed by ATP (5mM) for 1 hour. Caspase-1 activation was assessed by staining cells with a non-cytotoxic Fluorescent Labelled Inhibitor of Caspase-1 (FAM-FLICA) and DAPI (nuclear stain). Cells were treated with LPS alone (negative control), LPS+ATP (positive control), mouse IgG2a (1ug/ml, Ab control), or IL-1r/NLRP3 bi-specific Ab (1ug/ml, experimental). Representative confocal images are shown for each group. Green = active caspae-1 and blue=Dapi/nuclear stain.

Internalization of Inflamab – see Figure 35. THP1 cells, for Figure 35, were cultured in 96-well plates at 100,000cells/200ul complete media. Differentiation of THP1 cells was induced by PMA (100ug/ml for 72 hours). Following 24 hours of rest, differentiated THP1 cells were stimulated with LPS (1ug/ml) for 3 hours, treated with a pHrodo red labelled IL-1r/NLRP3 Ab (1ug/ml) for 30 minutes, followed by ATP (5mM) for 1 hour. The internalization of the Ab was tracked using a pHrodo red labelled bi-specific Ab that only fluoresces when internalized. (A) A representative confocal image shows the internalization of the pHrodo red labelled bi-specific Ab in a differentiated THP1 cell. (B) A representative confocal image shows significant reduction of caspase-1 activation (green) in THP1 cells that have internalized the bi-specific Ab (red, white arrow) as compared to THP1 cells that did not internalize the Ab (green only).

Example 9 – Targeting NLRP3 for Glaucoma

<u>Background and Significance</u>

Glaucoma is the leading cause of irreversible blindness worldwide, characterized by the progressive loss of retinal ganglion cells (RGCs). A recent study estimates that approximately 60 million people worldwide currently suffer from glaucoma and with the rapidly growing aging population this number is predicted to exceed 100 million by 2040 [1]. Unfortunately, there is no cure for glaucoma and intraocular pressure (IOP) reduction remains the only treatment strategy for all types of glaucoma [2]. However, while elevated IOP is a major risk factor for the development of glaucoma, lowering IOP alone does not prevent disease progression and many patients still experience significant vision loss even after IOP has been successfully lowered [3]. Moreover, the increasing incidence of normal tension glaucoma [4, 5] and the absence of neurodegeneration in some patients with elevated IOP [6] indicate that IOP-independent mechanisms also contribute to the development and progression of glaucoma and highlight the need for developing IOP-independent neuroprotective therapies to prevent disease progression and preserve vision.

Glaucoma is a complex multifactorial disease and while the exact mechanisms that mediate axon degeneration and death of RGCs are not well understood, there is growing evidence that axon damage in the optic nerve head (ONH) precedes death of the RGCs [7, 8]. Moreover, the axon

damage in the ONH has been linked to glial activation and inflammation [9, 10]. In human and experimental models of glaucoma, activated astrocytes [10, 11] and activated microglia [9, 12] are detected in ONH and coincides with increased expression of proinflammatory cytokines such as IL-1β and TNFα and neurotoxic mediators such as Nitric Oxide (NO), Reactive Oxygen Species (ROS), and Glutamate [12-14]. However, how elevated IOP triggers glial activation and how the inflammatory cascade is amplified and sustained is not well understood.

The NLRP3 inflammasome is an intracellular multi-protein complex that triggers inflammation in response to signals generated by infectious organisms, as well as endogenous signals associated with cell stress and tissue damage [15]. Dysregulation of the NLRP3 inflammasome has been implicated in several neurodegenerative diseases, including Alzheimer's disease and multiple sclerosis [16] but, most recently, activation of the NLRP3 inflammasome has been associated with the death of RGCs following retinal ischemia reperfusion injury and optic nerve crush [17, 18].

Results

Focusing specifically on the ONH region, where glial activation and inflammation has been linked to early axon damage, it has been demonstrated that NLRP3 is constitutively expressed in the ONH of mouse and human [Figure 36] and astrocyte-specific expression was confirmed by immunofluorescence showing co-localization of NLRP3 with the astrocyte-specific marker GFAP in the human ONH [Figure 37]. Using a microbead-induced mouse model of glaucoma in conjunction with a fluorescent reporter mouse to track inflammasome activation in vivo [19, 20], it has been demonstrated that NLRP3 inflammasome activation occurs early in the ONH following elevated IOP and coincides with induction of pro-inflammatory mediators in the ONH [Figure 38]. Using knockout mice that lacked the ASC adaptor protein (ASC KO), an essential component of the NLRP3 inflammasome, it has been demonstrated that early induction of inflammatory mediators and accumulation of Iba1+ immune cells in the ONH was dependent upon inflammasome activation [Figure 39]. Moreover, using mice that specifically lacked NLRP3 (NLRP3 KO) it was demonstrated that NLRP3 specifically mediated axon degeneration and death of RGCs in the microbead-induced mouse model of glaucoma [20] [Figure 40]. However, the most clinically relevant study revealed that systemic treatment of mice with a commercially available small molecule inhibitor of NLRP3 (MCC950) [21] prevented the death of RGCs in the microbead-induced mouse model of glaucoma [Figure 41]. This study provides proof-of-concept that pharmacologically targeting NLRP3 can serve as a neuroprotective therapy in glaucoma. However, due to a very short half-life, MCC950 had to be administered systemically every other day for the length of the study and as a potential treatment for glaucoma, systemically blocking the NLRP3 inflammasome in an aging population is not ideal, since the inflammasome plays an integral role in host defense against infection [15, 22]. In glaucoma, a disease that is limited to the RGCs and their axons, local delivery of the inhibitor into the vitreous would be ideal, but would require an inhibitor with a longer half-life in order to limit the number of intravitreal injections. Biologics are known to have longer half-lives than small molecules [23] and

there is strong precedence of using local administration of biologics in ophthalmology to treat eye diseases such as age-related macular degeneration.

In vitro studies clearly demonstrate the ability of Inflamab (NLRP3/IL1R1) to inhibit inflammasome activation (Figure 33a to Figure 35), and *in vivo* studies are now currently underway to assess the neuroprotective effect of InflaMab (NLRP3/IL1R1 bispecific antibody) when administered intravitreally in a microbead-induced mouse model of glaucoma. In the current study, WT C57BL/6J mice are receiving a single intravitreal injection of Inflamab (final vitreous concentration of 2.5, 25, or 250 ng/ml) on Day 0, immediately preceding the injection of microbeads and mice receiving saline only (no beads) are serving as a non-glaucoma control. RGC function is currently being measured by pERG using a fully integrated ERG system for rodents (Celeris). Changes in pERG amplitude is being measured in mice that received microbeads only and compared to mice that received microbeads plus Inflamab (final vitreous concentration of 2.5, 25, and 250 ng/ml) or saline only (no beads) [Figure 42]. In these studies, preliminary pERG results reveal a recovery of RGC function in the microbead-injected mice treated with Inflamab at a final vitreous concentration of 250 ng/ml as compared to mice receiving microbeads only or microbeads plus Inflamab at 2.5 or 25 ng/ml. Visual acuity using an optomotor reflex-based spatial frequency threshold test, as well as RGC and axon quantification, is performed to further corroborate the neuroprotective effect of Inflamab and including additional control groups (microbead-injected mice treated with the appropriate IgG control antibody).

NLRP3 is constitutively expressed in the mouse and human optic nerve head – see Figure 36. (A) Protein lysates were prepared from the ONH tissue of WT C57BL/6J mice and subjected to immunoblotting for NLRP3 (red) with actin (green) as a loading control. Conjunctiva (conj) from C57BL/6J WT mice was used as a positive control and ONH and conjunctival tissues from NLRP3 KO mice were used as negative controls. Western blot analysis demonstrates that NLRP3 is constitutively expressed in the non-glaucomatous mouse ONH. (B) Immunohistochemistry in sections of nonglaucomatous human optic nerve shows constitutive expression of NLRP3 (red) in the lamina cribosa region of the optic nerve head, with no constitutive expression in the myelinated portion of the optic nerve.

NLRP3 is constitutively expressed in optic nerve head astrocytes of normal (non-glaucomatous) human eyes – see Figure 37. Immunofluorescence in sections of human optic nerve shows co-localization of NLRP3 (red) with the astrocyte-specific marker GFAP (Green) in the unmeylinated lamina cribosa region of the optic nerve head. Dapi (blue) was used to identify all nucleated cells. Images are representative of staining performed on optic nerve sections obtained from three individual (non-glaucomatous) human optic nerves.

NLRP 3 inflammasome assembly in the ONH coincides with induction of inflammatory mediators at 7 days post microbead injection – see Figure 38. ASC-speck formation was monitored in vivo using a fluorescent reporter mouse (ASC citrine/Cre+). (A) At 7 days post microbead injection or saline as a control, frozen eye sections were stained for GFAP (astrocytes,

pink), MBP (myelin,red), and DAPI (blue). (B) The total number of ASC-citrine specks (green) was counted in the ONH region (top of the ONH to the myelinization zone (dotted line) and neural retina using Image J (N=5 sections per eye). A significant increase in the number of ASC-citrine specks was observed in the ONH, but not the retina, at 7 days post microbead as compared to saline. ***P<0.001, N=4 per group. (C) Staining with NLRP3 demonstrates constitutive expression of NLRP3 (red) and ASC (green) in the ONH of the saline injected control eye, but the ASC and NLRP3 do not co-localize (merged image). By contrast, at 7 days post microbead injection, the ASC-citrine specks co-localized with NLRP3 (yellow staining in merged image), indicating inflammasome assembly and activation of NLRP3. (D) Quantitative PCR on ONH and retinal tissue from 7 days post microbead injection revealed a significant increase in mRNA levels of GFAP, IL-1β, IL-18, and TNFα in the ONH but not retina when compared to uninjected contralateral eyes (fold of control). N = 5 mice/group (qPCR), *P<0.05, **P>0.001, ***P<0.001.

Macrophage infiltration and inflammatory gene expression in WT and ASC KO mice following elevated IOP – see Figure 39. (A) Frozen sections (3 sections per eye) were taken from WT and ASC KO eyes at 0, 7, and 14 days after microbead injection and the total number of Iba1+ cells (macrophage/microglia) was counted in the ONH region (top of the ONH to the myelinisation zone). (B) Results show a significant increase in the number of Iba1+ cells at D7 and D14 post microbead injection in WT but not ASC KO ONH. (C) Quantitative PCR on ONH tissue from 7 and 14 days post microbead injection revealed a significant increase in mRNA levels of IL-1β and IL-18 in WT as compared to uninjected contralateral eyes (fold of control). This increase in inflammatory gene expression was completely abrogated in ASC KO mice. N=5 mice/group (Immunofluorescence) and N = 6-8 mice/group (qPCR), *P<0.05, **P>0.001, ***P<0.001.

RGC and axon analysis in WT, ASC KO, and NLRP3 KO mice – see Figure 40. C57BL/6J WT, ASC KO, and NLRP3 KO mice received an anterior chamber injection of sterile polystyrene microbeads (7.2 x 106,15 µm) or saline as a control. Uninjected contralateral eyes were used as negative controls and IOP was followed every 3 days for 4 weeks using a rebound tonometer (TonoLab). (A) IOP analysis reveals a significant increase in IOP in microbead injected WT, ASC KO, and NLRP3 KO mice as compared to saline and uninjected contralateral controls, with no significant difference in the time course or magnitude of the microbead-induced IOP between WT, ASC KO, and NLRP3 KO mice. (B) At 4 weeks post microbead injection RGC density was quantitated in retinal flatmounts stained with β-III-tubulin (RGC specific marker) and data are presented as %RGC survival compared to uninjected contralateral eyes. (C) Axon density was quantitated in optic nerves stained with PPD and data presented as % axon survival compared to uninjected contralateral eyes a. N = 8-10/group, ****P<0.0001).

RGC analysis in WT mice treated with the NLRP3 inhibitor MCC950 – see Figure 1. To determine if treatment with MCC950 (small molecule NLRP3 inhibitor) can prevent RGC death in a microbead-induced mouse model of glaucoma, WT C57BL/6J mice received ip injections of MCC950 (10mg/kg, starting on Day 0) every other day for 4 weeks. Uninjected contralateral eyes and mice

receiving vehicle only served as negative controls. (A) IOP analysis reveals a significant increase in IOP in microbead injected WT mice treated with vehicle or MCC950 as compared to no beads control, with no significant difference in the time course or magnitude of the microbead-induced IOP between vehicle and MCC950 treated mice. (B) Representative confocal images of retinal flatmounts at 4 weeks post microbead injection stained with the RGC-specific marker Brn3a (red) and the nuclear marker Dapi (blue). (C) At 4 weeks post microbead injection RGC density was quantitated and showed a significant decrease in RGC density in microbead-injected WT mice that received vehicle only as compared to uninjected (no beads) contralateral eyes. By contrast, mice treated with MCC950 showed significant RGC protection with RGC densities equal to that of uninjected (no beads) controls. N = 5 per group, ***P<0.001).

RGC function in WT mice treated with the NLRP3 inhibitor InflaMab – see Figure 42. To determine if local treatment with InflaMab (NLRP3/IL1R1 bispecific antibody) can protect RGCs in a microbead-induced mouse model of glaucoma, WT C57BL/6J mice received a single intravitreal injection of Inflamab (final vitreous concentration of 2.5, 25, or 250 ng/ml) on Day 0, immediately preceding the injection of microbeads. Mice receiving saline only (no beads) served as a normal (no glaucoma) control. RGC function was measured by pERG using a fully integrated ERG system for rodents (Celeris) and changes in pERG amplitude was measured in mice that received microbeads only and compared to mice that received microbeads plus Inflamab (2.5, 25, and 250 ng/ml) or saline only (no beads). N = 4-5 per group. These studies are currently in progress. The preliminary pERG results presented herein reveal a recovery of RGC function in the microbead-injected mice treated with Inflamab at a final vitreous concentration of 250 ng/ml as compared to mice receiving microbeads only or microbeads plus Inflamab at 2.5 or 25 ng/ml. Visual acuity using an optomotor reflex-based spatial frequency threshold test, as well as RGC and axon quantification will also be performed to further corroborate the neuroprotective effect of Inflamab and additional control groups (microbead-injected mice treated with IgG control) will also be added.

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The invention is not limited to the embodiments described herein but can be amended or modified without departing from the scope of the present invention.

Claims

1. An NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3 for use in the treatment or prophylaxis of an inflammatory eye disease.

2. The NLRP3 inflammasome modulator for use according to claim 1, wherein the inflammatory eye disease is glaucoma.

3. The NLRP3 inflammasome modulator for use according to claim 1 or 2, wherein the modulator is also capable of binding to the PYD domain of NLRP3.

4. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is selected from the group comprising: a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, a fusion protein, or an aptamer molecule, a combination thereof, and fragments of each thereof.

5. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is a bi-antibody capable of binding to both of: IL-1R1 and NLRP3, wherein optionally, the modulator is a recombinant humanized bi-antibody capable of binding to both of: IL-1R1 and NLRP3.

6. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein modulator is a bi-antibody comprising one or more of the binding regions of a first antibody capable of binding IL-1R1 and one or more of the binding regions of a second antibody capable of binding NLRP3.

7. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody capable of binding IL-1R1 and one or more CDRs of a second antibody capable of binding NLRP3.

8. The NLRP3 inflammasome modulator for use according to claim 6 or 7, wherein the first and/or second antibody is a monoclonal antibody.

9. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is selected from an antibody fragment capable of binding to both: IL-1R1 and NLRP3, wherein optionally, the antibody fragment is selected from one or more of Fab, Fv, Fab’, (Fab’)2, scFv, bis-scFv, minibody, Fab2, and Fab3.

10. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is selected from a recombinant humanized antibody or antibody fragment capable of binding to both of: IL-1R1 and NLRP3.

11. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein modulator is an antibody or antibody fragment raised against one or more antigens selected

from both of IL-1R1 and NLRP3, optionally wherein the modulator is raised against one or more antigens selected from all or part of both of IL-1R1 and NLRP3.

12. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is an antibody or antibody fragment raised against one or more antigens selected from NLRP3, optionally NLRP3 conjugated to a carrier protein such as Keyhole Limpet Haemocyanin (KLH) (hereinafter, the NLRP3 immunogen), and IL-1R1, optionally recombinant IL-1R1.

