

generation of therapeutic antibodies, named nanobodies, which are suitable for intravenous oral or topical administration, can be readily manufactured in mono- or multi-valent forms exhibiting high potency and binding affinity to one or more targets.

Single domain VHH antibodies, including methods for their preparation, are described, for example, in WO2004062551.

Mice in which the λ (lambda) light (L) chain locus and/or the λ and κ (kappa) L chain loci have been functionally silenced and antibodies produced by such mice are described in U.S. Patent Nos. 7,541,513 and 8,367,888. Recombinant production of heavy-chain-only antibodies in mice and rats has been reported, for example, in WO2006008548; U.S. Application Publication No. 20100122358; Nguyen et al., 2003, *Immunology*; 109(1), 93-101; Brüggemann et al., *Crit. Rev. Immunol.*; 2006, 26(5):377-90; and Zou et al., 2007, *J Exp Med*; 204(13): 3271-3283. The production of knockout rats via embryo microinjections of zinc-finger nucleases is described in Geurts et al., 2009, *Science*, 325(5939):433. The characterization of immunoglobulin heavy chain knockout rats is reported by Ménoret et al., 2010, *European Journal of Immunology*, 40:2932-2941. Soluble heavy chain-only antibodies and transgenic rodents comprising a heterologous heavy chain locus producing such antibodies are described in U. S. Patent Nos. 8,883,150. CAR-T structures comprising single-domain antibodies as binding (targeting) domain are described, for example, in Iri-Sofla et al., 2011, *Experimental Cell Research* 317:2630-2641 and Jamnani et al., 2014, *Biochim Biophys Acta*, 1840:378-386.

Despite recent advances, there is a need for improved methods for the production of heavy chain-only antibodies, which have less propensity for aggregation and retain high affinity for their intended target.

SUMMARY

The present disclosure is based, at least in part, on the finding that heavy chain-only antibodies (HCABs) with less propensity for aggregation can be prepared by replacement of the native amino acid residue at the first position of the fourth framework region (FR4) of a HCAB by another amino acid residue that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position. Such hydrophobic patches are normally buried in the interface with the antibody light chain constant region but become surface exposed in HCABs and are, at least partially, for the unwanted aggregation and light chain association of HCABs.

Disclosed is an isolated human or chimeric heavy chain-only antibody (HCAB) comprising a heavy chain variable (VH) domain, comprising complementarity determining regions (CDRs) and framework regions (FRs), having binding affinity to a target antigen in the absence of an antibody light chain, wherein in said VH domain the native amino acid residue at the first position of the fourth framework region (FR4) of said HCAB is substituted by a different amino acid residue that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In one embodiment, the HCAB is a human antibody.

In another embodiment, in the HCAB the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue.

In yet another embodiment, in the HCAb the native amino acid residue at the first position of FR4 is substituted by a positively charged amino acid residue, such as, for example, lysine (K), arginine (R) or histidine (H), preferably arginine (R).

5 In a particular embodiment, the HCAb comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

In all embodiments, the HCABs may comprise one or more further mutations in one or more framework regions.

In all embodiments, the HCABs may have reduced propensity for aggregation relative to a corresponding antibody comprising the native amino acid residue at the first amino acid residue in FR4.

10 In all embodiments, the HCABs may have a binding affinity of about 1pM to about 1μM to its target antigen.

Disclosed is an isolated human or chimeric heavy chain-only antibody (HCAb) having binding affinity to a target antigen in the absence of an antibody light chain, comprising a heavy chain variable (VH) domain comprising complementarity determining regions (CDRs) and framework regions (FRs), wherein said HCAb comprises a tryptophane (T) to arginine (R) substitution at the first amino acid position in the fourth FR region (FR4) of the native human VH amino acid sequence.

15 In one embodiment, the HCAb further comprises a heavy chain constant (CH) domain, lacking a CH1 region, and can be an IgG antibody, such as an IgG1 antibody.

In another embodiment, the HCAb comprises one or more further mutations in one or more FR regions.

20 In yet another embodiment, the HCAb has reduced propensity for aggregation relative to a corresponding antibody comprising the native amino acid residue at the first amino acid residue in FR4.

Disclosed is a chimeric antigen receptor (CAR) comprising a heavy chain-only antibody as herein described. In one embodiment, the CAR comprises a single human VH domain.

25 Disclosed is an isolated autonomous human antibody heavy chain variable (VH) domain comprising complementary determining regions (CDRs) and framework regions (FR), having binding affinity to a target antigen comprising a substitution of a different amino acid residue, that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position, for the native amino acid residue at the first amino acid residue in the fourth framework (FR4) region.

