

IL-1R-I BINDING POLYPEPTIDE

Field of the invention

The present disclosure relates to a class of engineered polypeptides having a binding affinity for interleukin-1 receptor type-I (in the following referred to as IL-1R-I). The present disclosure also relates to the use of such an IL-1R-I binding polypeptide as a therapeutic, prognostic and/or diagnostic agent.

Background

The central role of the IL-1 system in the initiation and maintenance of inflammatory response, as well as its role in innate and adaptive branches of the immune system, makes it an attractive target for pharmaceutical intervention (Dinarello, 2009, *Annu Rev Immunol*, 27:519-50; Sims and Smith, 2010, *Nat Rev Immunol*.10(2):89-102). Since the IL-1 system in many cases plays the role of initiator and master cytokine in inflammatory response, many aspects of the progression of a pathological immune response can be modulated by such intervention. The biological activity of IL-1 β can be pharmacologically inhibited by antibodies that prevent, or modulate its binding to the type IL-1 receptor IL-1R-I.

The biological activity of the IL-1 system can also be modulated by antagonizing the IL-1R. The recombinant form of endogenous IL-1Ra denoted anakinra (Kineret[®]) is described in e.g. US 6599873, EP 0343684 and EP 0541920. Anakinra has shown clinical efficacy in rheumatoid arthritis, cryopyrin-associated periodic syndromes (CAPS; Koné-Paut and Galeotti, 2014, *Expert Rev Clin Immunol*,10(1):7-18), as well as in a large number of other inflammatory and autoimmune conditions (Dinarello *et al*, 2011, *Blood*, 117(14):3720-32; Dinarello *et al*, 2012, *Nat Rev Drug Discov*,11(8):633-52)).

The IL-1 receptor can also be antagonized by antibodies that bind to IL-1R-I subunit and prevent ligand-mediated IL-1 receptor activation and signaling. Several such antibodies are described in WO 2004/022718, which discloses IL-1R-I antagonists such as 15C4. Other examples of IL-1 receptor antagonizing antibodies binding to the IL-1R-I subunit are described in WO 2010/052505, which discloses IL-1R-I antagonists such as Antibody 9GL.

Yet another example of an IL-1 receptor antagonizing antibody, denoted 2D8 is described in WO 2005/023872.

The unpredictable and chronic nature of inflammatory diseases, as well as a high unmet medical need, warrants the development of new modes of treatment. Since tissue penetration rate is negatively associated with the size of the molecule, relatively large antibody molecules inherently have poor tissue distribution and penetration capacity.

Thus, the use of antibodies, or other large molecule drugs, is not always optimal for therapy and there is continued need for provision of agents with a high affinity for the IL-1 receptor.

Summary of the invention

It is an object of the present disclosure to provide new IL-1R-I binding agents, which could for example be used for therapeutic, prognostic and diagnostic applications.

It is an object of the present disclosure to provide a molecule allowing for efficient therapy targeting various forms of inflammatory, autoinflammatory and autoimmune diseases.

It is furthermore an object of the present disclosure to provide a molecule suitable for prognostic and diagnostic applications.

These and other objects, which are evident to the skilled person from the present disclosure, are met by different aspects of the invention as claimed in the appended claims and as generally disclosed herein.

Thus, in the first aspect of the disclosure, there is provided an IL-1R-I binding polypeptide, comprising an IL-1R-I binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EX_2X_3X_4X_5X_6X_7EIX_{10}X_{11}LPNLX_{16}RX_{18}QYX_{21}AFIX_{25}X_{26}LX_{28}D$
(SEQ ID NO:1686)

wherein, independently from each other,

X_2 is selected from A, D, E, F, H, I, L, Q, S, T and V;
 X_3 is selected from A, D, E, F, H, I, K, L, N, Q, R, S, T, V, W and Y;
 X_4 is selected from A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
 X_5 is selected from A, I and V;

- X₆ is selected from F, H, I, Q, R, T, V and Y;
 X₇ is selected from A, D, E, F, G, H, I, L, M, Q, S, T, V, W and Y;
 X₁₀ is selected from F and Y;
 X₁₁ is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and
 5 Y;
 X₁₆ is selected from N and T;
 X₁₈ is selected from K, R and S;
 X₂₁ is selected from Q, T and V;
 X₂₅ is selected from I, M, R, V and Y;
 10 X₂₆ is selected from K and S, and
 X₂₈ is selected from F, I, L and M,
 and
 ii) an amino acid sequence which has at least 96 % identity to the
 15 sequence defined in i) provided that X₅ is I or V.

The above definition of a class of sequence related, IL-1R-I binding
 polypeptides is based on a statistical analysis of a number of random
 polypeptide variants of a parent scaffold, that were selected for their
 20 interaction with IL-1R-I in several different selection experiments. The
 identified IL-1R-I binding motif, or "*BM*", corresponds to the target binding
 region of the parent scaffold, which region constitutes two alpha helices within
 a three-helical bundle protein domain. In the parent scaffold, the varied amino
 acid residues of the two *BM* helices constitute a binding surface for interaction
 25 with the constant Fc part of antibodies. In the present disclosure, the random
 variation of binding surface residues and subsequent selection of variants
 have replaced the Fc interaction capacity with a capacity for interaction with
 IL-1R-I.

As the skilled person will realize, the function of any polypeptide, such
 30 as the IL-1R-I binding capacity of the polypeptide of the present disclosure, is
 dependent on the tertiary structure of the polypeptide. It is therefore possible
 to make minor changes to the sequence of amino acids in a polypeptide
 without affecting the function thereof. Thus, the disclosure encompasses
 modified variants of the IL-1R-I binding polypeptide, which have retained IL-
 35 1R-I binding characteristics.

In this way, also encompassed by the present disclosure is an IL-1R-I
 binding polypeptide comprising an amino acid sequence with 96 % or greater

identity to a polypeptide as defined in i). Thus, certain sequence variants of the sequence defined in i) are encompassed by the above definition under the proviso that within such sequence variants X_5 is I or V. For example, it is possible that an amino acid residue belonging to a certain functional grouping of amino acid residues (e.g. hydrophobic, hydrophilic, polar etc) could be
5 exchanged for another amino acid residue from the same functional group.

In some embodiments, such changes may be made in any position of the sequence of the IL-1R-I binding polypeptide as disclosed herein. In other embodiments, such changes may be made only in the non-variable positions,
10 also denoted scaffold amino acid residues. In such cases, changes are not allowed in the variable positions, i.e. positions denoted with an "X" in sequence i).

The term "% identity", as used throughout the specification, may for example be calculated as follows. The query sequence is aligned to the target
15 sequence using the CLUSTAL W algorithm (Thompson *et al*, 1994, Nucleic Acids Research, 22: 4673-4680). A comparison is made over the window corresponding to the shortest of the aligned sequences. The shortest of the aligned sequences may in some instances be the target sequence. In other instances, the query sequence may constitute the shortest of the aligned
20 sequences. The amino acid residues at each position are compared and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

As used herein " X_n " and " X_m " are used to indicate amino acids in
25 positions n and m in the sequence i) as defined above, wherein n and m are integers which indicate the position of an amino acid within said sequence as counted from the N-terminal end of said sequence. For example, X_3 and X_7 indicate the amino acid in position three and seven, respectively, from the N-terminal end of sequence i).

In embodiments according to the first aspect, there are provided polypeptides wherein X_n in sequence i) is independently selected from a group of possible residues according to Table 1. The skilled person will appreciate that X_n may be selected from any one of the listed groups of possible residues and that this selection is independent from the selection of
35 amino acids in X_m , wherein $n \neq m$. Thus, any of the listed possible residues in position X_n in Table 1 may be independently combined with any of the listed possible residues in any other variable position in Table 1.