13. The NLRP3 inflammasome modulator for use according to claims 11 or 12, wherein IL-1R1 comprises the extracellular domain of IL-1R1 (hereinafter, the IL-1R1 immunogen) comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).

14. The NLRP3 inflammasome modulator for use according to claims 11 or 12, wherein NLRP3 comprises KLH-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), optionally KLH-linker-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), further optionally KLH- Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

15. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more of the binding regions of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

16. The NLRP3 inflammasome modulator for use according to any one of the preceding claims, wherein modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more CDRs of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

17. The NLRP3 inflammasome modulator for use according to claim 16, wherein the heavy chain CDRs of the first antibody comprise: GYPFTTAG (SEQ ID NO: 60); MNTQSEVP (SEQ ID NO: 61); and/or AKSVYFNWRYFDV (SEQ ID NO: 62); and/or wherein the light chain CDRs of the first antibody comprise: QSISDY (SEQ ID NO: 63); YAS; and/or QHGHSFPLT (SEQ ID NO: 64).

18. The NLRP3 inflammasome modulator for use according to claim 16 or 17, wherein the heavy chain CDRs of the second antibody comprise: GFTFSDYY (SEQ ID NO: 65); ISDGGTYT (SEQ ID NO: 66); and/or ARGWVSTMVKLLSSFPY (SEQ ID NO: 67); and/or wherein the light chain CDRs of the second antibody comprise: TGAVTTSNY (SEQ ID NO: 68); GTN; and/or ALWYSNYWV (SEQ ID NO: 69).

19. The NLRP3 inflammasome modulator for use according to any one of claims 5-18, wherein the light chain of the bi-antibody has the amino acid sequence:

MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57).

20. The NLRP3 inflammasome modulator for use according to any one of claims 5-17, wherein the heavy chain of the bi-antibody has the amino acid sequence:

MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ PK** (SEQ ID NO: 59).

21. A method for the treatment and/or prophylaxis of an inflammatory eye disease, the method comprising

providing a therapeutically effective amount of an NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3 and which suppresses activation and/or signalling of the NLRP3 inflammasome, and

administering the therapeutically effective amount of said compound to a subject in need of such treatment.

22. The method of claim 21, wherein the inflammatory eye disease is glaucoma.

23. The method of claim 21 or 22, wherein the modulator is also capable of binding to the PYD domain of NLRP3.

24. The method of any one of claims 21-23, wherein the modulator is selected from the group comprising: a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, a fusion protein, or an aptamer molecule, a combination thereof, and fragments of each thereof.

25. The method of any one of claims 21-24, wherein the modulator is a bi-antibody capable of binding to both of: IL-1R1 and NLRP3, wherein optionally, the modulator is a recombinant humanized bi-antibody capable of binding to both of: IL-1R1 and NLRP3.

26. The method of any one of claims 21-25, wherein modulator is a bi-antibody comprising one or more of the binding regions of a first antibody capable of binding IL-1R1 and one or more of the binding regions of a second antibody capable of binding NLRP3.

27. The method of any one of claims 21-26, wherein the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody capable of binding IL-1R1 and one or more CDRs of a second antibody capable of binding NLRP3.

28. The method of claim 26 or 27, wherein the first and/or second antibody is a monoclonal antibody.

29. The method of any one of claims 21-28, wherein the modulator is selected from an antibody fragment capable of binding to both: IL-1R1 and NLRP3, wherein optionally, the antibody fragment is selected from one or more of Fab, Fv, Fab’, (Fab’)2, scFv, bis-scFv, minibody, Fab2, and Fab3.

30. The method of any one of claims 21-29, wherein the modulator is selected from a recombinant humanized antibody or antibody fragment capable of binding to both of: IL-1R1 and NLRP3.

31. The method of any one of claims 21-30, wherein modulator is an antibody or antibody fragment raised against one or more antigens selected from both of IL-1R1 and NLRP3, optionally wherein the modulator is raised against one or more antigens selected from all or part of both of IL-1R1 and NLRP3.

32. The method of any one of claims 21-31, wherein the modulator is an antibody or antibody fragment raised against one or more antigens selected from NLRP3, optionally NLRP3 conjugated to a carrier protein such as Keyhole Limpet Haemocyanin (KLH) (hereinafter, the NLRP3 immunogen), and IL-1R1, optionally recombinant IL-1R1.

33. The method of claim 31 or 32, wherein IL-1R1 comprises the extracellular domain of IL-1R1 (hereinafter, the IL-1R1 immunogen) comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).

34. The method of claim 31 or 32, wherein NLRP3 comprises KLH-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), optionally KLH-linker-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), further optionally KLH- Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

35. The method of any one of claims 21-34, wherein the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD

GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more of the binding regions of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

36. The method of any one of claims 21-35, wherein modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more CDRs of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

37. The method of claim 36, wherein the heavy chain CDRs of the first antibody comprise: GYPFTTAG (SEQ ID NO: 60); MNTQSEVP (SEQ ID NO: 61); and/or AKSVYFNWRYFDV (SEQ ID NO: 62); and/or wherein the light chain CDRs of the first antibody comprise: QSISDY (SEQ ID NO: 63); YAS; and/or QHGHSFPLT (SEQ ID NO: 64).

38. The method of claim 36 or 37, wherein the heavy chain CDRs of the second antibody comprise: GFTFSDYY (SEQ ID NO: 65); ISDGGTYT (SEQ ID NO: 66); and/or ARGWVSTMVKLLSSFPY (SEQ ID NO: 67); and/or wherein the light chain CDRs of the second antibody comprise: TGAVTTSNY (SEQ ID NO: 68); GTN; and/or ALWYSNYWV (SEQ ID NO: 69).

39. The method of any one of claims 25-38, wherein the light chain of the bi-antibody has the amino acid sequence:

MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57).

40. The method of any one of claims 25-39, wherein the heavy chain of the bi-antibody has the amino acid sequence:

MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW
IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT
TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI
KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM
SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG
KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG
GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL
VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH
LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ
PK** (SEQ ID NO: 59).

41. Use of an NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3 in the preparation of a medicament for the treatment of an inflammatory eye disease.

42. The use of the NLRP3 inflammasome modulator according to claim 41, wherein the inflammatory eye disease is glaucoma.

43. The use of the NLRP3 inflammasome modulator according to claim 41 or 42, wherein the modulator is also capable of binding to the PYD domain of NLRP3.

44. The use of the NLRP3 inflammasome modulator according to any one of claims 41-43, wherein the modulator is selected from the group comprising: a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, a fusion protein, or an aptamer molecule, a combination thereof, and fragments of each thereof.

45. The use of the NLRP3 inflammasome modulator according to any one of claims 41-44, wherein the modulator is a bi-antibody capable of binding to both of: IL-1R1 and NLRP3, wherein optionally, the modulator is a recombinant humanized bi-antibody capable of binding to both of: IL-1R1 and NLRP3.

46. The use of the NLRP3 inflammasome modulator according to any one of claims 41-45, wherein modulator is a bi-antibody comprising one or more of the binding regions of a first antibody capable of binding IL-1R1 and one or more of the binding regions of a second antibody capable of binding NLRP3.

47. The use of the NLRP3 inflammasome modulator according to any one of claims 41-46, wherein the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody capable of binding IL-1R1 and one or more CDRs of a second antibody capable of binding NLRP3.

48. The use of the NLRP3 inflammasome modulator according to claim 46 or 47, wherein the first and/or second antibody is a monoclonal antibody.

49. The use of the NLRP3 inflammasome modulator according to any one of claims 41-48, wherein the modulator is selected from an antibody fragment capable of binding to both: IL-1R1 and NLRP3, wherein optionally, the antibody fragment is selected from one or more of Fab, Fv, Fab', (Fab')2, scFv, bis-scFv, minibody, Fab2, and Fab3.

50. The use of the NLRP3 inflammasome modulator according to any one of claims 41-49, wherein the modulator is selected from a recombinant humanized antibody or antibody fragment capable of binding to both of: IL-1R1 and NLRP3.

51. The use of the NLRP3 inflammasome modulator according to any one of claims 41-50, wherein modulator is an antibody or antibody fragment raised against one or more antigens selected from both of IL-1R1 and NLRP3, optionally wherein the modulator is raised against one or more antigens selected from all or part of both of IL-1R1 and NLRP3.

52. The use of the NLRP3 inflammasome modulator according to any one of claims 41-51, wherein the modulator is an antibody or antibody fragment raised against one or more antigens selected from NLRP3, optionally NLRP3 conjugated to a carrier protein such as Keyhole Limpet Haemocyanin (KLH) (hereinafter, the NLRP3 immunogen), and IL-1R1, optionally recombinant IL-1R1.

53. The use of the NLRP3 inflammasome modulator according to claim 51 or 52, wherein IL-1R1 comprises the extracellular domain of IL-1R1 (hereinafter, the IL-1R1 immunogen) comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).

54. The use of the NLRP3 inflammasome modulator according to claim 51 or 52, wherein NLRP3 comprises KLH-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), optionally KLH-linker-

EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), further optionally KLH- Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

55. The use of the NLRP3 inflammasome modulator according to any one of claims 41-54, wherein the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more of the binding regions of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

56. The use of the NLRP3 inflammasome modulator according to any one of claims 41-55, wherein modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more CDRs of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

57. The use of the NLRP3 inflammasome modulator according to claim 56, wherein the heavy chain CDRs of the first antibody comprise: GYPFTTAG (SEQ ID NO: 60); MNTQSEVP (SEQ ID NO:

61); and/or AKSVYFNWRYFDV (SEQ ID NO: 62); and/or wherein the light chain CDRs of the first antibody comprise: QSISDY (SEQ ID NO: 63); YAS; and/or QHGHSFPLT (SEQ ID NO: 64).

58. The use of the NLRP3 inflammasome modulator according to claim 56 or 57, wherein the heavy chain CDRs of the second antibody comprise: GFTFSDYY (SEQ ID NO: 65); ISDGGTYT (SEQ ID NO: 66); and/or ARGWVSTMVKLLSSFPY (SEQ ID NO: 67); and/or wherein the light chain CDRs of the second antibody comprise: TGAVTTSNY (SEQ ID NO: 68); GTN; and/or ALWYSNYWV (SEQ ID NO: 69).

59. The use of the NLRP3 inflammasome modulator according to any one claims 45-58, wherein the light chain of the bi-antibody has the amino acid sequence:

MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII
KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV
SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT
KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57).

60. The use of the NLRP3 inflammasome modulator according to any one claims 45-59, wherein the heavy chain of the bi-antibody has the amino acid sequence:

MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW
IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT
TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI
KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM
SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG
KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG
GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL
VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH
LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ
PK** (SEQ ID NO: 59).

61. A method to reduce or prevent or treat at least one symptom of an inflammatory eye disease in a subject comprising selectively inhibiting and / or reducing activation of the inflammasome pathway by the use of an NLRP3 inflammasome modulator which is capable of binding to both of IL-1R1 and NLRP3.

62. The method of claim 61, wherein the modulator is for use in the treatment or prevention of at least one symptom of an inflammatory eye disease in a subject comprising selectively inhibiting and or reducing activation of the inflammasome pathway by the use of the modulator.

63. The method of claim 61 or 62, wherein the inflammatory eye disease is glaucoma.

64. The method of any one of claims 61-63, wherein the modulator is also capable of binding to the PYD domain of NLRP3.

65. The method of any one of claims 61-64, wherein the modulator is selected from the group comprising: a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, a fusion protein, or an aptamer molecule, a combination thereof, and fragments of each thereof.

66. The method of any one of claims 61-65, wherein the modulator is a bi-antibody capable of binding to both of: IL-1R1 and NLRP3, wherein optionally, the modulator is a recombinant humanized bi-antibody capable of binding to both of: IL-1R1 and NLRP3.

67. The method of any one of claims 61-66, wherein modulator is a bi-antibody comprising one or more of the binding regions of a first antibody capable of binding IL-1R1 and one or more of the binding regions of a second antibody capable of binding NLRP3.

68. The method of any one of claims 61-67, wherein the modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody capable of binding IL-1R1 and one or more CDRs of a second antibody capable of binding NLRP3.

69. The method of claim 67 or 68, wherein the first and/or second antibody is a monoclonal antibody.

70. The method of any one of claims 61-69, wherein the modulator is selected from an antibody fragment capable of binding to both: IL-1R1 and NLRP3, wherein optionally, the antibody fragment is selected from one or more of Fab, Fv, Fab', (Fab')2, scFv, bis-scFv, minibody, Fab2, and Fab3.

71. The method of any one of claims 61-70, wherein the modulator is selected from a recombinant humanized antibody or antibody fragment capable of binding to both of: IL-1R1 and NLRP3.

72. The method of any one of claims 61-71, wherein modulator is an antibody or antibody fragment raised against one or more antigens selected from both of IL-1R1 and NLRP3, optionally wherein the modulator is raised against one or more antigens selected from all or part of both of IL-1R1 and NLRP3.

73. The method of any one of claims 61-72, wherein the modulator is an antibody or antibody fragment raised against one or more antigens selected from NLRP3, optionally NLRP3 conjugated to a carrier protein such as Keyhole Limpet Haemocyanin (KLH) (hereinafter, the NLRP3 immunogen), and IL-1R1, optionally recombinant IL-1R1.

74. The method of claim 72 or 73, wherein IL-1R1 comprises the extracellular domain of IL-1R1 (hereinafter, the IL-1R1 immunogen) comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD

GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1).

75. The method of claim 72 or 73, wherein NLRP3 comprises KLH-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), optionally KLH-linker-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30), further optionally KLH- Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

76. The method of any one of claims 61-75, wherein the modulator is a bi-antibody comprising one or more of the binding regions of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP
GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI
SRSPGK* (SEQ ID NO: 1),

and one or more of the binding regions of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

77. The method of any one of claims 61-76, wherein modulator is a bi-antibody comprising one or more complementary determining regions (CDRs) of a first antibody raisable, optionally raised, against IL-1R1 immunogen and comprising the sequence:

MKVLLRLICFIALLISSLEADKCKEREEKIILVSSANEIDVRPCPLNPNEHKGTITWYKDDSKTPVSTEQ
ASRIHQHKEKLWFVPAKVEDSGHYYCVVRNSSYCLRIKISAKFVENEPNLCYNAQAIFKQKLPVAGD
GGLVCPYMEFFKNENNELPKLQWYKDCKPLLLDNIHFSGVKDRLIVMNVAEKHRGNYTCHASYTYL
GKQYPITRVIEFITLEENKPTRPVIVSPANETMEVDLGSQIQLICNVTGQLSDIAYWKWNGSVIDEDDP
VLGEDYYSVENPANKRRSTLITVLNISEIESRFYKHPFTCFAKNTHGIDAAYIQLIYPVTNFQKLEGGPS
VFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSHLPI
QHQDWMSGKEFKCKVNNKDLPSPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNP

GDISVEWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRHEGLKNYYLKKTI SRSPGK* (SEQ ID NO: 1),

and one or more CDRs of a second antibody raised against NLRP3 immunogen comprising the sequence:

KLH-Hydrazide-Ahx-EDYPPQKGCIPLPRGQTEKADHVD (SEQ ID NO: 30).

78. The method of claim 77, wherein the heavy chain CDRs of the first antibody comprise: GYPFTTAG (SEQ ID NO: 60); MNTQSEVP (SEQ ID NO: 61); and/or AKSVYFNWRYFDV (SEQ ID NO: 62); and/or wherein the light chain CDRs of the first antibody comprise: QSISDY (SEQ ID NO: 63); YAS; and/or QHGHSFPLT (SEQ ID NO: 64).

79. The method of claim 77 or 78, wherein the heavy chain CDRs of the second antibody comprise: GFTFSDYY (SEQ ID NO: 65); ISDGGTYT (SEQ ID NO: 66); and/or ARGWVSTMVKLLSSFPY (SEQ ID NO: 67); and/or wherein the light chain CDRs of the second antibody comprise: TGAVTTSNY (SEQ ID NO: 68); GTN; and/or ALWYSNYWV (SEQ ID NO: 69).

80. The method of any one of claims 66-79, wherein the light chain of the bi-specific antibody has the amino acid sequence:

MVSSAQFLGLLLLCFQGTRCDIVMTQSPATLSVTPGDRVSLSCRASQSISDYLSWYQQRSHESPRLII KYASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQHGHSFPLTFGSGTKLELKRADAAPTV SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLT KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC** (SEQ ID NO: 57).