30 In one embodiment, in the isolated autonomous human VH domain the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue.

In another embodiment, the native amino acid residue at the first position of FR4 is substituted by a positively charged amino acid residue, such as a lysine (K), arginine (R) or histidine (H), residue, preferably an arginine (R) residue.

35 In a further embodiment, the isolated autonomous human VH domain comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

In a still further embodiment, the isolated autonomous human VH domain comprises one or more further mutations in one or more framework regions.

Disclosed is a multi-valent binding protein containing multiple antigen binding domains that include at least one human VH domain comprising complementarity determining regions (CDRs) and framework regions (FRs), having binding affinity to a target antigen, wherein in said VH domain the native amino acid residue at the first position of the fourth framework region (FR4) of said multi-valent binding protein is substituted by a different amino acid residue that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In one embodiment, in the multi-valent binding protein the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue.

In another embodiment, in the multi-valent binding protein the native amino acid residue at the first position of FR4 is substituted by a positively charged amino acid residue, such as a lysine (K), arginine (R) or histidine (H) residue, preferably an arginine (R) residue.

In a further embodiment, the multi-valent binding protein comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

In all embodiments, the multi-valent binding protein may comprise one or more further mutations in one or more framework regions.

In a further embodiment, the disclosure concerns a recombinant heavy chain-only immunoglobulin (Ig) locus comprising one or more human V gene segments, one or more human D gene segments, and one or more human J gene segments, which when recombined with each other in the genome of a non-human animal, and following affinity maturation, encode a heavy chain variable (VH) region comprising complementarity determining regions (CDRs) and framework (FR) regions, wherein at least one of said human J segments comprises a codon encoding a non-native amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In one aspect, there is provided a recombinant heavy chain-only immunoglobulin (Ig) locus comprising one or more human V gene segments, one or more human D gene segments, and one or more human J gene segments, which when recombined with each other in the genome of a non-human animal, and following affinity maturation, encode a heavy chain variable (VH) region comprising complementarity determining regions (CDRs) and framework (FR) regions, wherein at least one of said human J segments comprises a codon encoding a different amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In one embodiment, the recombinant heavy chain-only Ig locus further comprises a constant (C) region gene segment, encoding an immunoglobulin constant effector region lacking CH1 functionality.

In various embodiments, the recombinant heavy chain-only Ig locus comprises two to 40 D gene segments, and/or two to 20 J gene segments.

In another embodiment, more than one of the human J segments comprise a codon encoding a non-native amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In yet another embodiment, in the recombinant heavy chain-only Ig locus of all of the human J segments comprise a codon encoding a non-native amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.

In a further embodiment, in the encoded heavy VH region the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue, such as a lysine (K), arginine (R) or histidine (H), preferably an arginine (R) residue.

In a still further embodiment, the encoded VH region comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

In yet another embodiment, the recombinant heavy chain-only Ig locus comprises a J4 gene segment in which the codon for W is replaced by R.

5 In all embodiments, the recombinant heavy chain-only Ig locus encodes a VH region which comprises one or more further mutations in one or more framework regions.

In a further embodiment, the recombinant heavy chain-only Ig locus encodes a human or humanized heavy chain-only antibody comprising a VH region as hereinabove described.

10 In another aspect, there is provided a transgenic non-human animal comprising a recombinant heavy chain-only Ig locus as hereinabove described.

In various embodiment, the transgenic non-human animal is a non-human mammal, as non-human vertebrate, a rodent, a mouse, or a rat, such as a UniRat™.

15 In another aspect, there is provided a transgenic non-human animal that does not express any functional immunoglobulin light chain genes and comprises a heterologous heavy chain-only Ig locus comprising one or more V gene segments, one or more D gene segments, and one or more J gene segments, which when recombined with each other and following affinity maturation encode a VH domain comprising complementarity determining regions (CDRs) and framework regions (FRs), in which the native amino acid residue at the first position of the fourth framework region (FR4) of said VH domain is substituted by a different amino acid residue that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position, and one or more constant effector region gene segments, each of which encodes an antibody constant effector region including CH1 functionality, wherein the gene segments are arranged such that a V, a D and a J gene segment and a constant region gene segment recombine to produce a rearranged affinity matured heavy chain-only gene locus encoding a heavy chain-only antibody (HCAb).

20 In one embodiment, in the VH domain of said transgenic non-human animal the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue, or a positively charged amino acid residues, such as lysine (K), arginine (R) or histidine (H) residue, preferably an arginine (R) residue.

In another embodiment, in the transgenic non-human animal the VH domain comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

30 In yet another embodiment, in the transgenic non-human animal the heterologous heavy chain-only locus comprises a J4 segment in which a codon for W is replaced by a codon for R.