The skilled person will appreciate that Table 1 is to be read as follows:

In one embodiment according to the first aspect, there is provided a polypeptide wherein amino acid residue "X_n" in sequence i) is selected from "Possible residues". Thus, Table 1 discloses several specific and

- 5 individualized embodiments of the first aspect of the present disclosure. For example, in one embodiment according to the first aspect, there is provided a polypeptide wherein X₄ in sequence i) is selected from A, D, E, I, K, Q, S and V, and in another embodiment according to the first aspect, there is provided a polypeptide wherein X₄ in sequence i) is selected from A, E, I, K, Q and V.
- 10 For avoidance of doubt, the listed embodiments may be freely combined in yet other embodiments. For example, one such combined embodiment is a polypeptide in which X₄ is selected from A, E, I, K, Q and V, while X₆ is selected from H, Q, T and Y, and X₁₁ is selected from H, R and Y.

- 15 Table 1: Embodiments of the first aspect of the present disclosure

Residue X _n	Possible residues
X ₂	A, E, F, H, I, L, Q, S, T, V
X ₂	A, F, H, I, L, Q, S, T, V
X ₂	A, H, I, L, Q, S, T, V
X ₂	A, H, I, L, Q, T, V
X ₂	A, H, I, L, T, V
X ₂	A, F, I, L, T, V
X ₂	A, I, L, T, V
X ₂	A, I, T, V
X ₂	A, I, L, T
X ₂	A, L, T, V
X ₂	A, I, L, V
X ₂	I, L, T, V
X ₂	A, I, T
X ₂	A, I, V
X ₂	A, T, V
X ₂	I, T, V

X ₂	I, L, T
X ₂	I, L, V
X ₂	L, T, V
X ₂	I, V
X ₂	I, L
X ₂	I, T
X ₂	I, V
X ₂	L, T
X ₂	L, V
X ₂	T, V
X ₂	A, I
X ₂	A, T
X ₂	A, V
X ₂	L
X ₂	T
X ₂	V
X ₂	I
X ₂	A
X ₃	A, D, E, F, H, I, K, L, Q, R, S, T, V, W, Y

X ₃	A, D, E, F, H, I, K, L, N, Q, R, S, T, V, Y
X ₃	A, D, E, H, I, K, L, N, Q, R, S, T, V, Y
X ₃	A, D, E, F, H, I, K, Q, R, S, T, V, Y
X ₃	A, D, E, F, H, I, K, Q, R, V, Y
X ₃	D, E, H, I, K, L, Q, T, V, Y
X ₃	D, E, H, I, K, Q, T, V, Y
X ₃	D, E, H, I, K, Q, R, S, V, Y
X ₃	D, E, H, I, K, Q, S, V, Y
X ₃	D, E, H, I, K, Q, R, V, Y
X ₃	D, E, I, K, Q, R, V, Y
X ₃	D, E, H, I, K, Q, V, Y
X ₃	D, E, H, I, Q, V, Y
X ₃	D, E, I, Q, R, V, Y
X ₃	D, E, I, Q, V, Y
X ₃	D, E, H, Q, Y
X ₃	D, E, Q, V, Y
X ₃	D, E, V, Y
X ₃	D, E, R, V
X ₃	D, E, K, V
X ₃	E, I, V, Y
X ₃	E, V, Y
X ₃	D, E, V
X ₃	D, E, Y
X ₃	E, V
X ₃	D, E
X ₃	E, Y
X ₃	E, I

X ₃	E, K
X ₃	E
X ₄	A, D, E, F, H, I, K, L, N, Q, R, S, T, V, W, Y
X ₄	A, D, E, F, H, I, K, L, N, Q, R, S, T, V, Y
X ₄	A, D, E, F, H, I, K, L, Q, R, S, T, V, W, Y
X ₄	A, D, E, F, H, I, K, Q, R, S, T, V, W, Y
X ₄	A, D, E, F, H, K, L, Q, R, S, T, V, W, Y
X ₄	A, D, E, H, I, K, L, Q, R, S, T, V, W, Y
X ₄	A, D, E, H, I, K, L, N, Q, R, S, T, V, Y
X ₄	A, D, E, I, K, Q, R, S, T, V
X ₄	A, D, E, I, K, Q, T, V
X ₄	A, D, E, H, I, K, N, Q, R, S, T, V, Y
X ₄	A, D, E, H, I, K, Q, R, S, T, V, W, Y
X ₄	A, D, E, I, K, Q, R, S, T, V
X ₄	A, E, I, K, Q, R, T, V, Y
X ₄	A, D, E, H, I, K, Q, R, S, T, V
X ₄	A, D, E, H, I, K, R, S, V
X ₄	A, D, E, H, K, Q, V, W
X ₄	A, D, E, H, I, R, S, T, V
X ₄	A, D, E, I, K, Q, S, V
X ₄	A, D, E, I, Q, R, V
X ₄	A, D, E, K, Q, S, T, V
X ₄	A, D, E, I, Q, V

X ₄	A, D, E, K, Q, V
X ₄	A, E, I, K, Q, V
X ₄	A, E, H, K, Q, V
X ₄	A, D, E, I, V
X ₄	A, D, E, I, R
X ₄	D, E, R, T, V
X ₄	A, D, E, K, Q
X ₄	A, D, E, Q, V
X ₄	A, D, E, K, V
X ₄	A, E, Q, V
X ₄	A, E, I, R
X ₄	A, D, E, I
X ₄	A, D, E, Q
X ₄	A, D, E, V
X ₄	A, D, E, K
X ₄	A, E, K, V
X ₄	A, D, E
X ₄	A, E, V
X ₄	A, D
X ₄	A, E
X ₄	D, E
X ₄	E, V
X ₄	E
X ₄	D
X ₄	A
X ₄	V
X ₅	A, V
X ₅	I, V
X ₅	A, I
X ₅	A
X ₅	I
X ₅	V
X ₆	F, H, Q, R, V, Y
X ₆	H, Q, T, Y
X ₆	F, H, Q, Y

X ₆	A, H, Q, W, Y
X ₆	H, Q, Y
X ₆	Q, Y
X ₆	Y
X ₆	Q
X ₇	A, D, E, F, H, I, L, M, Q, S, V, W, Y
X ₇	A, D, E, F, H, L, M, Q, R, W, Y
X ₇	A, D, E, F, H, I, L, M, Q, W, Y
X ₇	D, E, F, H, L, M, Q, W, Y
X ₇	D, F, H, L, M, Q, W, Y
X ₇	F, H, I, L, M, Q, V, W, Y
X ₇	F, H, I, L, M, Q, V, Y
X ₇	D, F, H, L, M, Q, W, Y
X ₇	F, H, I, L, M, Q, Y
X ₇	F, L, M, Q, W, Y
X ₇	F, M, Q, W, Y
X ₇	F, L, M, W, Y
X ₇	F, I, L, M, Y
X ₇	F, H, M, W, Y
X ₇	F, L, M, Y
X ₇	F, M, W, Y
X ₇	F, L, M, W
X ₇	D, F, H, M
X ₇	D, F, M
X ₇	F, M, Y
X ₇	M, W, Y
X ₇	F, M, W
X ₇	F, L, M
X ₇	L, M, Y
X ₇	F, L, Y
X ₇	F, M