81. The method of any one of claims 66-80, wherein the heavy chain of a bi-specific antibody has the amino acid sequence:

MGWTLVFLFLLSVTAGVHSQIQLVQSGPELRKPGETVRISCKASGYPFTTAGLQWVQKMSGKGLKW IGWMNTQSEVPKYAEEFKGRIAFSLETAASTAYLQINNLKTEDTATYFCAKSVYFNWRYFDVWGAGT TVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKI KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWM SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG KGSAGGSGGDSEVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVATISDG GTYTYYPDSVKGRFTISRDNAKNNLYLQMNSLKSEDTAMYYCARGWVSTMVKLLSSFPYWGQGTL VTVSAGGGGSGGGGSGGGGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDH LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNYWVFGGGTKLTVLGQ PK** (SEQ ID NO: 59).

Figure 1:

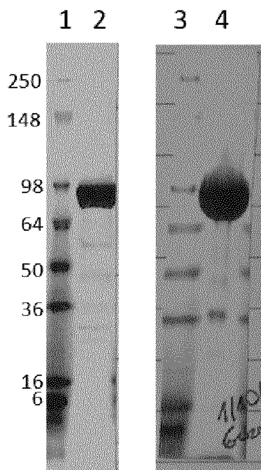
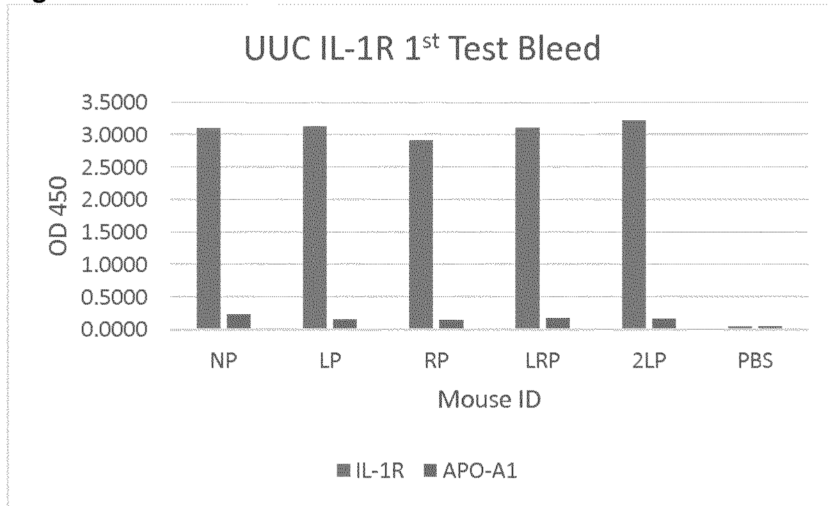


Figure 2:



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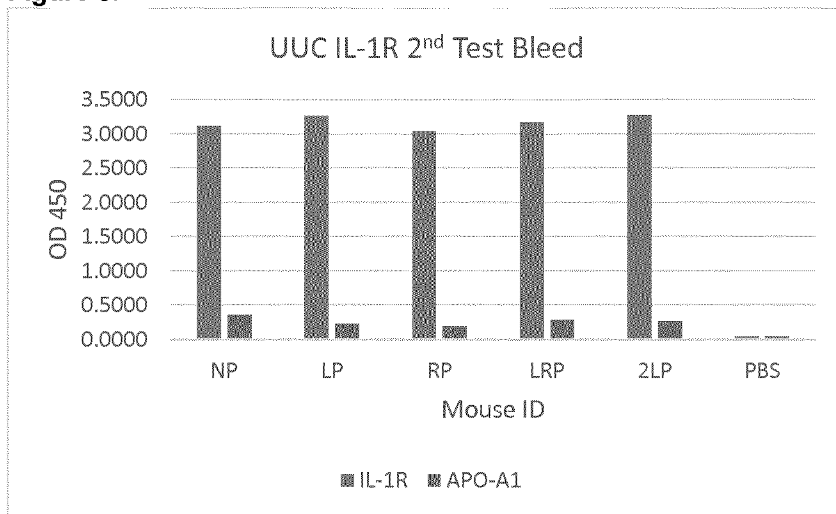

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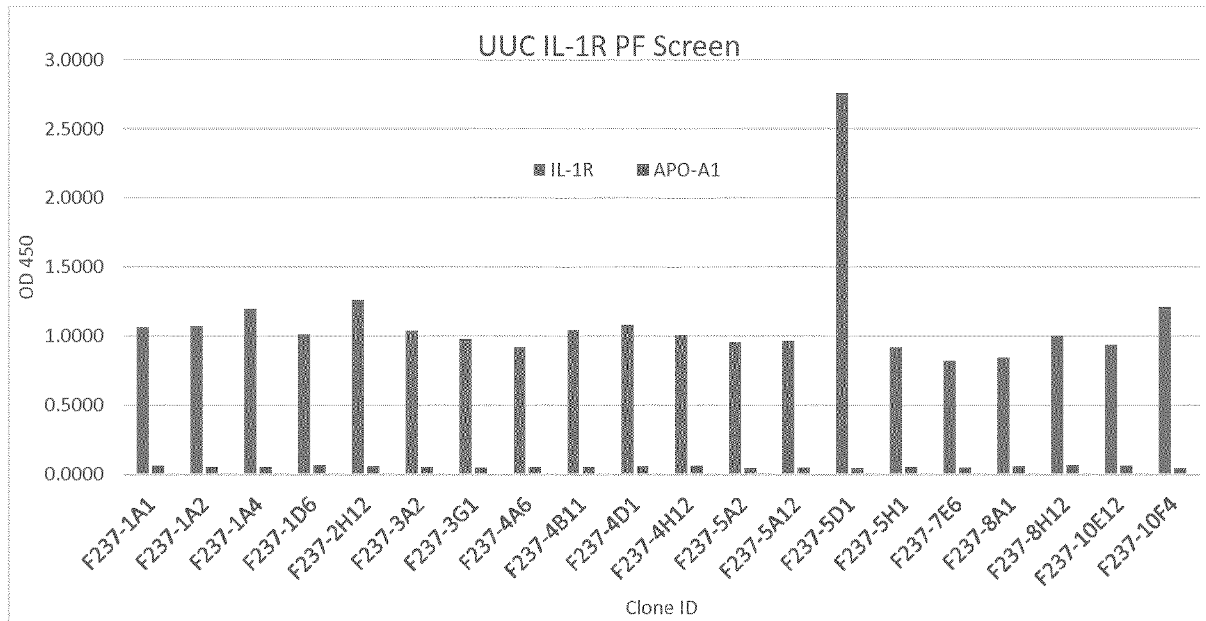

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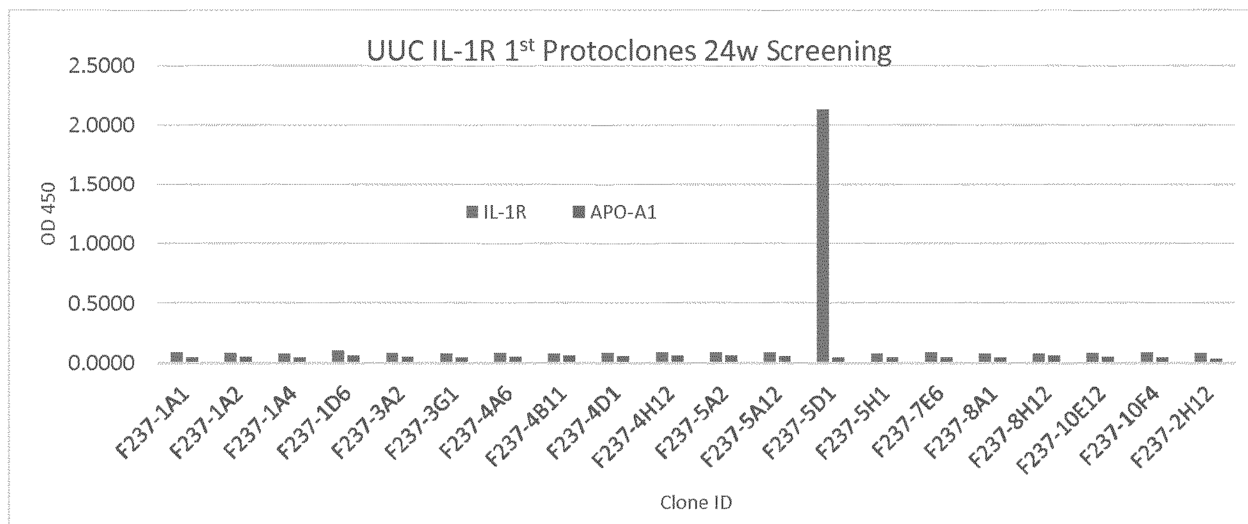

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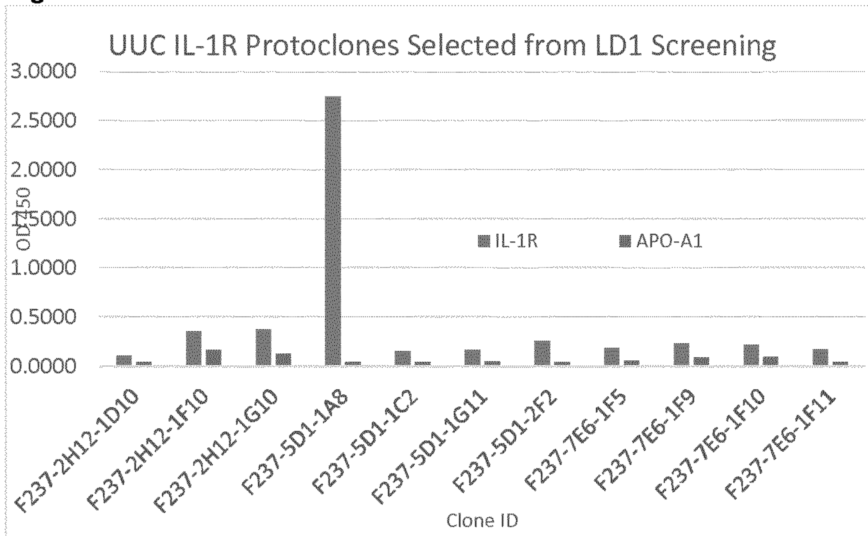

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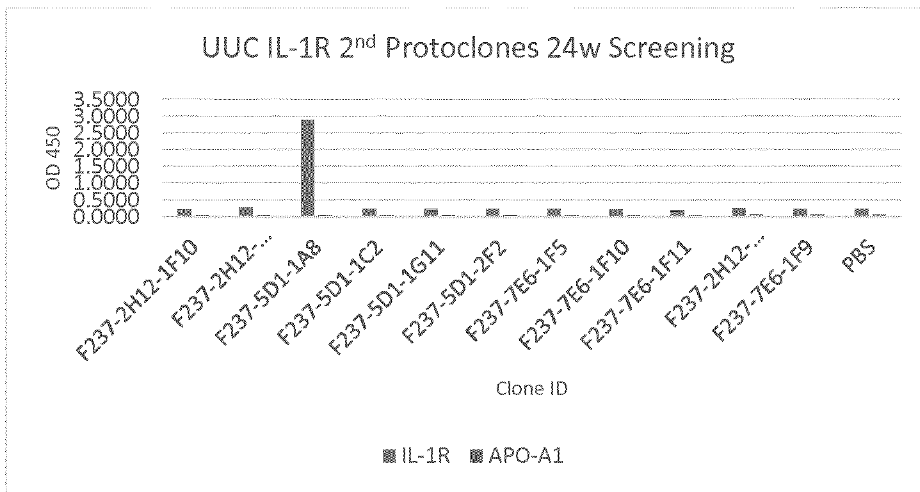

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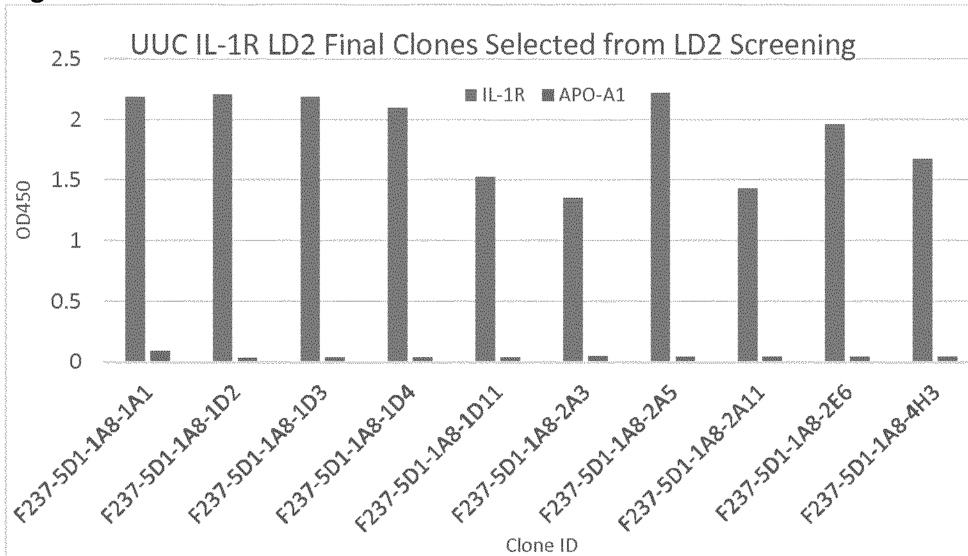

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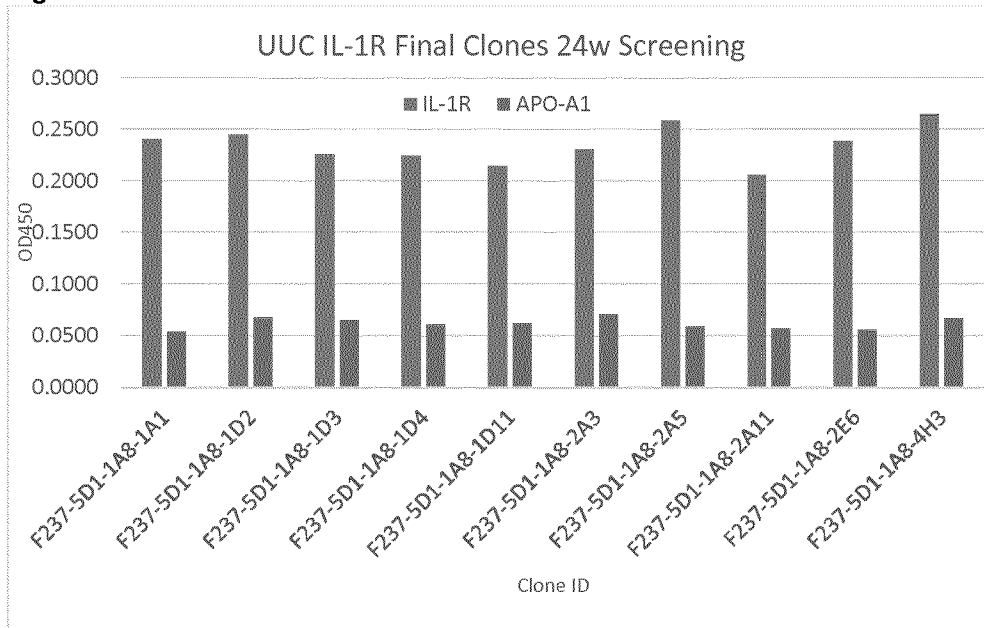