In all embodiments, the encoded heavy chain-only antibody comprises one or more further mutations in one or more framework regions.

In a further embodiment, the transgenic non-human animal is a mammal, a vertebrate, a rodent, a mouse or a rat, such as a UniRat™.

35 In all aspects, in certain embodiments, the heavy chain-only antibodies herein do not contain mutations in other framework regions, including the FR1, FR2, and FR3 regions.

In all aspect, in certain embodiments, the heavy chain-only antibodies herein do not contain additional framework mutations typically present in camelids, such as camel, llama, dromedary, alpaca or guanaco.

In all aspects, in certain embodiments, the heavy chain-only antibodies herein may comprise one or more further mutations in one or more framework regions, including the FR1, FR2, FR3 and/or FR4 regions, such as, for example, in the FR2 region or in the FR2 and FR4 regions.

In all aspects and embodiments, the target antigens to which the HCAs of the present disclosure has binding affinity include, without limitation, cell surface receptors and tumor antigens, such as, for example, EGFR, ErbB2 (HER2), ErbB3 (HER3), ErbB4 (HER4), CTLA-4/CD152, RANKL, TNF- α , CD20, IL-12/IL-23, IL1- β , IL-17A, IL-17F, CD38, NGF, IGF-1, IL-12, CD20, CD30, CD39, CD73, CD40, PD-1, PDL-1, PD-L2, BCMA, BTLA, thymic stromal lymphopoietin (TSP), Follicle Stimulating Hormone Receptor (FSHR), Prostate Specific Membrane Antigen (PSMA), Prostate Stem Cell Antigen (PSCA), CD137, OX-40, and IL-33.

In all aspects and embodiments, HCAb binding domains may be part of a multi-specific binding protein that bind to multiple different epitopes of the same target antigen or multiple different epitopes on more than one target antigen. Multi-specific, such as bispecific HCAs are specifically included, including, for example, bispecific HCAb structures having the following binding affinities: epithelial cell adhesion molecule (EpCam) $\times$ CD3; CD19 $\times$ CD3; EpCam $\times$ CD3; TNF- α $\times$ IL-17; IL-1 α $\times$ IL-1 β ; CD30 $\times$ CD16A; human epidermal growth factor receptor 2 (HER2) $\times$ HER3; IL-4 $\times$ IL-13; angiopoietin 2 (Ang-2) $\times$ vascular endothelial growth factor a (VEGF-A); Factor IXa $\times$ Factor X; epidermal growth factor receptor (EGFR) $\times$ HER3; IL-17A $\times$ IL-17F; HER2 $\times$ HER3; carcinoembryonic antigen $\times$ CD3; CD20 $\times$ CD3; CD123 $\times$ CD3; BCMA $\times$ CD3, PSMA $\times$ PSCA $\times$ CD3, PSMA $\times$ CD3, PSCA $\times$ CD3, CD19 $\times$ CD22 $\times$ CD3, CD22 $\times$ CD3, CD38 $\times$ PD1, CD38 $\times$ PD-L1, CD38 $\times$ CD73, CD38 $\times$ CD39, PD1 $\times$ CD39 $\times$ CD73, and PD1 $\times$ CD73.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of the human transgene with a J4 gene segment (SEQ ID NO: 40) expressing a R at position 101 in UniRatTM (SEQ ID NO: 41) that express heavy chain-only antibodies, as described in the examples.

FIG. 2 is a diagram of the human transgene with all J segments (SEQ ID NOS 42-47, respectively, in order of appearance) expressing Rs at position 101 in UniRatTM that express heavy chain-only antibodies, as described in the examples.

FIG. 3 shows the J gene usage in UniRatTM and OmniFlicTM, where the latter is a transgenic rat with the same human V gene cluster as UniRatTM but expresses a fixed kappa light chains.

FIG. 4 illustrates that a W $\rightarrow$ R mutation at the first amino acid residue of the fourth framework region (FR4) in heavy chain-only human antibodies inhibits association with lambda light chain.

FIG. 5 Free lambda protein association with heavy chain antibodies in the same CDR3 family. The figure shows a multiple sequence alignment of 11 VH sequences from heavy chain antibodies in the same CDR3 family (SEQ ID NOS 48-58, respectively, in order of appearance). All of these sequences contain a W at position 101.