X ₇	L, M
X ₇	M, Y
X ₇	F, L
X ₇	L, Y
X ₇	F, Y
X ₇	F, W
X ₇	M, W
X ₇	L
X ₇	Y
X ₇	F
X ₇	M
X ₇	W
X ₁₀	F
X ₁₀	Y
X ₁₁	A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, Y
X ₁₁	A, D, E, F, G, H, K, L, N, Q, R, S, T, V, W, Y
X ₁₁	A, D, E, F, G, H, I, K, N, Q, R, S, T, V, Y
X ₁₁	A, D, E, F, G, H, K, L, N, Q, R, S, T, V, Y
X ₁₁	A, D, E, F, G, H, K, L, Q, R, S, T, V, W, Y
X ₁₁	A, D, E, F, G, H, K, L, N, Q, R, S, Y
X ₁₁	A, D, E, F, G, H, K, L, Q, R, S, T, V, Y
X ₁₁	A, D, E, F, G, H, K, L, Q, R, S, T, Y
X ₁₁	A, D, E, F, G, H, K, L, N, Q, R, S, T, Y
X ₁₁	A, D, E, F, G, H, K, N, Q, R, S, T, V, Y
X ₁₁	A, D, E, F, G, H, K, N, Q, R, S, V, Y

X ₁₁	A, D, E, F, G, H, K, Q, R, S, V, W, Y
X ₁₁	A, D, E, F, G, H, K, Q, R, S, V, Y
X ₁₁	A, D, E, G, H, K, N, Q, R, S, Y
X ₁₁	A, D, E, F, G, H, I, L, M, Q, S, T, V, W, Y
X ₁₁	A, D, E, G, K, N, Q, R, S, T, Y
X ₁₁	A, D, E, F, G, H, Q, R, S, Y
X ₁₁	A, D, E, G, H, Q, S, Y
X ₁₁	A, D, E, F, G, H, K, Q, R, S, Y
X ₁₁	A, D, G, H, K, Q, R, S, Y
X ₁₁	A, E, G, H, K, N, Q, R, S, Y
X ₁₁	A, E, G, H, K, N, R, S, Y
X ₁₁	A, E, G, H, K, N, S, Y
X ₁₁	A, E, G, H, N, R, S, Y
X ₁₁	A, D, G, H, K, Q, R, S
X ₁₁	A, D, G, H, Q, R, S, Y
X ₁₁	A, E, G, H, R, S, Y
X ₁₁	A, D, G, H, Q, R, S
X ₁₁	A, G, H, Q, R, S, Y
X ₁₁	A, G, H, Q, R, S
X ₁₁	A, E, G, H, S, Y
X ₁₁	A, E, G, N, S, Y
X ₁₁	A, H, R, S, Y
X ₁₁	A, D, G, H, S, Y
X ₁₁	A, D, G, H, R, Y
X ₁₁	A, E, N, R, Y
X ₁₁	A, E, G, H, Y

X ₁₁	A, G, H, S, Y
X ₁₁	A, E, G, S, Y
X ₁₁	A, E, G, H, S
X ₁₁	A, E, H, R, Y
X ₁₁	A, G, H, R, Y
X ₁₁	A, H, R, S, Y
X ₁₁	A, E, G, N, S
X ₁₁	A, H, N, R, Y
X ₁₁	A, D, G, H
X ₁₁	A, G, S, Y
X ₁₁	A, G, H, Y
X ₁₁	A, G, H, S
X ₁₁	A, E, G, Y
X ₁₁	A, E, G, S
X ₁₁	A, E, G, H
X ₁₁	A, H, R, Y
X ₁₁	G, H, R, Y
X ₁₁	A, G, R, Y
X ₁₁	A, G, H, Y
X ₁₁	A, G, H, R
X ₁₁	A, E, G, N
X ₁₁	A, E, G, S
X ₁₁	A, E, G
X ₁₁	A, G, H
X ₁₁	A, G, S
X ₁₁	A, G, Y
X ₁₁	A, H, R
X ₁₁	A, R, Y
X ₁₁	A, H, Y
X ₁₁	H, R, Y
X ₁₁	A, G
X ₁₁	A, H
X ₁₁	A, R
X ₁₁	A, Y
X ₁₁	H, R

X ₁₁	H, Y
X ₁₁	R, Y
X ₁₁	A, E
X ₁₁	A,
X ₁₁	G
X ₁₁	R
X ₁₁	Y
X ₁₁	H
X ₁₁	E
X ₁₆	N
X ₁₆	T
X ₁₈	K, R
X ₁₈	R
X ₁₈	K
X ₂₁	T, V
X ₂₁	T
X ₂₁	V
X ₂₅	I, M, R
X ₂₅	I, R
X ₂₅	R
X ₂₅	I
X ₂₆	K
X ₂₆	S
X ₂₈	F, I, L
X ₂₈	F, L
X ₂₈	F
X ₂₈	L

In one embodiment of the present disclosure there is provided an IL-1R-I binding polypeptide, wherein in i)

- 5 X₂ is selected from A, E, F, H, I, L, Q, S, T and V;
 X₃ is selected from A, D, E, F, H, I, K, L, N, Q, R, S, T, V, W and Y;
 X₄ is selected from A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
 X₅ is selected from A, I and V;
 X₆ is selected from F, H, Q, R, V and Y;
 X₇ is selected from A, D, E, F, G, H, I, L, M, Q, S, T, V, W and Y;
10 X₁₀ is selected from F and Y;
 X₁₁ is selected from A, D, E, F, G, H, I, L, M, Q, S, T, V, W and Y;
 X₁₆ is selected from N and T;
 X₁₈ is selected from K and R;
 X₂₁ is selected from T and V;
15 X₂₅ is selected from I and R;
 X₂₆ is selected from K and S, and
 X₂₈ is selected from F and L.

In one embodiment of the present disclosure there is provided an IL-1R-I binding polypeptide, wherein in i)

- 20 X₂ is selected from A, E, F, H, I, L, Q, S, T and V;
 X₃ is selected from A, D, E, F, H, I, K, L, Q, R, S, T, V, W and Y;
 X₄ is selected from A, D, E, F, H, I, K, L, N, Q, R, S, T, V, W and Y;
 X₅ is selected from A, I and V;
25 X₆ is selected from H, Q, R, and Y;
 X₇ is selected from A, D, E, F, G, H, I, L, M, Q, S, V, W and Y;
 X₁₀ is selected from F and Y;
 X₁₁ is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
 X₁₆ is selected from N and T;
30 X₁₈ is selected from K and R;
 X₂₁ is selected from T and V;
 X₂₅ is selected from I and R;
 X₂₆ is selected from K and S, and
 X₂₈ is selected from F and L.

In one embodiment of the present disclosure there is provided an IL-1R-I binding polypeptide, wherein in i)

- X₂ is selected from A, I, L, T and V;
- X₃ is selected from E, I, V and Y;
- 5 X₄ is selected from A, D, E, F, H, I, K, L, Q, R, S, T, V, W and Y;
- X₅ is selected from A, I and V;
- X₆ is selected from Q and Y;
- X₇ is selected from F, H, I, L, M, Q, V, W and Y;
- X₁₀ is selected from F and Y;
- 10 X₁₁ is selected from A, D, E, F, G, H, K, L, Q, R, S, T, V, W and Y;
- X₁₆ is selected from N and T;
- X₁₈ is selected from K and R;
- X₂₁ is selected from T and V;
- X₂₅ is selected from I and R;
- 15 X₂₆ is selected from K and S, and
- X₂₈ is selected from F and L.