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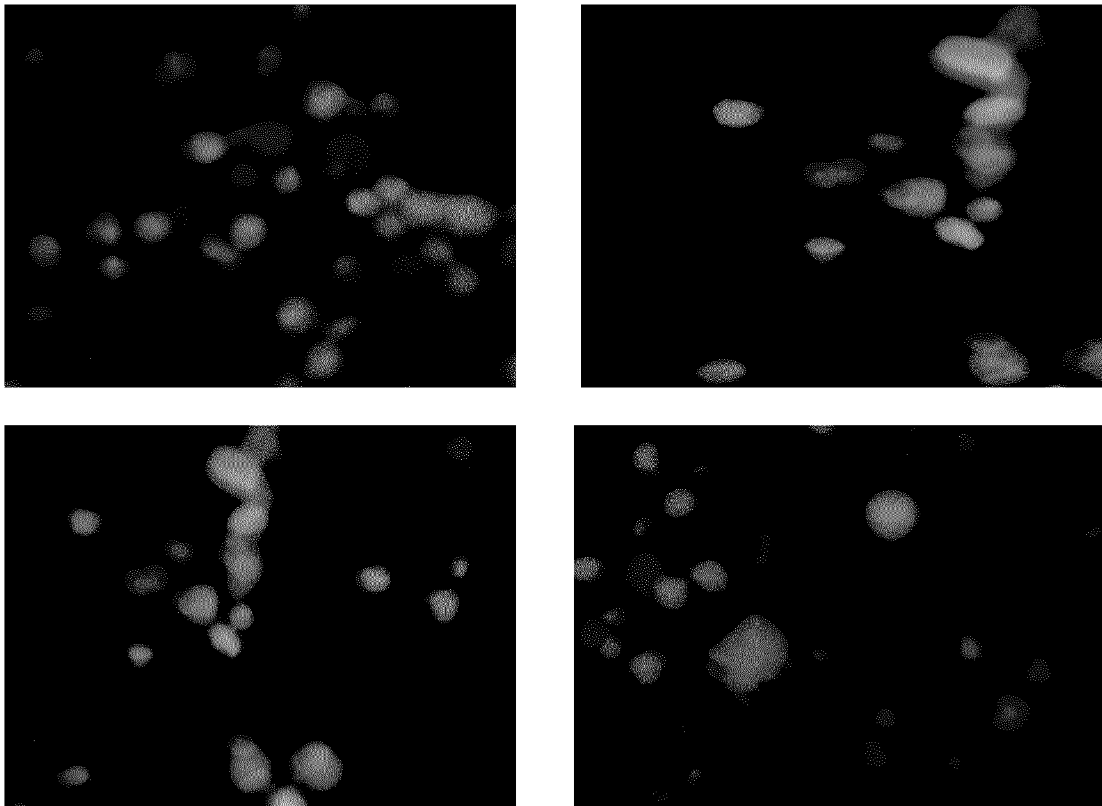

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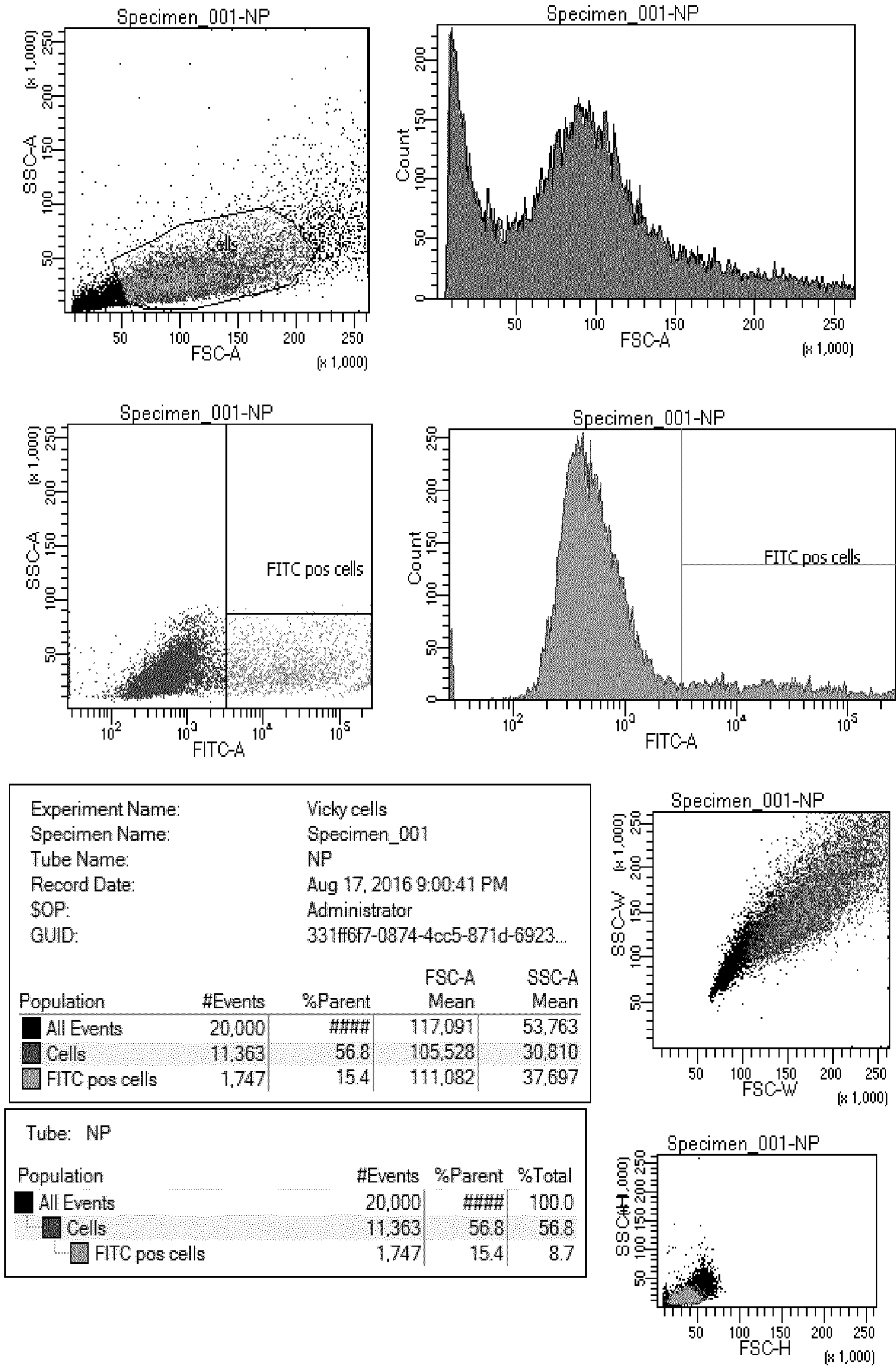
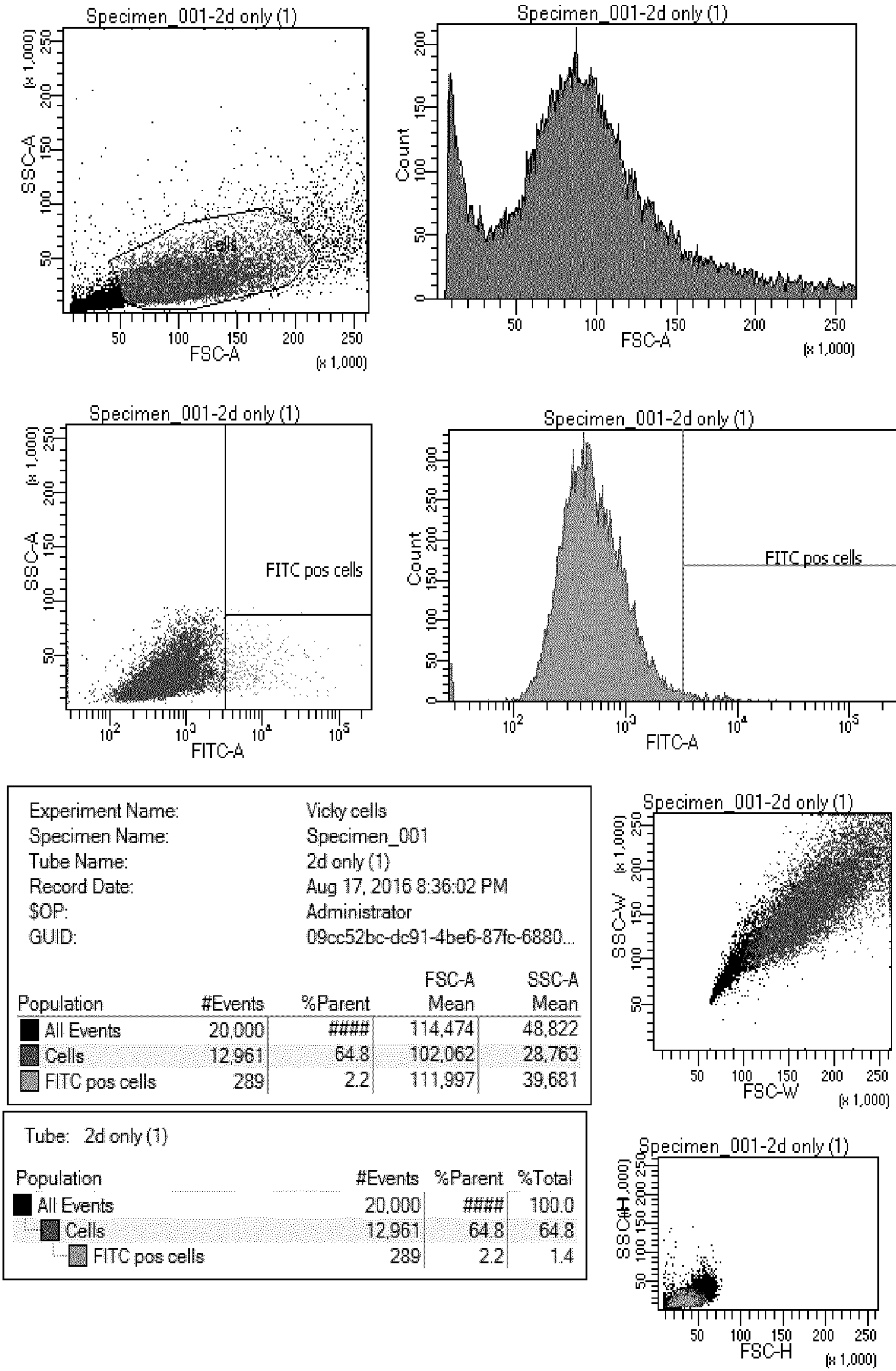


Figure 11 (ii):



Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	20,000	####	114,474	48,822
Cells	12,961	64.8	102,062	28,763
FITC pos cells	289	2.2	111,997	39,681

Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
Cells	12,961	64.8	64.8
FITC pos cells	289	2.2	1.4

Figure 12:

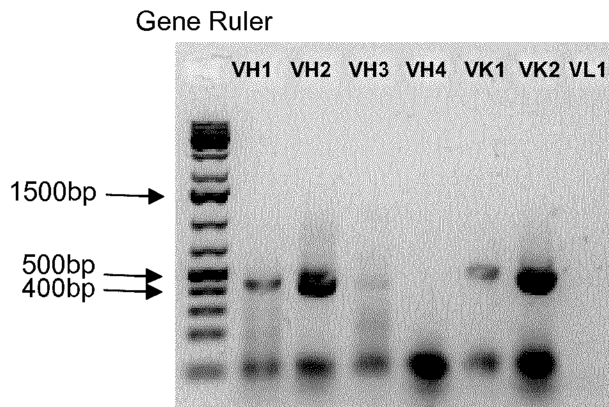


Figure 13:

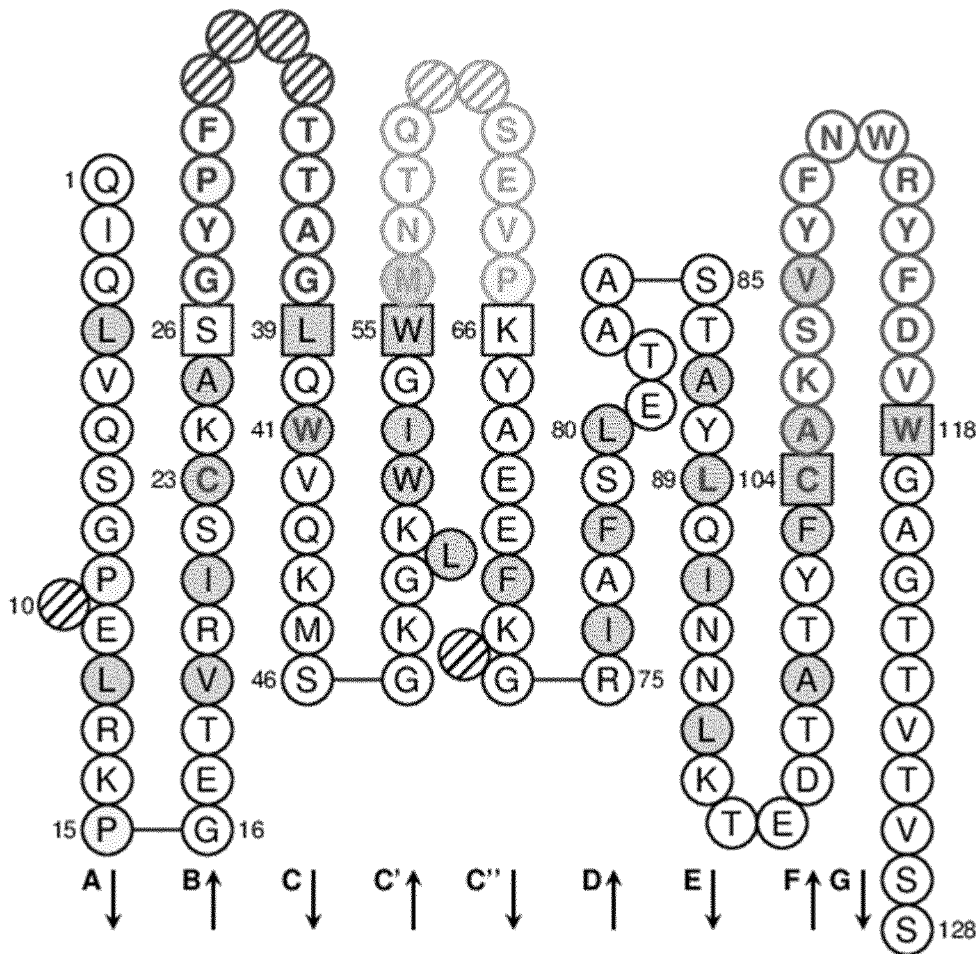
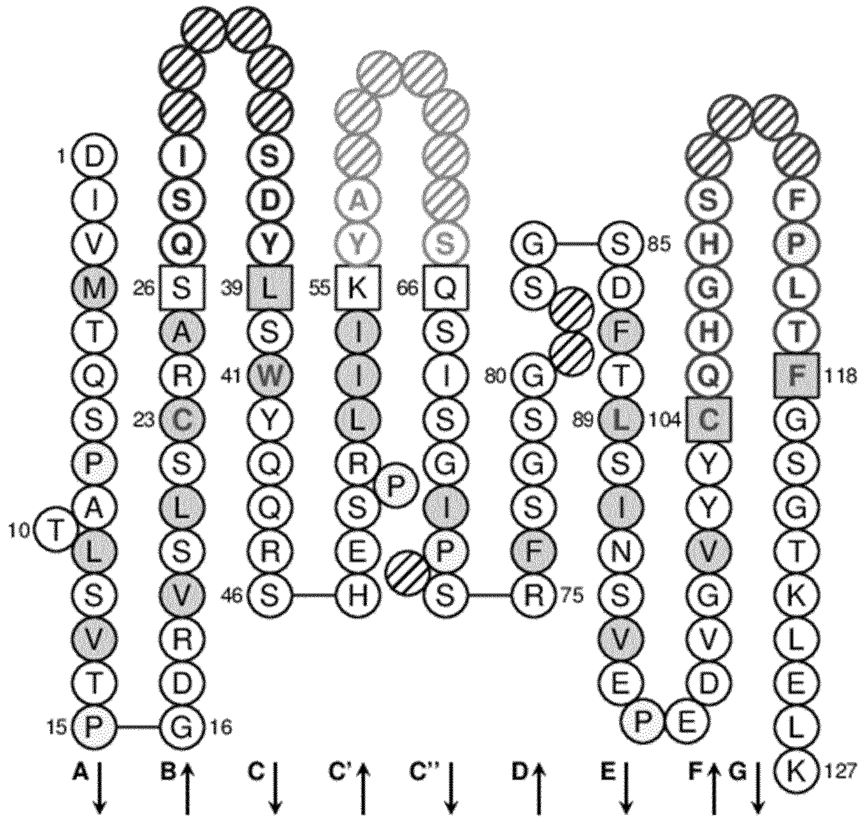


Figure 14:



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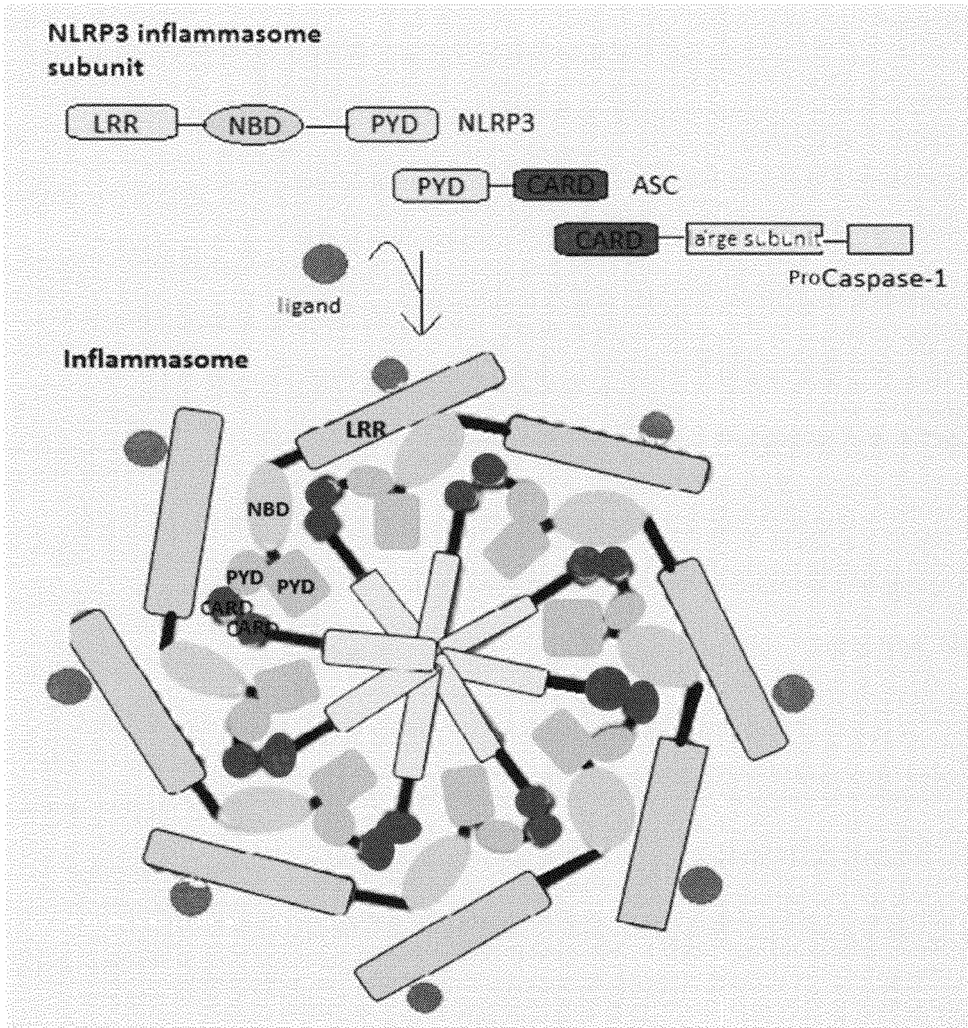
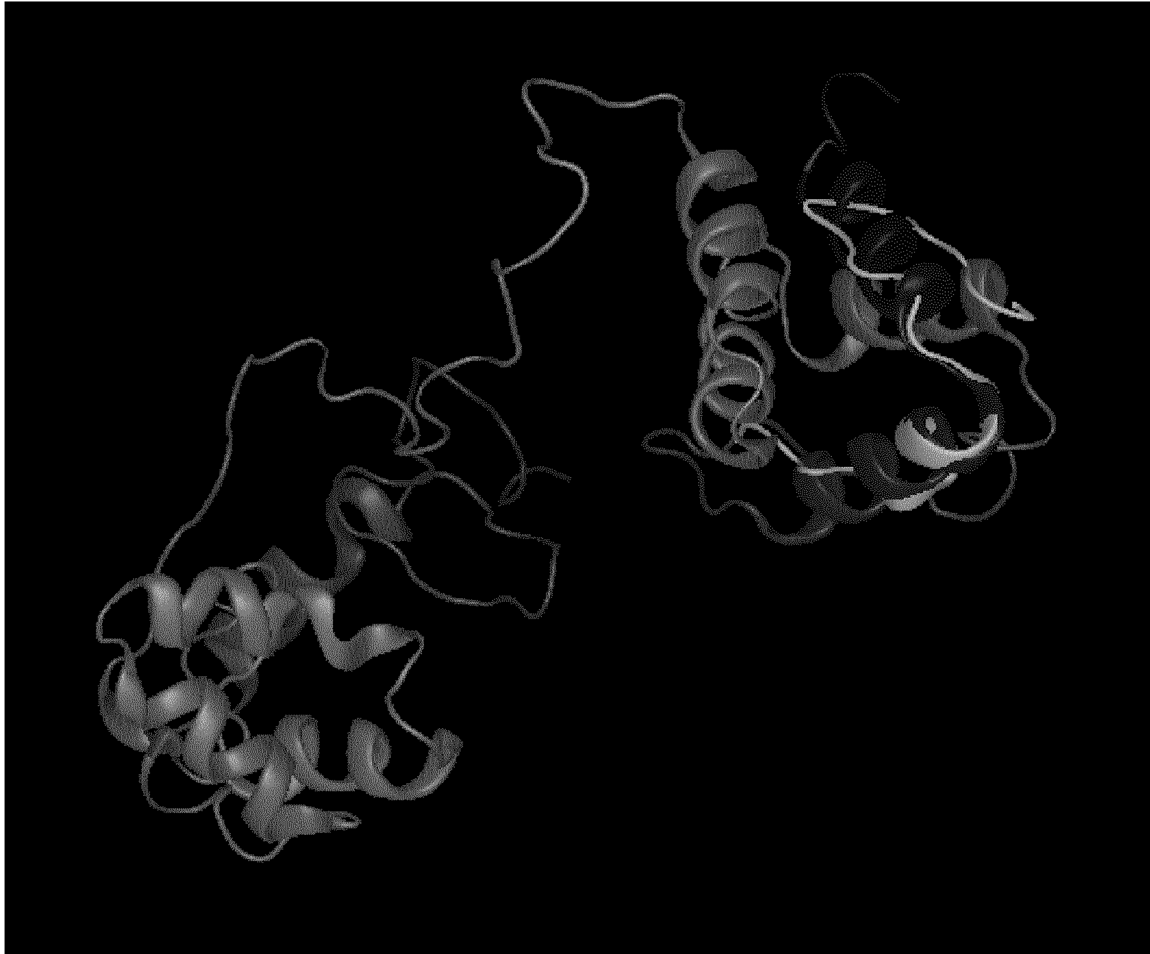


Figure 16:

Sequence Analysis

```
SP|Q96P20|NALP3_HUMAN KMASTRCKLARYLEDLEDVDLKKFKMHLEDYPPQKGCIPLPRGQTEKADHVDLATLMIDF 61
SP|Q8R4B8|NALP3_MOUSE -MTSVRCKLAQYLEDLEDVDLKKFKMHLEDYPPEKGCIPVPRGQMEKADHLDLATLMIDF 59
SP|Q9C000|NALP1_HUMAN MAGGAWGRLACYLEFLKKEELKEFQLLLANKAHSRSSSGETPAQPEKTSGMEVASYLVAQ 60
SP|Q9NX02|NALP2_HUMAN SSAQMGFNLQALLEQLSQDELSKFKYLITTFSLAHELQKIPHKEVDKADGKQLVEILTTH 62
SP|Q8WX94|NALP7_HUMAN TSPQLEWTLQTLLEQLNEDELKSFKSLLWAFPLEDVLQKTPWSEVEEADGKKLAEILVNT 61
SP|Q96MN2|NALP4_HUMAN ASFFSDFGLMWYLEELKKEEFRKFKEHLKQMTLQLELKQIPWTEVKKASREELANLLIKH 62
SP|P59047|NALP5_HUMAN SLTFSSYGLQWCLYELDKEEFQTFKELLKKKSSESTTCSIPQFEIENANVECLALLLHEY 116
SP|P59046|NAL12_HUMAN AGRDGLCRLSTYLEELEAVELKKFKLYLGTA-TELGEGKIPWGSMEKAGPLEMAQLLITH 63
SP|Q86W24|NAL14_HUMAN SSFFPDFGLLLYLEELNKEELNTFKLFLKETM-EPEHGLTPWNEVKKARREDLANLMKKY 65
SP|Q86W28|NALP8_HUMAN PGSPCENGVMLYMRNVSHEELQRFKQLLLTEL-STGTMPITWDQVETASWAEVVHLLIER 91
SP|Q7RTR0|NALP9_HUMAN ESFFSDFGLLWYLKELRKEEFWKFKELLKQPLEKFELKPIPWAELKKASKEDVAKLLDKH 62
SP|P59044|NALP6_HUMAN RLAVARELLLAALEELSQEQLKRFRHKLRDVGPDG--RSIPWGRLERADAVDLAEQLAQF 71
SP|Q86W26|NAL10_HUMAN KARKPREALLWALSDLEENDFKKLKFYLRDMTLSEGQPPLARGELEGLIPVDLAELLISK 64
SP|P59045|NAL11_HUMAN ESDSTDFDLLWYLENLSDKEFQSFKKYLARKILDFKL---PQFPLIQMTKEELANVLPIS 59
```

Figure 17:



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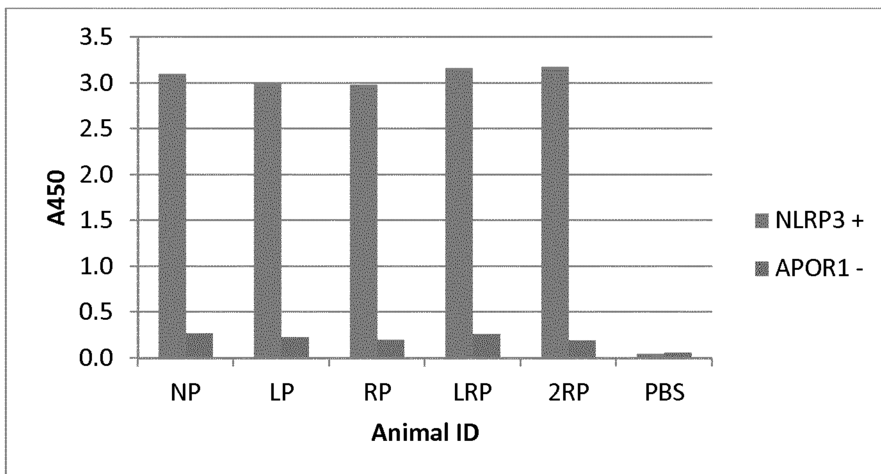

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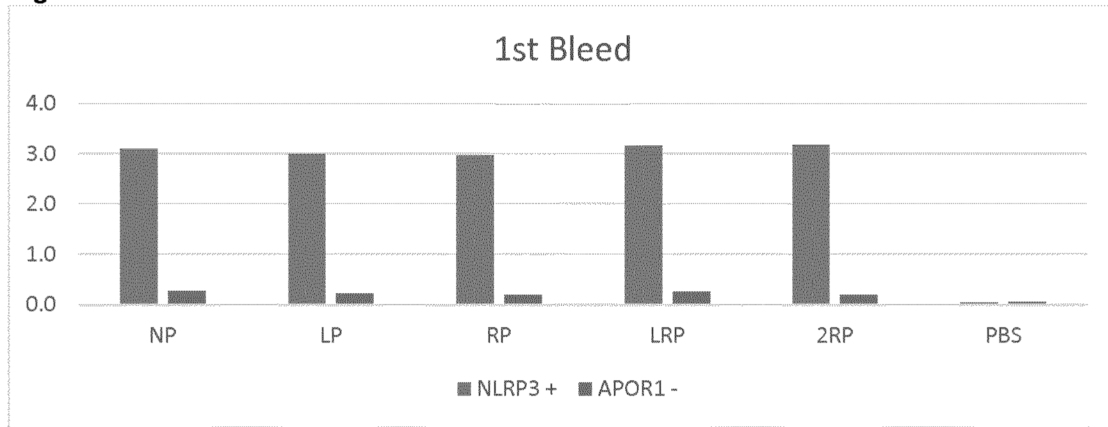

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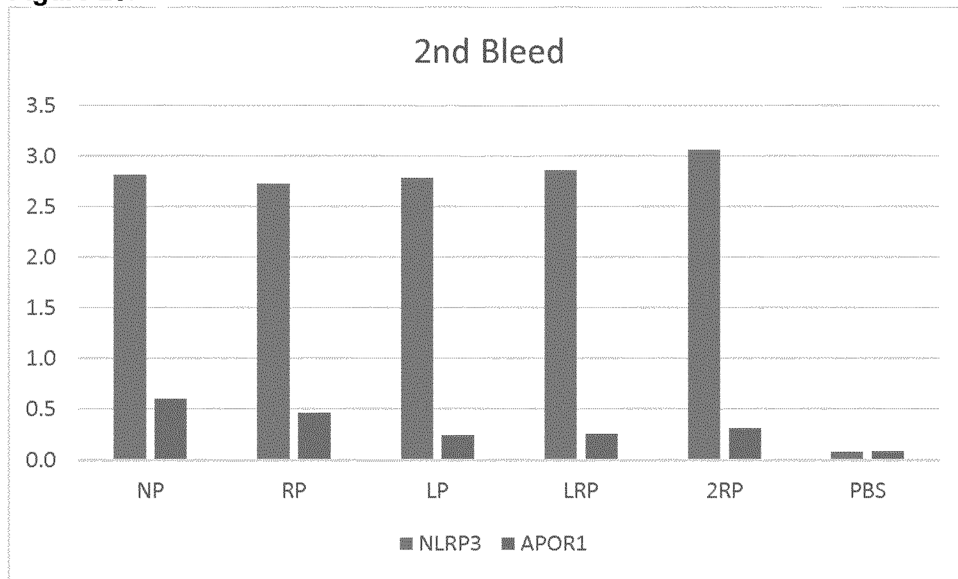

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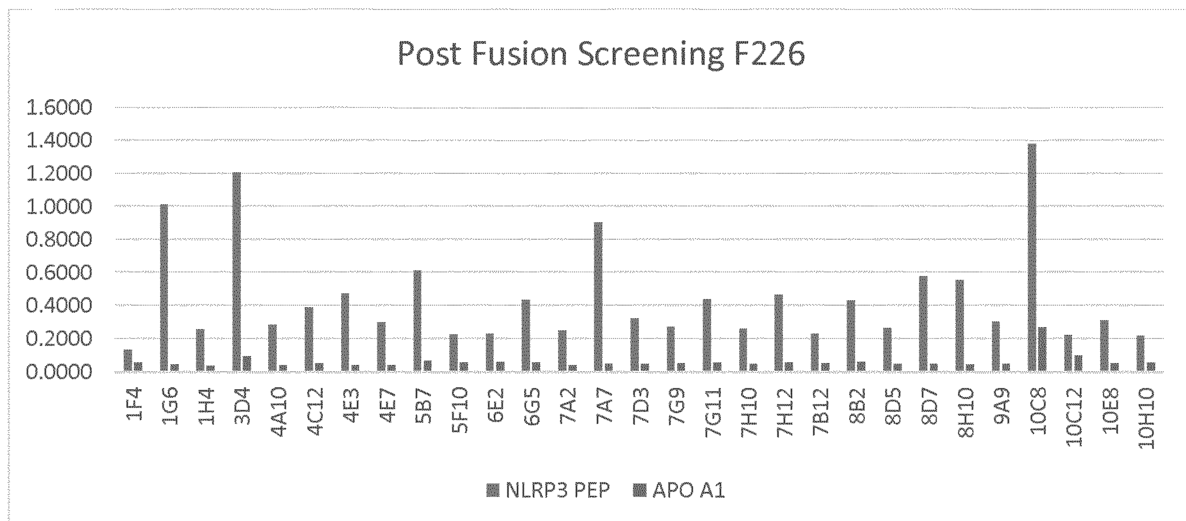