CLAIMS:

1. A recombinant heavy chain-only immunoglobulin (Ig) locus comprising one or more human V gene segments, one or more human D gene segments, and one or more human J gene segments, which when recombined with each other in the genome of a non-human animal, and following affinity maturation, encode a heavy chain variable (VH) region comprising complementarity determining regions (CDRs) and framework (FR) regions, wherein at least one of said human J segments comprises a codon encoding a different amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.
2. The recombinant heavy chain-only Ig locus of claim 1, further comprising a constant (C) region gene segment, encoding an immunoglobulin constant effector region lacking CH1 functionality.
3. The recombinant heavy chain-only Ig locus of claim 1 or 2, comprising 2 to 40 D gene segments, and 2 to 20 J gene segments.
4. The recombinant heavy chain-only Ig locus of claim 3, wherein more than one of said human J gene segments comprise a codon encoding a different amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.
5. The recombinant heavy chain-only Ig locus of claim 4, wherein all of said human J gene segments comprise a codon encoding a different amino acid residue at the first position of the fourth framework region (FR4) that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position.
6. The recombinant heavy chain-only Ig locus of claim 5, wherein in the encoded VH region, the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue.
7. The recombinant heavy chain-only Ig locus of claim 5, wherein in the encoded VH region, the native amino acid residue at the first position of FR4 is substituted by a positively charged amino acid residue.

8. The recombinant heavy chain-only Ig locus of claim 7, wherein in the encoded VH region, the positively charged amino acid residue is selected from the group consisting of lysine (K), arginine (R) and histidine (H).
9. The recombinant heavy chain-only Ig locus of claim 8, wherein in the encoded VH region, the positively charged amino acid residue is arginine (R).
10. The recombinant heavy chain-only Ig locus of claim 9, wherein the encoded VH region comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.
11. The recombinant heavy chain-only Ig locus of claim 10, comprising a J4 gene segment in which the codon for W is replaced by R.
12. The recombinant heavy chain-only Ig locus of any one of claims 1 to 11, wherein the encoded VH region comprises one or more further mutations in one or more framework regions.
13. The recombinant heavy chain-only Ig locus of any one of claims 1 to 12, encoding a human or humanized heavy chain-only antibody comprising said VH region.
14. A transgenic non-human animal comprising a recombinant heavy chain-only Ig locus of any one of claims 1 to 13.
15. The transgenic non-human animal of claim 14, which is a non-human mammal.
16. The transgenic non-human animal of claim 15, which is a rodent.
17. The transgenic non-human animal of claim 16, which is a rat or a mouse.
18. The transgenic non-human animal of claim 17, which is a rat.
19. The transgenic non-human animal of claim 18, which is a UniRat™.
20. A transgenic non-human animal that does not express any functional immunoglobulin light chain genes and comprises a heterologous heavy chain-only Ig locus comprising one or more V gene segments, one or more D gene segments, and one or more J gene segments, which when recombined with each other and following affinity maturation encode a VH domain comprising complementarity determining regions (CDRs) and framework regions (FRs), in which the native

amino acid residue at the first position of the fourth framework region (FR4) of said VH domain is substituted by a different amino acid residue that is capable of disrupting a surface-exposed hydrophobic patch comprising or associated with the native amino acid residue at that position, and one or more constant effector region gene segments, each of which encodes an antibody constant effector region including CH1 functionality, wherein the gene segments are arranged such that a V, a D and a J gene segment and a constant region gene segment recombine to produce a rearranged affinity matured heavy chain-only gene locus encoding a heavy chain-only antibody (HCAb).

21. The transgenic non-human animal of claim 20, wherein in said VH domain, the native amino acid residue at the first position of FR4 is substituted by a polar amino acid residue.

22. The transgenic non-human animal of claim 20, wherein in said VH domain, the native amino acid residue at the first position of FR4 is substituted by a positively charged amino acid residue.

23. The transgenic non-human animal of claim 22, wherein in said VH domain, the positively charged amino acid residue is selected from the group consisting of lysine (K), arginine (R) and histidine (H).

24. The transgenic non-human animal of claim 23, wherein in said VH domain, the positively charged amino acid residue is arginine (R).

25. The transgenic non-human animal of claim 24, wherein said VH domain comprises a tryptophan (W) to arginine (R) substitution at the first amino acid residue in the fourth framework (FR4) region.

26. The transgenic non-human animal of claim 25, wherein said heterologous heavy chain-only locus comprises a J4 segment in which a codon for W is replaced by a codon for R.

27. The transgenic non-human animal of any one of claims 20 to 26, wherein the encoded heavy chain-only antibody comprises one or more further mutations in one or more framework regions.

28. The transgenic non-human animal of any one of claims 20 to 27, which is a mammal.

29. The transgenic non-human animal of claim 28, which is a rodent.

30. The transgenic non-human animal of claim 29, which is a rat or a mouse.
31. The transgenic non-human animal of claim 30, which is a rat.
32. The transgenic non-human animal of claim 31, which is a UniRat™.