In another embodiment of the present disclosure there is provided an IL-1R-I binding polypeptide, wherein said IL-1R-I binding motif consists of an amino acid sequence selected from a sequence wherein in i)

- 20 X₂ is selected from A, I, L, T and V;
- X₃ is selected from E and Y;
- X₄ is selected from A, E, I, K, Q, R, T, V and Y;
- X₅ is selected from I and V;
- 25 X₆ is selected from Q and Y;
- X₇ is selected from F and M;
- X₁₀ is selected from F and Y;
- X₁₁ is selected from A, D, E, F, G, H, K, L, Q, R, S, T, V and Y;
- X₁₆ is selected from N and T;
- 30 X₁₈ is selected from K and R;
- X₂₁ is selected from T and V;
- X₂₅ is selected from I and R;
- X₂₆ is selected from K and S, and
- X₂₈ is selected from F and L,
- 35 and an amino acid sequence which has at least 93 % identity to the sequence defined above.

In another embodiment of the present disclosure there is provided an IL-1R-I binding polypeptide, wherein in i)

- X₂ is selected from A, I, L, T and V;
- X₃ is selected from E and Y;
- 5 X₄ is selected from A, D, E, F, H, K, L, Q, R, S, T, V, W and Y;
- X₅ is selected from A, I and V;
- X₆ is selected from Q and Y;
- X₇ is selected from F, H, I, L, M, Q, V, W and Y, such as F, H, I, L, Q, V, W and Y;
- 10 X₁₀ is selected from F and Y;
- X₁₁ is selected from A, D, E, F, G, H, K, Q, R, S, V, W and Y;
- X₁₆ is selected from N and T;
- X₁₈ is selected from K and R;
- X₂₁ is T;
- 15 X₂₅ is selected from I and R;
- X₂₆ is selected from K and S, and
- X₂₈ is selected from F and L.

In a more specific embodiment defining a sub-class of IL-1R-I binding polypeptides, sequence i) fulfills at least five of the ten conditions I-X:

- I. X₃ is E;
- II. X₅ is selected from I and V;
- III. X₆ is selected from Q and Y;
- 25 IV. X₇ is selected from F and M;
- V. X₁₀ is selected from F and Y;
- VI. X₁₈ is selected from K and R;
- VII. X₂₁ is T;
- VIII. X₂₅ is R;
- 30 IX. X₂₆ is selected from K and S; and
- X. X₂₈ is selected from F and L.

In some examples of an IL-1R-I binding polypeptide according to the first aspect, sequence i) fulfills at least six of the ten conditions I-X. More specifically, sequence i) may fulfil at least seven of the ten conditions I-X, such as at least eight of the ten conditions I-X, such as at least nine of the ten conditions I-X, such as all of the ten conditions I-X.

In some embodiments of an IL-1R-I binding polypeptide according to the first aspect, there is provided an IL-1R-I binding polypeptide, wherein $X_2X_3X_6$ is VEQ or VEY. In some embodiments there is provided an IL-1R-I binding polypeptide, wherein $X_2X_3X_6$ is IEQ or VEQ. In some embodiments X_6X_{10} is selected from the group consisting of QF, QY, YF and YY. In some embodiments X_6X_{10} is QF. In some embodiments $X_{10}X_{18}$ is selected from the group consisting of FK, FR, YK and YR. In some embodiments $X_{10}X_{18}$ is FK or FR. In some embodiments $X_{18}X_{25}X_{28}$ is KRL or RRL. In some embodiments X_5X_7 is AM, IM or VM. In some embodiments X_5X_7 is IM or VM.

10 In one embodiment, X_5 is selected from I and V. As demonstrated in the appended Example 4, the unintentional alteration in position X_5 of the BM resulted in the finding of an IL-1R-I binding polypeptide (Z18557, SEQ ID NO:1205) displaying high affinity for IL-1R-I (see Table 11). The exchange of alanine in this position to valine or isoleucine allows for generation of IL-1R-I
15 binding polypeptides with high affinity for IL-1R-I.

As described in detail in the experimental section to follow, the selection of IL-1R-I binding polypeptide variants has led to the identification of a number of individual IL-1R-I binding motif (*BM*) sequences. These sequences constitute individual embodiments of sequence i) according to this
20 aspect. The sequences of individual IL-1R-I binding motifs correspond to amino acid positions 8-36 of the amino acid sequences listed as SEQ ID NOs 1-1632 and 1679 presented in Figure 1. Thus, in one embodiment, of the IL-1R-I binding polypeptide according to this aspect, sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the
25 group consisting of SEQ ID NO:1-1632 and 1679, such as the group consisting SEQ ID NO:20-1632, and 1679. In one embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1206-1632 and 1679, such as the group consisting of SEQ ID NO:1210-1632 and 1679.

30 In one embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1205 and 1250-1632.

In one embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of
35 SEQ ID NO:1206-1252 and 1679.

In another embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of

SEQ ID NO:1253-1632. In another embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1253-1441 or the group consisting of SEQ ID NO:1442-1632.

- 5 In one embodiment, sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:158, 227, 268, 294, 325, 1176, 1205, 1251, 1252, 1270, 1284, 1285, 1298, 1307, 1308, 1311, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1353, 1361, 1362, 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 10 1422, 1423, 1435, 1471, 1472, 1571, 1594 and 1662, such as from the group consisting of SEQ ID NO:268, 1205, 1252, 1270, 1284, 1285, 1298, 1307, 1308, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1361, 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 1422, 1423 and 1571, such as from the group consisting of SEQ ID NO:1252, 1285, 1298, 1308, 1324, 1328, 1330, 15 1331, 1340, 1361, 1393, 1415, 1420 and 1421.

- In one particular embodiment sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1679. In one embodiment sequence i) corresponds to 20 the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1252, 1328, 1435 and 1679, such as the group consisting of SEQ ID NO:1252, 1328 and 1679; the group consisting of SEQ ID NO:1252, 1435, 1679; the group consisting of SEQ ID NO:1252 and 1679; or the group consisting of SEQ ID NO:1328 and 1435. In one 25 embodiment, sequence i) corresponds to the sequence from position 8 to position 36 in sequence selected from the group consisting of SEQ ID NO:1252, 1328 and 1435.

- Figure 1 thus exemplifies polypeptide variants whose IL-1R-I binding capacity has been verified experimentally (at least by virtue of being isolated 30 in an affinity based assay), which variants have led to the identification of a number of individual IL-1R-I binding motif (*BM*) sequences which constitute individual embodiments of *BM* which exhibit 93 %, where applicable, or 96 % identity with sequence i).

- In some embodiments of the present disclosure, the *BM* as defined 35 above “forms part of” a three-helix bundle protein domain. This is understood to mean that the sequence of the *BM* is “inserted” into or “grafted” onto the sequence of the original three-helix bundle domain, such that the *BM*

replaces a similar structural motif in the original domain. For example, without wishing to be bound by theory, the *BM* is thought to constitute two of the three helices of a three-helix bundle, and can therefore replace such a two-helix motif within any three-helix bundle. As the skilled person will realize, the replacement of two helices of the three-helix bundle domain by the two *BM* helices has to be performed so as not to affect the basic structure of the polypeptide. That is, the overall folding of the C α backbone of the polypeptide according to this embodiment of the invention is substantially the same as that of the three-helix bundle protein domain of which it forms a part, e.g. having the same elements of secondary structure in the same order etc. Thus, a *BM* according to the present disclosure “forms part” of a three-helix bundle domain if the polypeptide according to this embodiment has the same fold as the original domain, implying that the basic structural properties are shared, those properties e.g. resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant.