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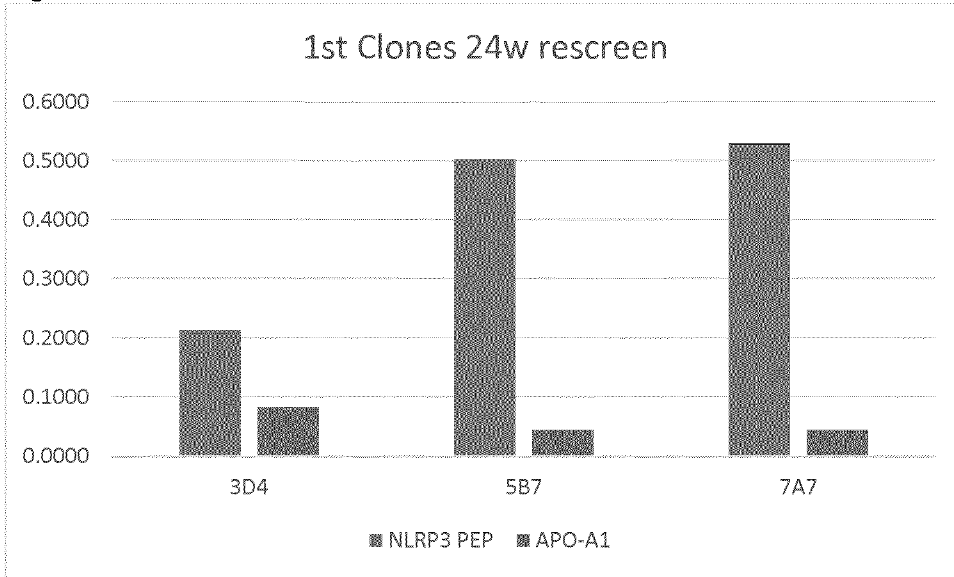

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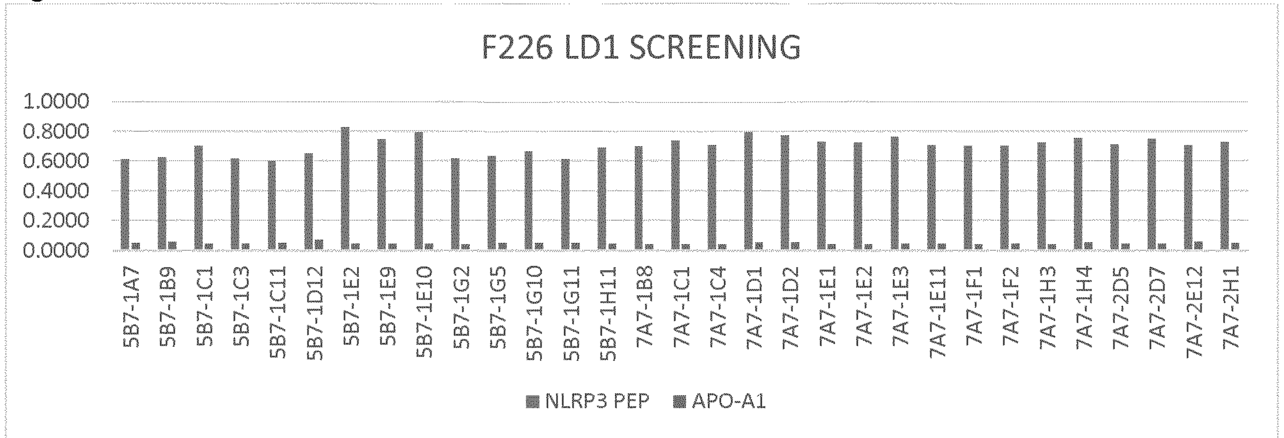

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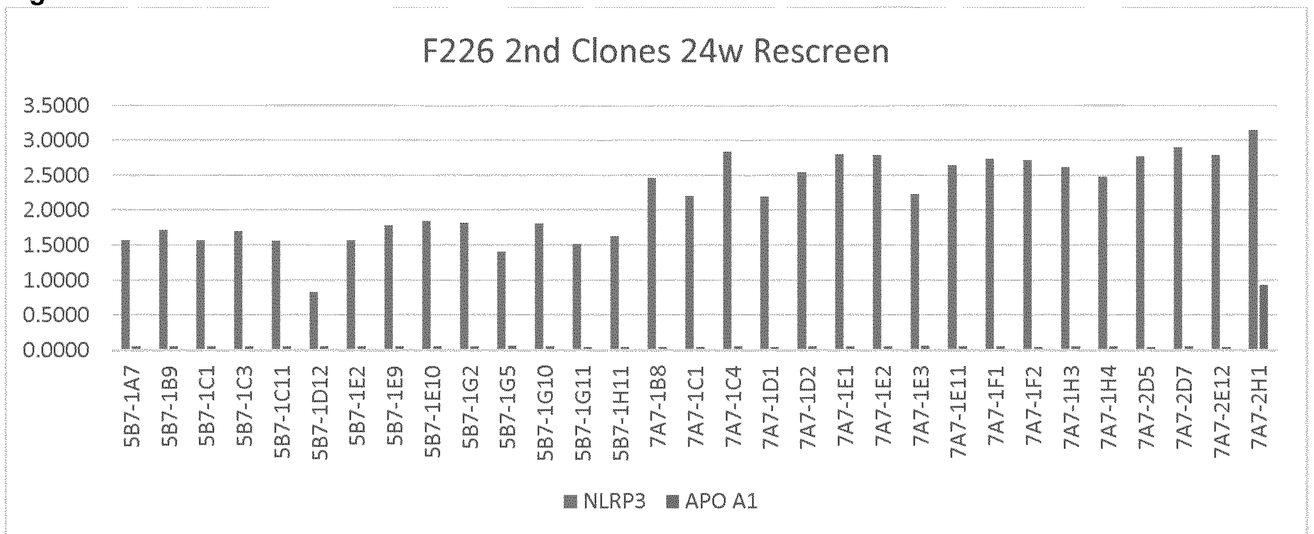

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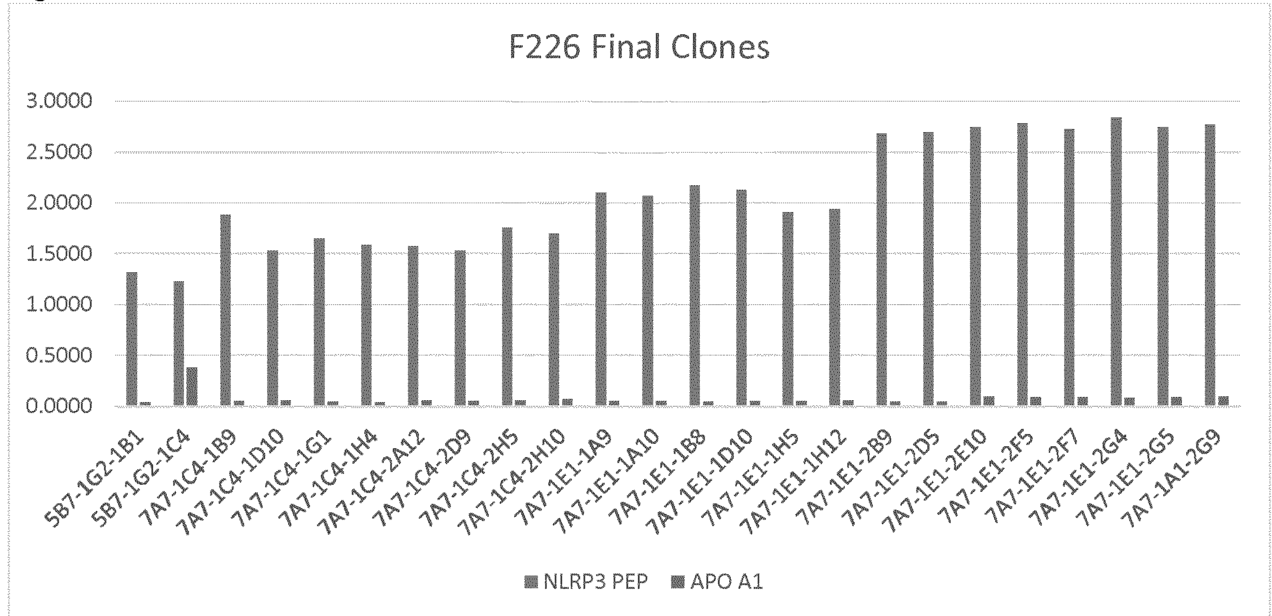

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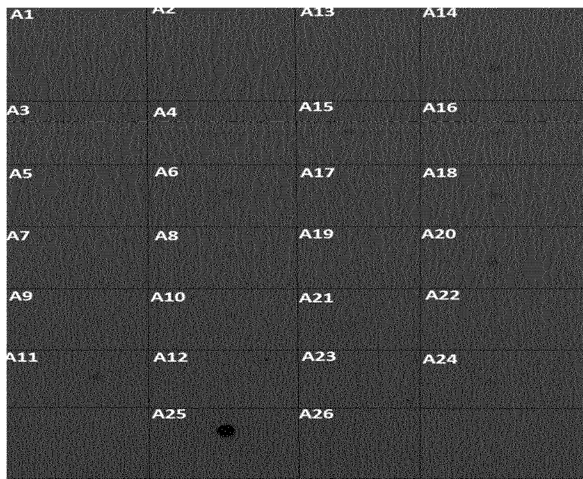

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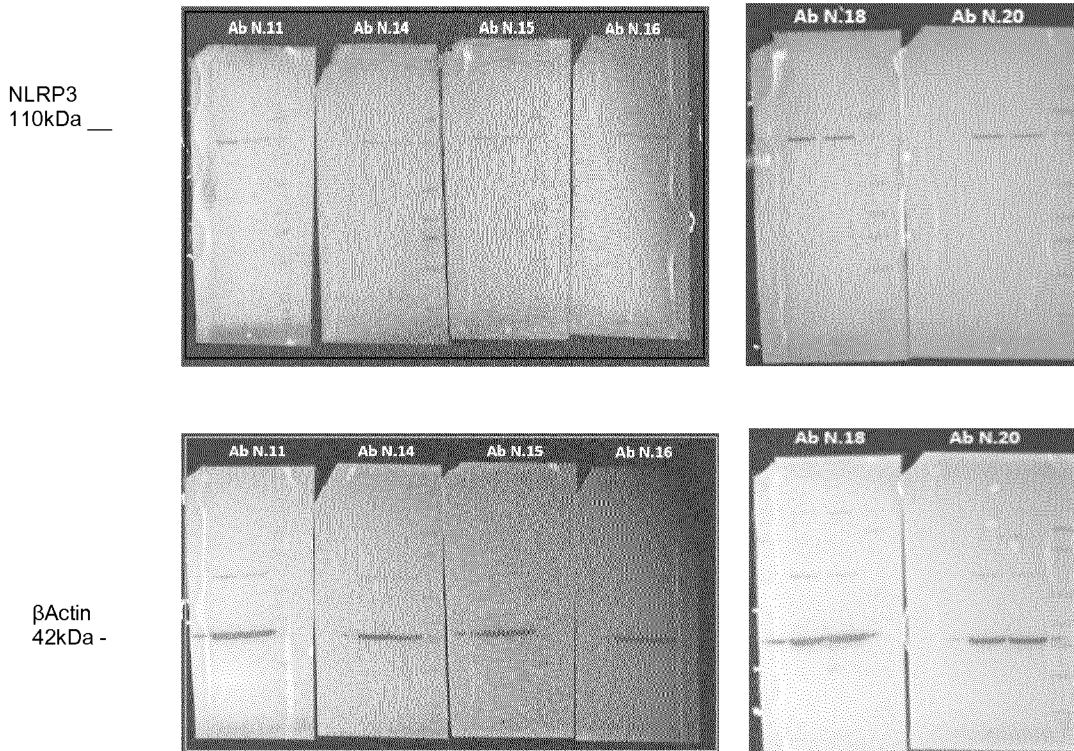

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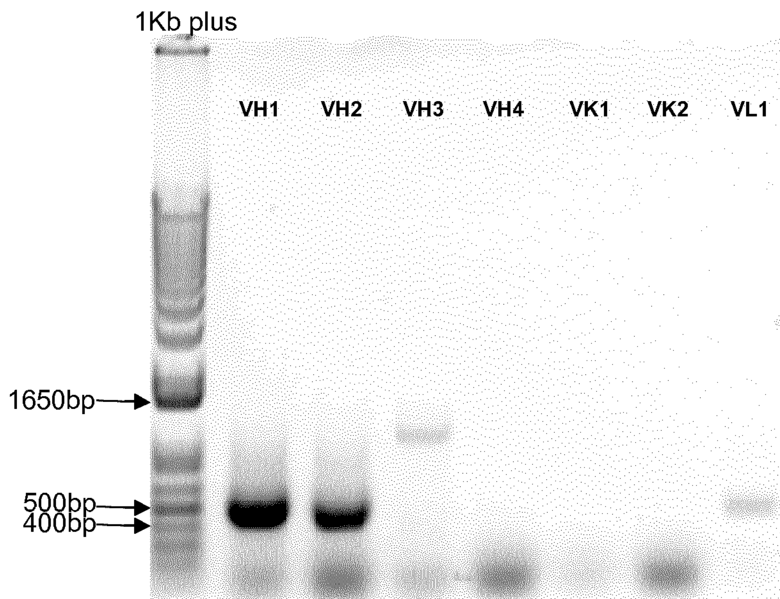

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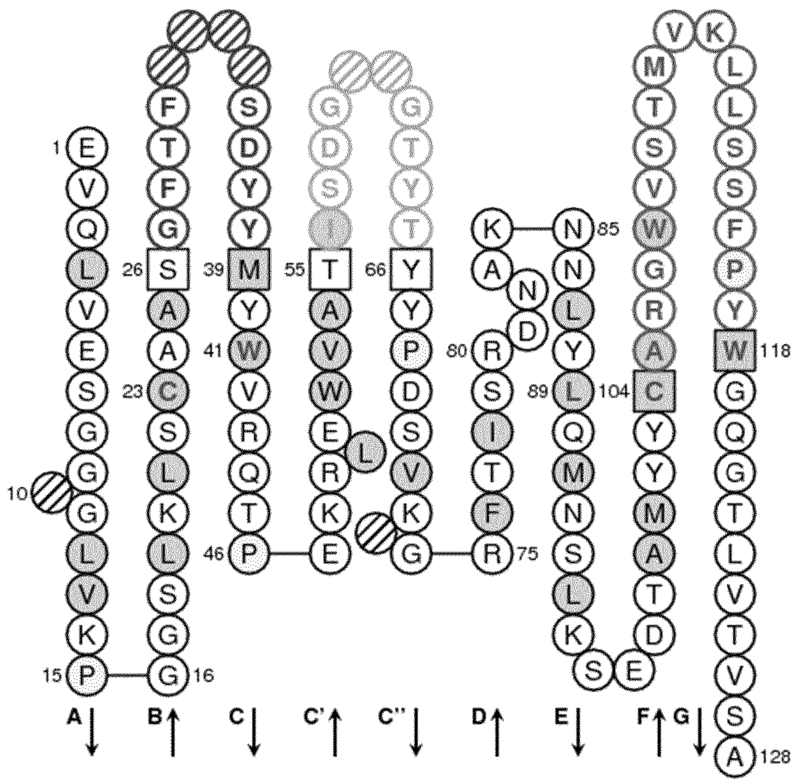
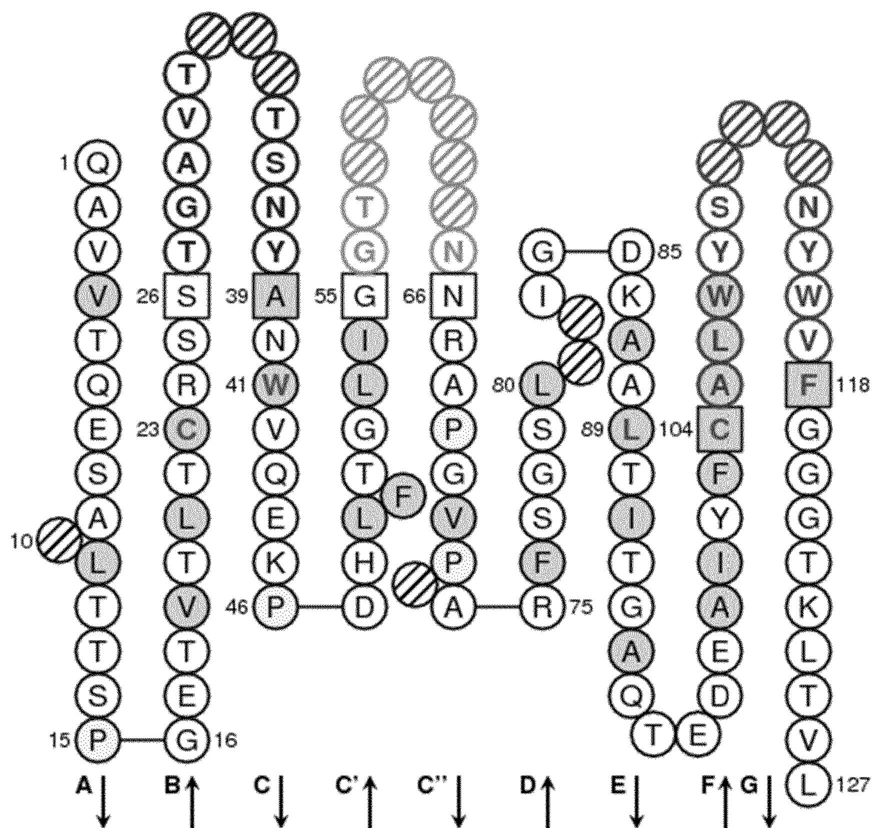


Figure 30:



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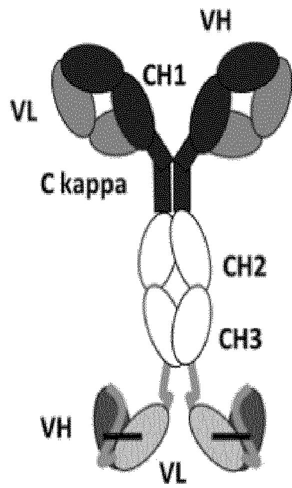
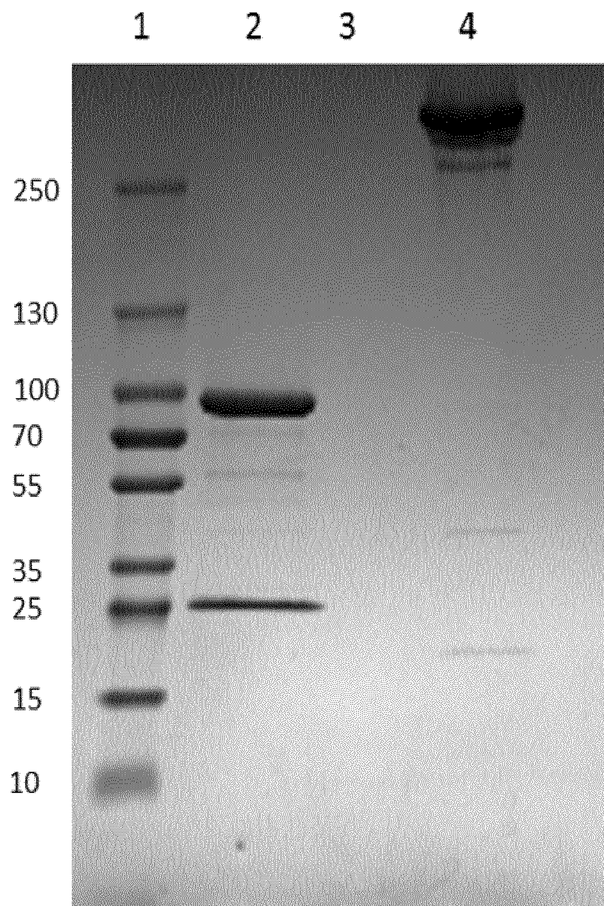


Ab896
pD2610-v5:287423
11471 bp

P_EBNA-human
Ori_pUC
M_Kanamycin-r_'
P_EM7
E_CMV_IE1_Hs
P_CMV_IE1_Hs
CMV_IE1_exon1
Intron_CMVa_IE1_Hs
Ab896
pA_GH-Bt(min)
OriP_EBV
pA_EBNA
M_EBNA_'

1
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000

Figure 32:



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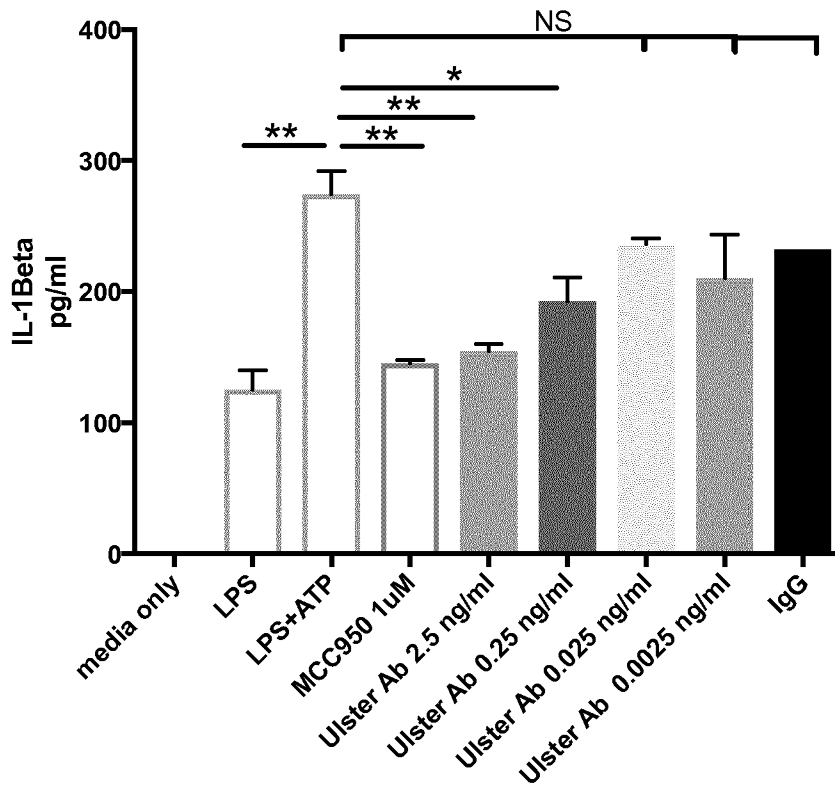
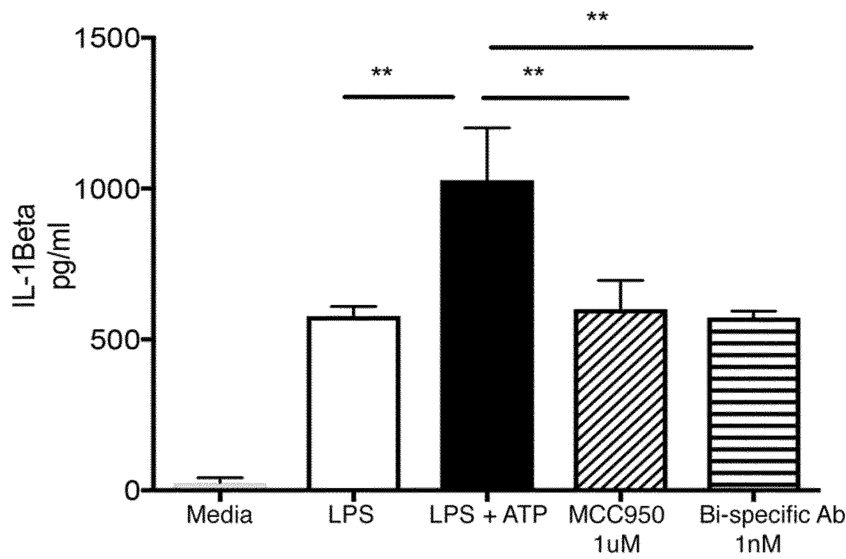


Figure 33b:



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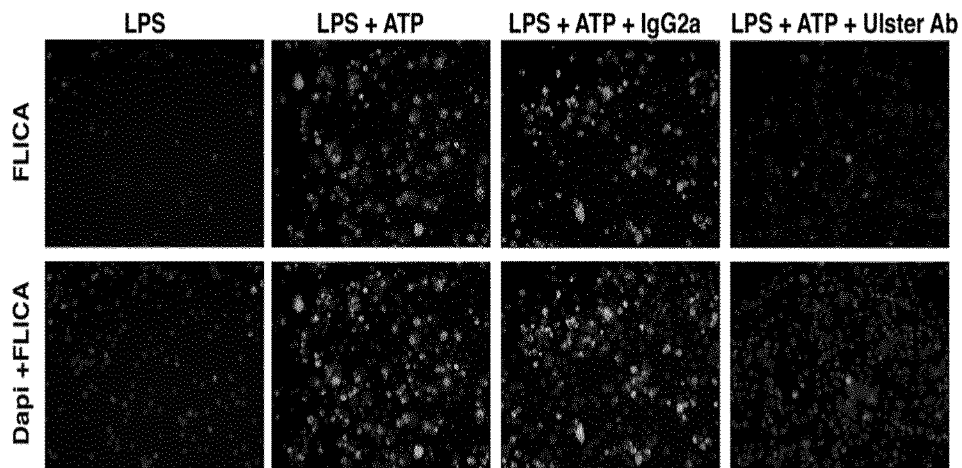
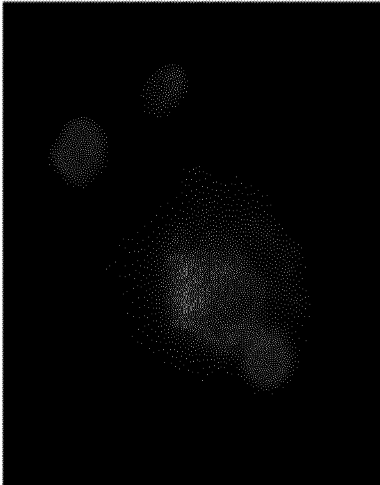
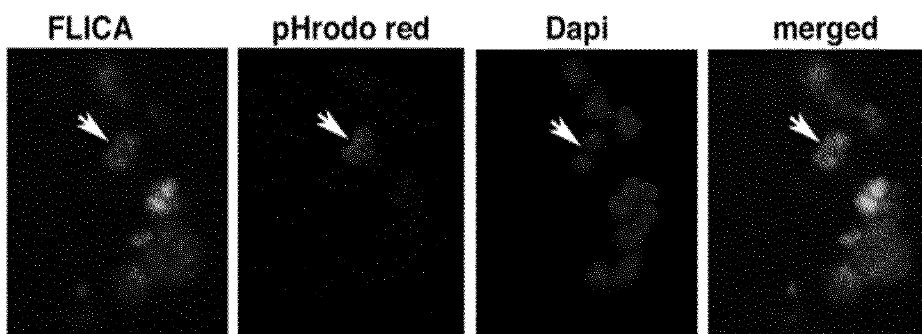


Figure 35:

A.



B.



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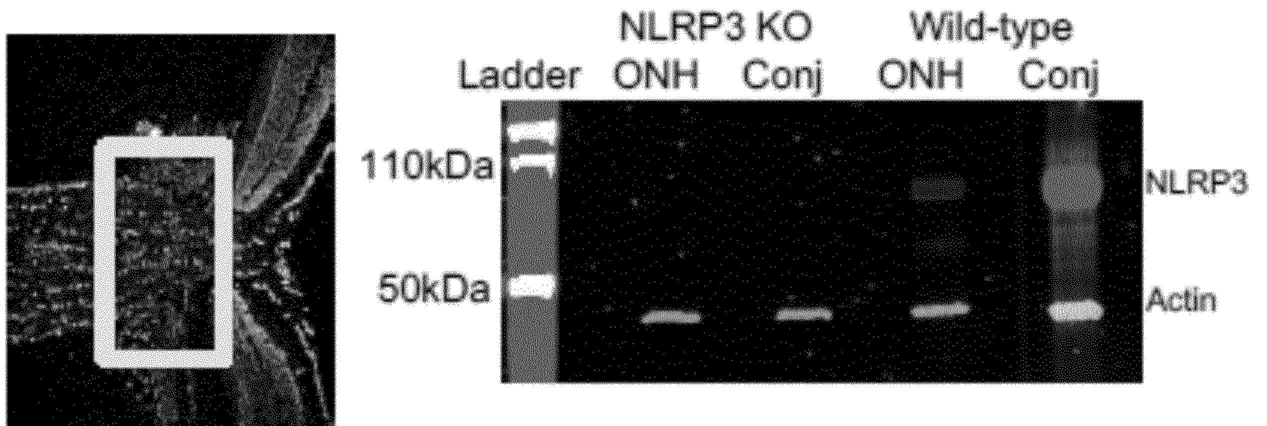

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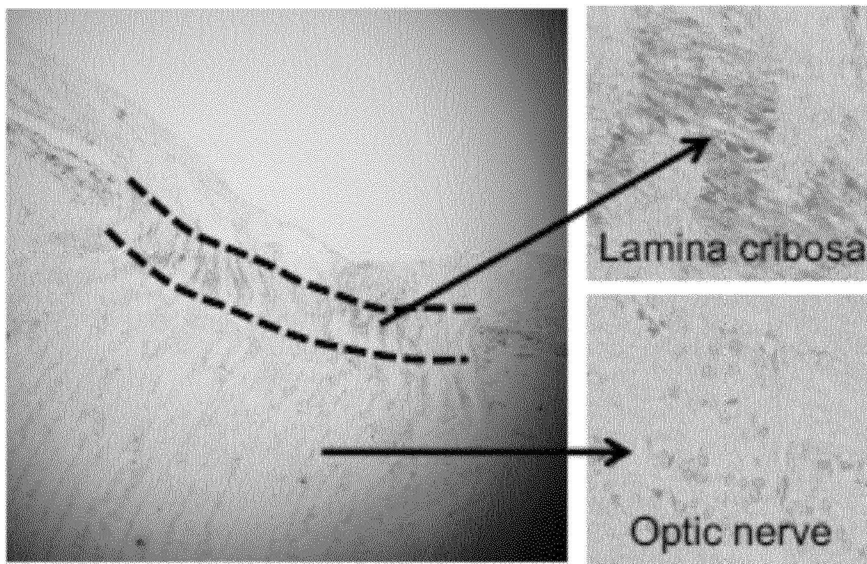

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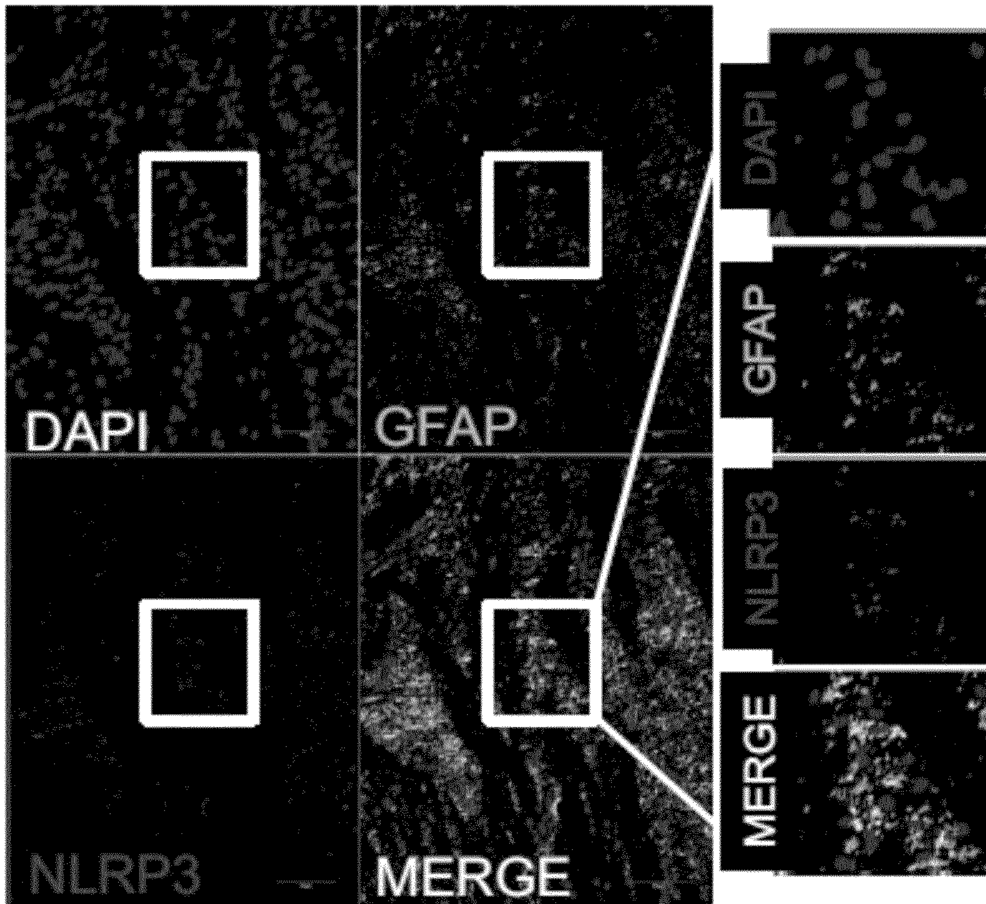