In particular embodiments, the IL-1R-I binding motif (*BM*) thus forms part of a three-helix bundle protein domain. For example, the *BM* may essentially constitute two alpha helices with an interconnecting loop, within said three-helix bundle protein domain. In particular embodiments, said three-helix bundle protein domain is selected from domains of bacterial receptor proteins. Non-limiting examples of such domains are the five different three-helical domains of Protein A from *Staphylococcus aureus*, such as domain B, and derivatives thereof. In some embodiments, the three-helical bundle protein domain is a variant of protein Z, which is derived from domain B of staphylococcal Protein A.

In some embodiments where the IL-1R-I binding polypeptide as disclosed herein forms part of a three-helix bundle protein domain, the IL-1R-I binding polypeptide may comprise a binding module (*BMod*), the amino acid sequence of which is selected from:

iii) K-[*BM*]-DPSQSX_aX_bLLX_cEAKKLX_dX_eX_fQ;
(SEQ ID NO:1687)

wherein

[*BM*] is an IL-1R-I binding motif as defined herein;
X_a is selected from A and S;
X_b is selected from N and E;

X_c is selected from A, S and C;
 X_d is selected from E, N and S;
 X_e is selected from D, E and S;
 X_f is selected from A and S; and

5

- iv) an amino acid sequence which has at least 91 % identity to a sequence defined by iii).

10 In some embodiments, said polypeptides may beneficially exhibit high structural stability, such as resistance to chemical modifications, to changes in physical conditions and to proteolysis, during production and storage, as well as *in vivo*.

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences, which do not largely affect the tertiary structure and the function of the polypeptides, are also within the scope of the present disclosure. Thus, in some embodiments, sequence iv) 15 has at least at least 93 %, such as at least 95 %, such as at least 97 % identity to a sequence defined by iii). In particular embodiments, such minor changes as exemplified above may be present only in the flanking sequence, i.e. the parts of the sequence flanking the BM in iii) above. In other 20 embodiments, minor changes can be present in the BM and/or in the flanking sequences.

In one embodiment, X_a in sequence iii) is A.
 In one embodiment, X_a in sequence iii) is S.
 25 In one embodiment, X_b in sequence iii) is N.
 In one embodiment, X_b in sequence iii) is E.
 In one embodiment, X_c in sequence iii) is A.
 In one embodiment, X_c in sequence iii) is S.
 In one embodiment, X_c in sequence iii) is C.
 30 In one embodiment, X_d in sequence iii) is E.
 In one embodiment, X_d in sequence iii) is N.
 In one embodiment, X_d in sequence iii) is S.
 In one embodiment, X_e in sequence iii) is D.
 In one embodiment, X_e in sequence iii) is E.
 35 In one embodiment, X_e in sequence iii) is S.
 In one embodiment, X_dX_e in sequence iii) is selected from EE, ES, SE and SS.

In one embodiment, X_dX_e in sequence iii) is ES.

In one embodiment, X_dX_e in sequence iii) is SE.

In one embodiment, X_f in sequence iii) is A.

In one embodiment, X_f in sequence iii) is S.

5 In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A and X_f is A.

In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C and X_f is A.

10 In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S and X_f is S.

In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C and X_f is S.

In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is ND and X_f is A.

15 In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is ND and X_f is A.

In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is ND and X_f is S.

20 In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is ND and X_f is S.

In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is SE and X_f is A.

In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is SE and X_f is A.

25 In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is SE and X_f is S.

In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is SE and X_f is S.

30 In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is ES and X_f is A.

In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is ES and X_f is A.

In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is ES and X_f is S.

35 In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is ES and X_f is S.

In one embodiment, sequence iii) or iv) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-1638 and 1667-1670.

In yet a further embodiment, sequence iii) corresponds to the
5 sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-1638, 1667-1668 and 1670-1679; such as the group consisting of SEQ ID NO:20-1638 and 1670-1679. In one embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID
10 NO:1206-1638 and 1670-1679, such as the group consisting of SEQ ID NO:1206-1632 and 1670-1679, such as the group consisting of SEQ ID NO:1210-1638 and 1670-1679, such as the group consisting of SEQ ID NO:1210-1632 and 1670-1679.

In one embodiment sequence iii) corresponds to the sequence from
15 position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1205, 1250-1632 and 1670-1679.

In one embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1206-1252, 1672 and 1679.

20 In another embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1253-1632, 1670-1671 and 1673-1678. In another embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID
25 NO:1253-1441, 1670-1671 and 1674-1678 or the group consisting of SEQ ID NO:1442-1632 and 1673.

In one embodiment, sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:158, 227, 268, 294, 325, 1176, 1205, 1251, 1252, 1270, 1284,
30 1285, 1298, 1307, 1308, 1311, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1353, 1361, 1362, 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 1422, 1423, 1435, 1471, 1472, 1571, 1594, 1662 and 1670-1679, such as from the group consisting of SEQ ID NO:268, 1205, 1252, 1270, 1284, 1285, 1298, 1307, 1308, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1361,
35 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 1422, 1423, 1571, 1670-1672, 1674, and 1676-1679, such as from the group consisting of SEQ ID

NO:1252, 1285, 1298, 1308, 1324, 1328, 1330, 1331, 1340, 1361, 1393, 1415, 1420, 1421, 1670-1672, 1674, 1676-1677 and 1679.

In one particular embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1670-1679. In one embodiment sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1252, 1328, 1435, 1672, 1675-1676 and 1679 such as the group consisting of SEQ ID NO:1252, 1328 and 1679; the group consisting of SEQ ID NO:1252, 1435 and 1679; or the group consisting of SEQ ID NO:1328 and 1435. In one embodiment, sequence i) corresponds to the sequence from position 7 to position 55 in sequence selected from the group consisting of SEQ ID NO:1252, 1328 and 1435.

In one embodiment, sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1672, 1675-1676 and 1679, such as the group consisting of SEQ ID NO:1672 and 1675-1676.

Also, in a further embodiment, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid sequence selected from:

v) YA-[BMod]-AP; (SEQ ID NO:1688)

wherein [BMod] is an IL-1R-I binding module as defined herein; and

vi) an amino acid sequence which has at least 90 % identity to a sequence defined in v).

Alternatively, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid sequence selected from:

vii) FN-[BMod]-AP; (SEQ ID NO:1689)

wherein [BMod] is an IL-1R-I binding module as defined herein; and

viii) an amino acid sequence which has at least 90 % identity to a sequence defined in vii).

In one embodiment there is provided an IL-1R-I binding polypeptide, which comprises an amino acid selected from the group consisting of

ix) FNK-[BM]-DPSQS ANLLX_c EAKKL NDAQA P; (SEQ ID NO:1690)

wherein [BM] is an IL-1R-I binding motif as defined above and X_c is selected from A and C; and

x) an amino acid sequence which has at least 90 % identity to a sequence defined in ix).

In another embodiment, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid selected from the group consisting of

xi) FAK-[BM]-DPSQS SELLX_c EAKKL SESQA P; (SEQ ID NO:1691)

wherein [BM] is an IL-1R-I binding motif as defined above and X_c is selected from A, S and C; and

xii) an amino acid sequence which has at least 90 % identity to a sequence defined in xi).

In another embodiment, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid selected from the group consisting of

xiii) FAK-[BM]-DPSQS SELLX_c EAKKL NDSQA P; (SEQ ID NO:1692)

wherein [BM] is an IL-1R-I binding motif as defined above and X_c is selected from A, S and C;

xiv) an amino acid sequence which has at least 90 % identity to a sequence defined in xiii).

In yet another embodiment, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid selected from the group consisting of

xv) YAK-[BM]-DPSQS SELLX_c EAKKL X_dX_eSQA P; (SEQ ID NO:1693)

wherein [BM] is an IL-1R-I binding motif as defined above and X_c is selected from A, S and C, X_d is selected from E, N and S, and X_e is selected from D, E and S;

xvi) and an amino acid sequence which has at least 90 % identity to a sequence defined in xv).