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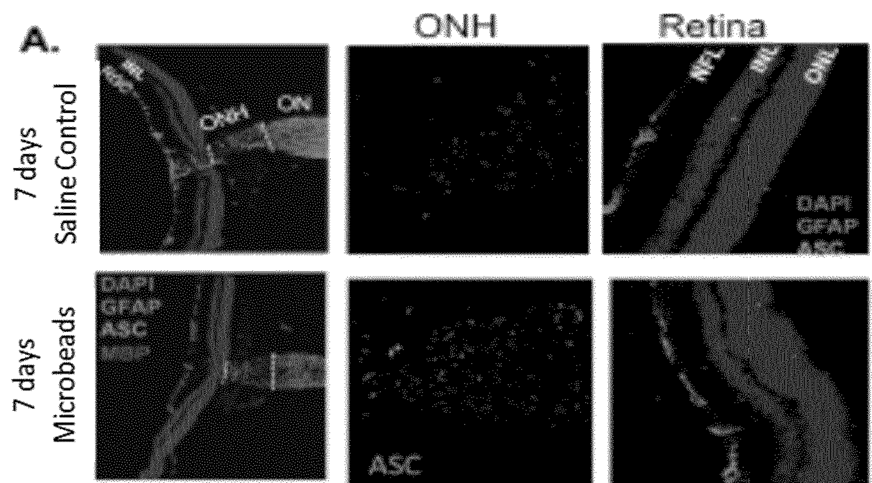

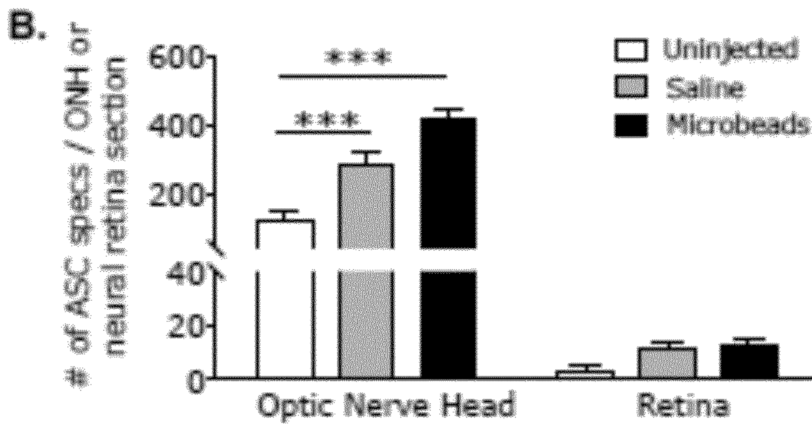

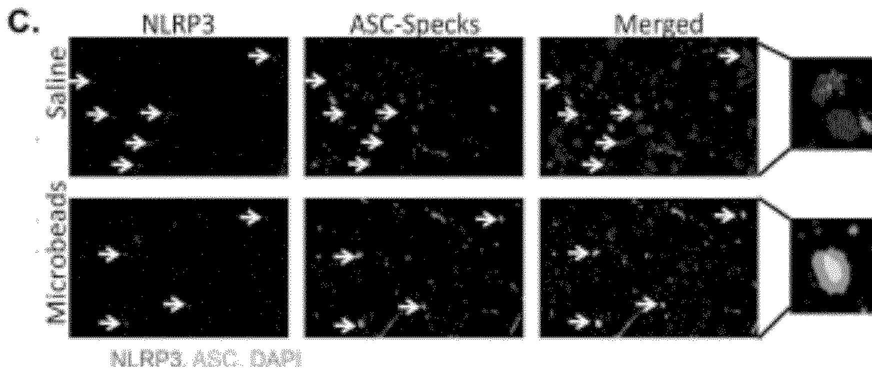

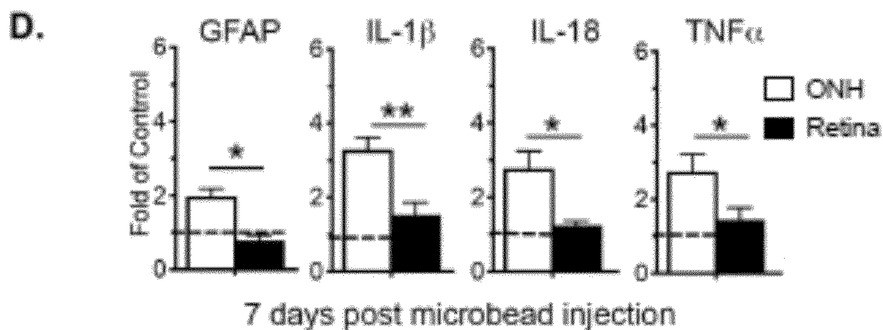

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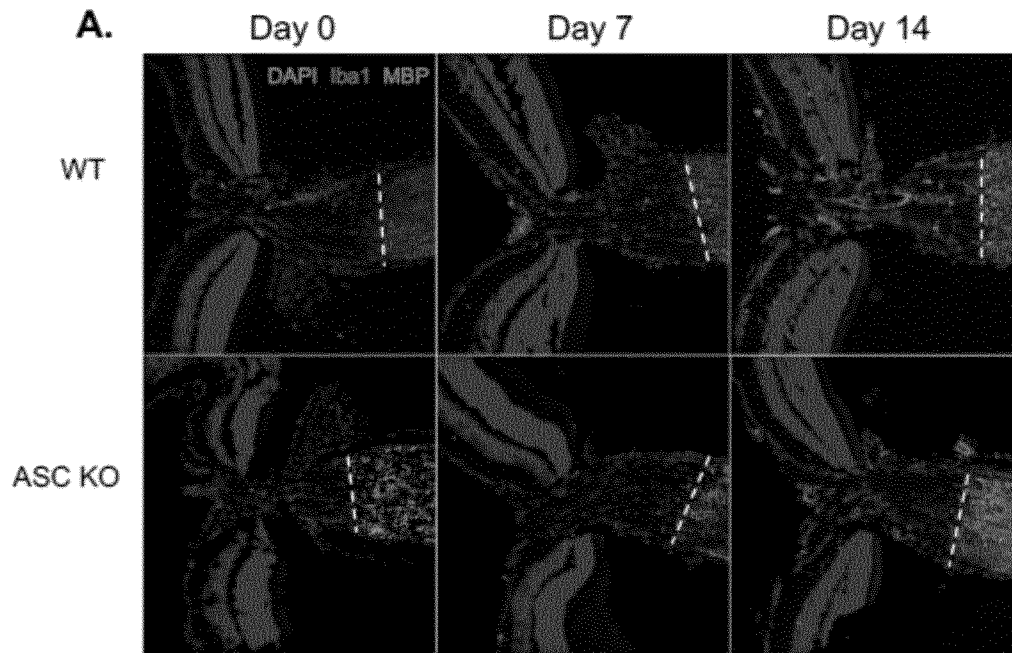

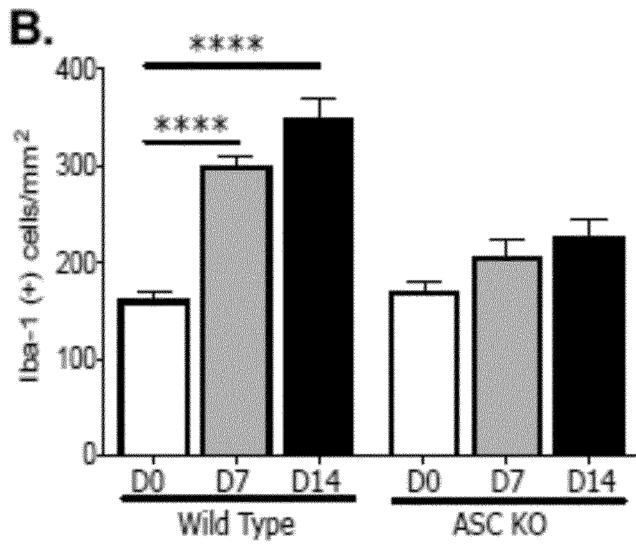

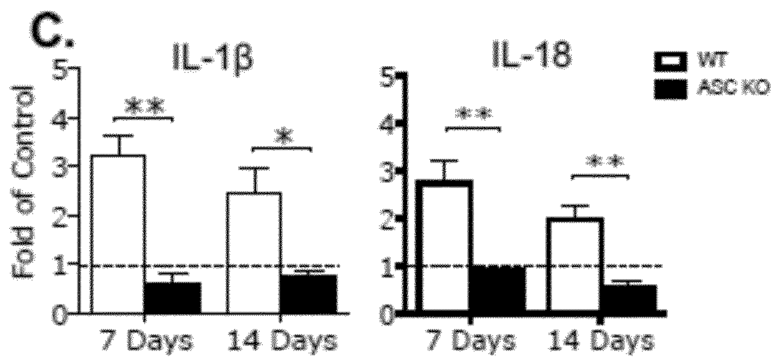

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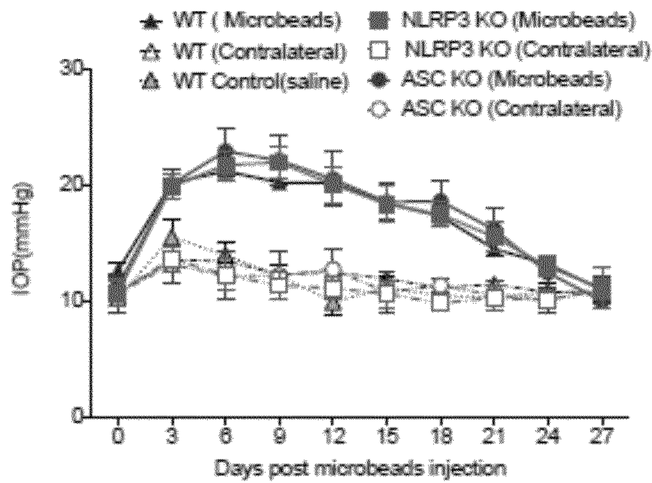
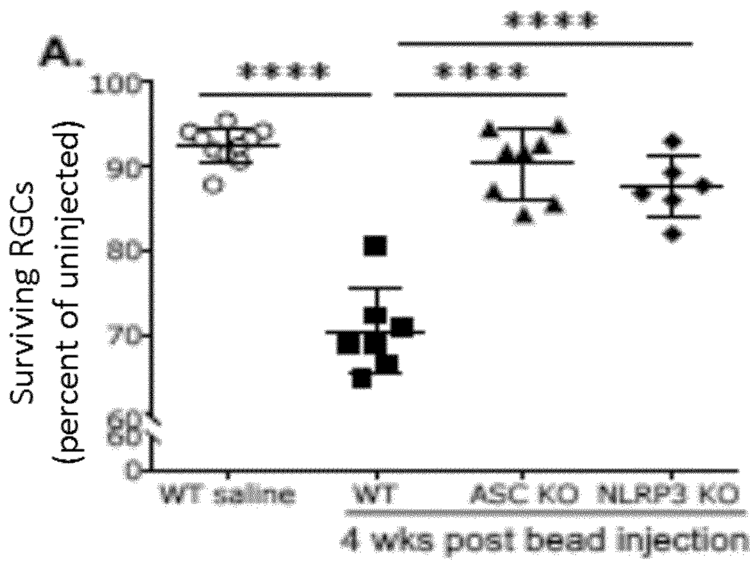




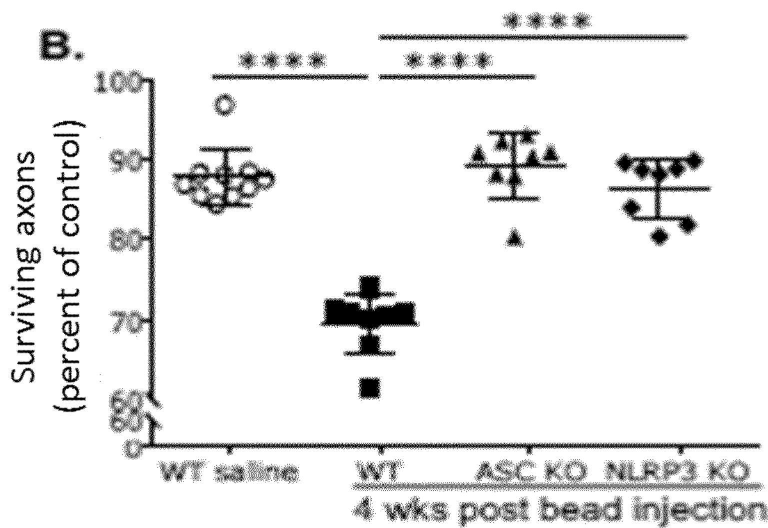


Figure 41:

A.

Beads + vehicle
Beads + MCC950
IOP (mmHg)
40
30
20
10
0
normal IOP range
0 3 7 11 14 18 21 28
Days post microbead injection

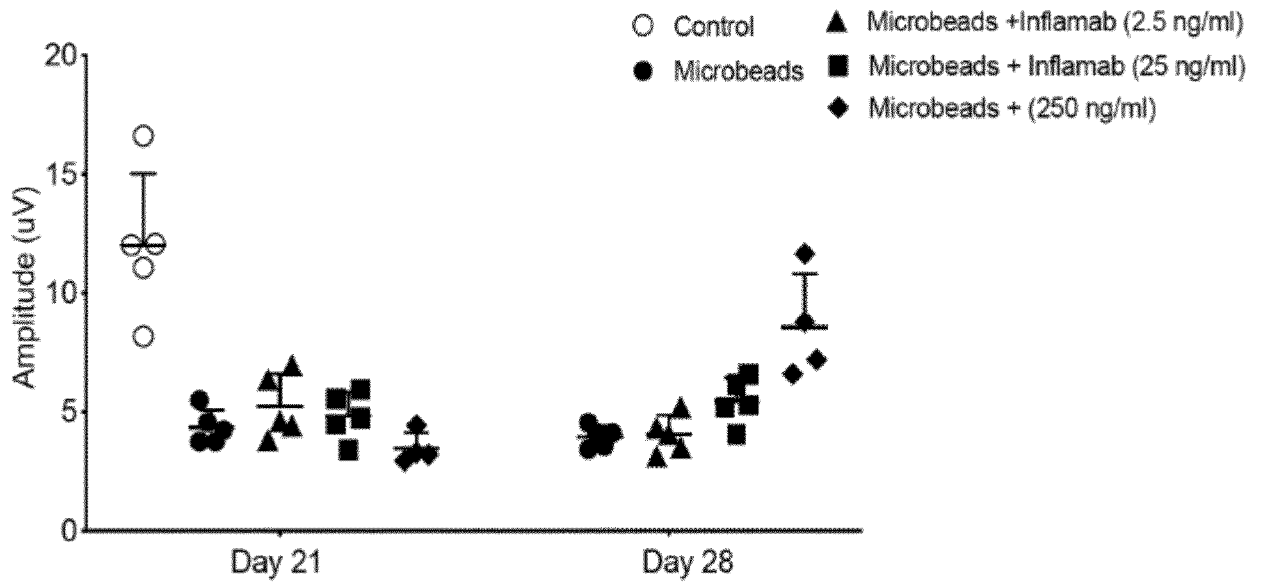
B.

Control (no beads)
Microbeads
Vehicle
MCC950
DAPI (all cells) BRN3a (RGCs)

C.

***
***
RGC density /mm^2 retina
5000
4000
3000
2000
1000
0
Uninjected Beads Uninjected Beads
Vehicle
MCC950 (10mg/kg)

Figure 42:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/074744

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P29/00 C07K16/18 C07K16/28
ADD. A61K39/00

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)
A61P A61K C07K

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, Sequence Search, 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	LIANG FENG ET AL: "NLRP3 inflammasome in retinal ganglion cell loss in optic neuropathy", NEURAL REGENERATION RESEARCH, vol. 11, no. 7, 1 July 2016 (2016-07-01), pages 1077-1078, XP55645694, CN ISSN: 1673-5374, DOI: 10.4103/1673-5374.187036 page 1077 last paragraph -----	1-81

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[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
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"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
29 November 2019	10/12/2019

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 2015/123493 A2 (UNIV NORTHWESTERN [US]) 20 August 2015 (2015-08-20) the whole document	1-81
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International application No
PCT/EP2019/074744

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