In yet another embodiment, there is provided an IL-1R-I binding polypeptide, which comprises an amino acid selected from the group consisting of

xvii) KYAK-[BM]-DPSQS SELLX_c EAKKL X_dX_eSQA P; (SEQ ID NO:1694)

wherein *[BM]* is an IL-1R-I binding motif as defined above and X_c is selected from A, S and C, X_d is selected from E, N and S, and X_e is selected from D, E and S;

- xviii) and an amino acid sequence which has at least 90 % identity to
5 a sequence defined in xvii).

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences, which do not largely affect the tertiary structure and the function of the polypeptide, also fall within the scope
10 of the present disclosure. Thus, in some embodiments, sequence vi), viii), x), xii), xiv), xvi) or xviii) may for example be at least 90 %, such as at least 92 %, such as at least 94 %, such as at least 96 %, such as at least 98 % identical to a sequence defined by v), vii), ix), xi), xiii), xv) and xvii), respectively. In particular embodiments, such minor changes as exemplified above can be
15 present only in flanking sequences, i.e. the parts of the sequences flanking the *BM* in v), vii), ix), xi), xiii), xv) and xviii). In other embodiments, minor changes can be present in the *BM* and/or in the flanking sequences.

- In some embodiments, the IL-1R-I binding motif may form part of a
20 polypeptide comprising an amino acid sequence selected from:
- | | |
|---|-----------------|
| ADNNFNK- <i>[BM]</i> -DPSQSANLLSEAKKLNESQAPK | SEQ ID NO:1695; |
| ADNKFNK- <i>[BM]</i> -DPSQSANLLAEAKKLNDQAQPK | SEQ ID NO:1696; |
| ADNKFNK- <i>[BM]</i> -DPSVSKEILAEAKKLNDQAQPK | SEQ ID NO:1697; |
| ADAQQNNFNK- <i>[BM]</i> -DPSQSTNVLGEAKKLNESQAPK | SEQ ID NO:1698; |
| 25 AQHDE- <i>[BM]</i> -DPSQSANVLGEAQKLNDQAPK | SEQ ID NO:1699; |
| VDNKFNK- <i>[BM]</i> -DPSQSANLLAEAKKLNDQAQPK | SEQ ID NO:1700; |
| AEAKYAK- <i>[BM]</i> -DPSESSELLSEAKKLNKSQAPK | SEQ ID NO:1701; |
| VDAKYAK- <i>[BM]</i> -DPSQSSELLAEAKKLNDQAQPK | SEQ ID NO:1702; |
| VDAKYAK- <i>[BM]</i> -DPSQSSELLAEAKKLNDQAPK | SEQ ID NO:1703; |
| 30 AEAKYAK- <i>[BM]</i> -DPSQSSELLSEAKKLNDQAPK | SEQ ID NO:1704; |
| AEAKYAK- <i>[BM]</i> -DPSQSSELLSEAKKLNDQAP | SEQ ID NO:1705; |
| AEAKFAK- <i>[BM]</i> -DPSQSSELLSEAKKLNDQAPK | SEQ ID NO:1706; |
| AEAKFAK- <i>[BM]</i> -DPSQSSELLSEAKKLNDQAP | SEQ ID NO:1707; |
| AEAKYAK- <i>[BM]</i> -DPSQSSELLAEAKKLNDQAQPK | SEQ ID NO:1708; |
| 35 AEAKYAK- <i>[BM]</i> -DPSQSSELLSEAKKLSESQAPK | SEQ ID NO:1709; |
| AEAKYAK- <i>[BM]</i> -DPSQSSELLSEAKKLSESQAP | SEQ ID NO:1710; |
| AEAKFAK- <i>[BM]</i> -DPSQSSELLSEAKKLSESQAPK | SEQ ID NO:1711; |

	AEAKFAK-[BM]-DPSQSSELLSEAKKLSAQAP	SEQ ID NO:1712;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLSAQAPK	SEQ ID NO:1713;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLESSQAPK	SEQ ID NO:1714;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLESSQAP	SEQ ID NO:1715;
5	AEAKYAK-[BM]-DPSQSSELLAEAKKLESAQAPK	SEQ ID NO:1716;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAPK	SEQ ID NO:1717;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAP	SEQ ID NO:1718;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLSDSQAPK	SEQ ID NO:1719;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLSDAQAPK	SEQ ID NO:1720;
10	VDAKYAK-[BM]-DPSQSSELLSEAKKLNDSDQAPK	SEQ ID NO:1721;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLNDSDAQAPK	SEQ ID NO:1722;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLSAQAPK	SEQ ID NO:1723;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSAQAPK	SEQ ID NO:1724;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLESSQAPK	SEQ ID NO:1725;
15	VDAKYAK-[BM]-DPSQSSELLAEAKKLESAQAPK	SEQ ID NO:1726;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAPK	SEQ ID NO:1727;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSDSQAPK	SEQ ID NO:1728;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSDAQAPK	SEQ ID NO:1729;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK	SEQ ID NO:1730;
20	AEAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK	SEQ ID NO:1731,
	and	
	ADAKYAK-[BM]-DPSQSSELLSEAKKLNDSDQAPK	SEQ ID NO:1732,

wherein [BM] is an IL-1R-I binding motif as defined herein.

In one embodiment, said IL-1R-I binding polypeptide comprises an amino acid sequence which has at least 89 % identity to any one of the sequences defined above.

In one embodiment, the IL-1R-I binding polypeptide comprises an amino acid sequence selected from:

xix) VDAKYAK-[BM]-DPSQSSELLSEAKKLNDSDQAPK (SEQ ID NO:1721)

wherein [BM] is an IL-1R-I binding motif as defined herein; and

xx) an amino acid sequence which has at least 89 % identity to the sequence defined in xix).

In one embodiment, the IL-1R-I binding polypeptide comprises an amino acid sequence selected from:

xxi) AEAKEYAK-[BM]-DPSQSSELLSEAKKLSAQAPK (SEQ ID NO:1709)

wherein *[BM]* is an IL-1R-I binding motif as defined herein; and

xxii) an amino acid sequence which has at least 89 % identity to the sequence defined in xxi).

In one particular embodiment, said IL-1R-I binding polypeptide
5 comprises an amino acid sequence selected from the group consisting of
SEQ ID NO:1667-1668 and 1670-1679.

In one embodiment, the IL-1R-I binding polypeptide comprises an
amino acid sequence selected from:

xxiii) AEAKFAK-*[BM]*-DPSQSSELLSEAKKLSAQAPK (SEQ ID
10 NO:1711) wherein *[BM]* is an IL-1R-I binding motif as defined herein; and
xxiv) an amino acid sequence which has at least 89 % identity to the
sequence defined in xxiii).

Again, polypeptides comprising minor changes as compared to the
above amino acid sequences, which do not largely affect the tertiary structure
15 and the function of the polypeptide, also fall within the scope of the present
disclosure. Thus, in some embodiments, sequence xx), xxii) or xxiv) may for
example be at least 89 %, such as at least 91 %, such as at least 93 %, such
as at least 94 %, such as at least 96 %, such as at least 98 % identical to a
sequence defined by xix), xxi) or xxiii) respectively. Once again, in particular
20 embodiments such minor changes as exemplified above can be present only
in the sequences flanking the BM as set out in xix), xxi) or xxiii). In other
embodiments, minor changes can be present in the BM and/or in the flanking
sequences.

25 Sequence xix)-xxiv) in such IL-1R-I binding polypeptide may be
selected from the sequence from position 1 to position 58 in a sequence
selected from the group consisting of SEQ ID NO:1-1638, 1667-1668 and
1670-1679 as presented in Figure 1.

In one embodiment of the IL-1R-I binding polypeptide according to this
30 aspect, sequence xix), xxi) or xxiii) corresponds to the sequence from position
1 to position 58 in a sequence selected from the group consisting of SEQ ID
NO:1-1632, 1667-1668 and 1670-1679; such as the group consisting of SEQ
ID NO:20-1638, 1667-1668 and 1670-1679. In one embodiment sequence
xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in
35 a sequence selected from the group consisting of SEQ ID NO:1206-1632,
1667-1668 and 1670-1679, such as the group consisting of SEQ ID NO:1210-
1632, 1667-1668 and 1670-1679.

In one embodiment sequence xvii), xix) or xxi) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1205, 1250-1632 and 1670-1679.

5 In one embodiment sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1206-1252, 1672 and 1679.

In another embodiment sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1253-1632, 1670-1671 and 1673-1678. In
10 another embodiment sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1253-1441, 1670-1671 and 1674-1678 or the group consisting of 1442-1632 and 1673.

In one embodiment sequence xix), xxi) or xxiii) corresponds to the
15 sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:158, 227, 268, 294, 325, 1176, 1205, 1251, 1252, 1270, 1284, 1285, 1298, 1307, 1308, 1311, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1353, 1361, 1362, 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 1422, 1423, 1435, 1471, 1472, 1571, 1594, 1662 and
20 1670-1679, such as from the group consisting of SEQ ID NO:268, 1205, 1252, 1270, 1284, 1285, 1298, 1307, 1308, 1324, 1328, 1330, 1331, 1334, 1339, 1340, 1343, 1361, 1364, 1367, 1368, 1375, 1393, 1415, 1420, 1421, 1422, 1423, 1571, 1670-1672, 1674 and 1676-1679, such as from the group consisting of SEQ ID NO:1252, 1285, 1298, 1308, 1324, 1328, 1330, 1331,
25 1340, 1361, 1393, 1415, 1420, 1421, 1670-1672, 1674, 1676-1677 and 1679.

In one particular embodiment sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1670-1679. In one embodiment sequence xix),
30 xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1252, 1328, 1435 1672, 1675-1676 and 1679, such as the group consisting of SEQ ID NO:1252, 1328 and 1679; the group consisting of SEQ ID NO:1252, 1435 and 1679; or the group consisting of SEQ ID NO:1328 and 1435. In one
35 embodiment, sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1252, 1328 and 1435.

In one embodiment, sequence xix), xxi) or xxiii) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1672, 1675-1676 and 1679, such as from the group consisting of SEQ ID NO:1672, 1675 and 1676.

5

In one embodiment, said IL-1R-I binding polypeptide has a melting temperature of above 45 °C.

Binding of a polypeptide as defined herein to IL-1R-I may interfere with signaling via IL-1R-I either *in vivo* or *in vitro*. Thus, in one embodiment, there is provided an IL-1R-I binding polypeptide as defined herein which is capable of blocking IL-1R-I dependent signaling.

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance for inhibiting a specific quantifiable biological or biochemical function. This quantitative measure indicates how much of a particular substance is needed to inhibit a specific biological function by 50 % and is commonly used in the art. In one particular embodiment, there is provided an IL-1R-I binding polypeptide as defined herein capable of blocking IL-1R-I signaling such that the half maximal inhibitory concentration (IC_{50}) of the blocking is at most 1×10^{-7} M, such as at most 5×10^{-8} M, such as at most 2×10^{-8} M, such as at most 1×10^{-8} M, such as at most 5×10^{-9} M, such as at most 2×10^{-9} M, such as at most 1×10^{-9} M, such as at most 5×10^{-10} M, such as at most 4×10^{-10} M, such as at most 3×10^{-10} M. In one embodiment, the half maximal inhibitory concentration (IC_{50}) of the blocking is between 1×10^{-15} M and 1×10^{-7} M, such as between 1×10^{-15} M and 1×10^{-8} M, such as between 1×10^{-15} M and 1×10^{-9} M, such as between 1×10^{-15} M and 1×10^{-10} M, such as between 1×10^{-15} M and 1×10^{-11} M, such as between 1×10^{-15} M and 1×10^{-12} M.

The terms "IL-1R-I binding" and "binding affinity for IL-1R-I" as used in this specification refer to a property of a polypeptide which may be tested for example by ELISA, Biolayer Interferometry (BLI) or the use of surface plasmon resonance (SPR) technology. For example as described in the examples below, IL-1R-I binding affinity may be tested in an experiment in which samples of the polypeptide are captured on antibody-coated ELISA plates and biotinylated IL-1R-I is added followed by streptavidin-conjugated HRP. TMB substrate is added and the absorbance at 450 nm is measured using a multi-well plate reader, such as Victor³ (Perkin Elmer). The skilled person may then interpret the results obtained by such experiments to

establish at least a qualitative measure of the binding affinity of the polypeptide for IL-1R-I. If a quantitative measure is desired, for example to determine the EC_{50} value (the half maximal effective concentration) for the interaction, ELISA may also be used. The response of the polypeptide against
5 a dilution series of biotinylated IL-1R-I is measured using ELISA as described above. The skilled person may then interpret the results obtained by such experiments, and EC_{50} values may be calculated from the results using for example GraphPad Prism 5 and non-linear regression.

IL-1R-I binding affinity may also be tested in an experiment in which IL-
10 1R-I, or a fragment thereof, is immobilized on a sensor chip of a surface plasmon resonance (SPR) instrument, and the sample containing the polypeptide to be tested is passed over the chip. Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing IL-1R-I, or a fragment thereof, is passed over the chip. The skilled
15 person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the polypeptide for IL-1R-I. If a quantitative measure is desired, for example to determine a K_D value for the interaction, surface plasmon resonance methods may also be used. Binding values may for example be defined in a Biacore
20 (GE Healthcare) or ProteOn XPR 36 (Bio-Rad) instrument. IL-1R-I is suitably immobilized on a sensor chip of the instrument, and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected in random order. K_D values may then be calculated from the results using for example the 1:1 Langmuir binding model of the
25 BIAevaluation 4.1 software, or other suitable software, provided by the instrument manufacturer.

Thus, in one embodiment, there is provided an IL-1R-I binding polypeptide as defined herein, which is capable of binding to IL-1R-I such that the EC_{50} value of the interaction is at most 1×10^{-7} M, such as at most $5 \times$
30 10^{-8} M, such as at most 1×10^{-8} M, such as at most 5×10^{-9} M, such as at most 1×10^{-9} M, such as at most 5×10^{-10} M, such as at most 4×10^{-10} M, such as at most 3×10^{-10} M, such as at most 2×10^{-10} M. In one embodiment, the EC_{50} value of the interaction is between 1×10^{-15} M and 1×10^{-7} M, such as between 1×10^{-15} M and 1×10^{-8} M, such as between 1×10^{-15} M and $1 \times$
35 10^{-9} M, such as between 1×10^{-15} M and 5×10^{-10} M, such as between 1×10^{-15} M and 1×10^{-10} M, such as between 1×10^{-15} M and 5×10^{-11} M, such as

between 1×10^{-15} M and 1×10^{-11} M, such as between 1×10^{-15} M and 5×10^{-12} M, such as between 1×10^{-15} M and 1×10^{-12} M.

In one particular embodiment, there is provided an IL-1R-I binding polypeptide which is capable of binding to IL-1R-I such that the K_D value of the interaction with IL-1R-I is at most 1×10^{-6} M, such as at most 1×10^{-7} M, such as at most 5×10^{-8} M, such as at most 4×10^{-8} M, such as at most 3×10^{-8} M, such as at most 2×10^{-8} M, such as at most 1×10^{-8} M, such as at most 7×10^{-9} M, such as at most 5×10^{-9} M, such as at most 4×10^{-9} M, such as at most 3×10^{-9} M, such as at most 2×10^{-9} M, such as at most 1×10^{-9} M, such as at most 9×10^{-10} M, such as at most 7×10^{-10} M, such as at most 5×10^{-10} M. In one embodiment, the K_D value of the interaction with IL-1R-I is between 1×10^{-15} M and 1×10^{-6} M, between 1×10^{-15} M and 1×10^{-7} M, such as between 1×10^{-15} M and 1×10^{-8} M, such as between 1×10^{-15} M and 1×10^{-9} M, such as between 1×10^{-15} M and 1×10^{-10} M, such as between 1×10^{-15} M and 1×10^{-11} M, such as between 1×10^{-15} M and 1×10^{-12} M.

In one embodiment, there is provided an IL-1R-I binding polypeptide as defined herein, wherein said IL-1R-I is human IL-1R-I or cynomolgus IL-1R-I, such as human IL-1R-I.

The IL-1R-I binding polypeptide as defined herein may be capable of blocking the interaction of IL-1R-I with IL-1 cytokines, such as the interaction of IL-1R-I with IL-1 α and/or IL-1 β . Thus, in one embodiment said IL-1R-I binding polypeptide is capable of blocking the interaction of IL-1R-I with IL-1 cytokines, such as the interaction of IL-1R-I with IL-1 α and/or IL-1 β .

The skilled person will understand that various modifications and/or additions can be made to an IL-1R-I binding polypeptide according to any aspect disclosed herein in order to tailor the polypeptide to a specific application without departing from the scope of the present disclosure.

For example, in one embodiment there is provided an IL-1R-I binding polypeptide as described herein, which polypeptide has been extended by and/or comprises additional amino acids at the C terminus and/or N terminus. Such a polypeptide should be understood as a polypeptide having one or more additional amino acid residues at the very first and/or the very last position in the polypeptide chain. Thus, an IL-1R-I binding polypeptide may comprise any suitable number of additional amino acid residues, for example at least one additional amino acid residue. Each additional amino acid residue may individually or collectively be added in order to, for example, improve production, purification, stabilization *in vivo* or *in vitro*, coupling or detection of

the polypeptide. Such additional amino acid residues may comprise one or more amino acid residues added for the purpose of chemical coupling. One example of this is the addition of a cysteine residue. Additional amino acid residues may also provide a "tag" for purification or detection of the polypeptide, such as a His₆ tag, a (HisGlu)₃ tag ("HEHEHE" tag), a "myc" (c-myc) tag or a "FLAG" tag for interaction with antibodies specific to the tag or for immobilized metal affinity chromatography (IMAC) in the case of a His₆-tag.

The further amino acids as discussed above may be coupled to the IL-1R-I binding polypeptide by means of chemical conjugation (using known organic chemistry methods) or by any other means, such as expression of the IL-1R-I binding polypeptide as a fusion protein or joined in any other fashion, either directly or via a linker, for example an amino acid linker.

The further amino acids as discussed above may for example comprise one or more polypeptide domain(s). A further polypeptide domain may provide the IL-1R-I binding polypeptide with another function, such as for example yet another binding function, an enzymatic function, a toxic function, a fluorescent signaling function or combinations thereof.

A further polypeptide domain may moreover provide another IL-1R-I binding moiety with the same IL-1R-I binding function. Thus, in a further embodiment, there is provided an IL-1R-I binding polypeptide in a multimeric form. Said multimer is understood to comprise at least two IL-1R-I binding polypeptides as disclosed herein as monomer units, the amino acid sequences of which may be the same or different. Multimeric forms of the polypeptides may comprise a suitable number of domains, each having an IL-1R-I binding motif, and each forming a monomer within the multimer. These domains may have the same amino acid sequence, but alternatively, they may have different amino acid sequences. In other words, the IL-1R-I binding polypeptide of the invention may form homo- or heteromultimers, for example homo- or heterodimers. In one embodiment, there is provided an IL-1R-I binding polypeptide, wherein said monomeric units are covalently coupled together. In one embodiment, there is provided an IL-1R-I binding polypeptide, wherein said monomeric units are non-covalently coupled together. In another embodiment, said IL-1R-I binding polypeptide monomer units are expressed as a fusion protein. In one embodiment, there is provided an IL-1R-I binding polypeptide in dimeric form. One example of an IL-1R-I binding polypeptide which will form a disulphide bonded dimer after

production is the polypeptide having the sequence set out in SEQ ID NO:1658. The hinge region of IgG1 Fc (disclosed in Figure 1 as position 1-16 of SEQ ID NO:1662) may be used to accomplish dimeric forms of IL-1R-I binding polypeptides.

5 Additionally, "heterogenic" fusion polypeptides or proteins, or conjugates, in which an IL-1R-I binding polypeptide as described herein, or a multimer thereof, constitutes a first domain, or first moiety, and the second and further moieties have other functions than binding to IL-1R-I, are also contemplated and fall within the ambit of the present disclosure. The second
10 and further moiety/moieties of the fusion polypeptide or conjugate in such a protein suitably also have a desired biological activity.

Thus, in a second aspect of the present disclosure, there is provided a fusion protein or a conjugate, comprising a first moiety consisting of an IL-1R-I binding polypeptide according to the first aspect, and a second
15 moiety consisting of a polypeptide having a desired biological activity. In another embodiment, said fusion protein or conjugate may additionally comprise further moieties, comprising desired biological activities that can be either the same or different from the biological activity of the second moiety.

Thus, in one embodiment, said IL-1R-I binding polypeptide comprises
20 an IL-1R-I binding motif *BM* as set out in sequence i) or ii); a binding module *BMod* as set out in sequence iii) or iv), or an amino acid sequence as set out in any one of sequences v)-xxiv). In particular, said IL-1R-I binding motifs may correspond to an amino acid sequence from position 8 to 36; said binding modules may correspond to an amino acid sequence from position 7 to
25 position 55; and said sequence xix), xxi), or xxiii) may be selected from the group consisting of sequences selected from SEQ ID NO:s shown in Figure 1 and listed above. Preferred embodiments of IL-1R-I binding polypeptides are disclosed in the first aspect.

Non-limiting examples of a desired biological activity comprise a
30 therapeutic activity, a binding activity, and an *in vivo* half-life increasing activity.

In one embodiment, said desired biological activity is an *in vivo* half-life increasing activity such that said second moiety increases *in vivo* half-life of the fusion protein or conjugate.

35 An *in vivo* half-life increasing activity should in this context be understood as an activity which increases the *in vivo* half-life of the fusion protein or conjugate. The half-life of a first moiety (and possibly additional

moieties) is increased by fusion or conjugation to a second moiety having an *in vivo* half-life increasing activity. It will be appreciated that the corresponding fusion protein or conjugate may comprise a first IL-1R-I binding moiety as described above, a second moiety having an *in vivo* half-life increasing activity, and optionally one or more additional moieties, such as an additional IL-1R-I binding moiety. For example, said fusion protein or conjugate may comprise two IL-1R-I binding polypeptides and a second moiety which increases *in vivo* half-life compared to the fusion or conjugate comprising the two IL-1R-I binding polypeptides only. Examples of fusion proteins comprising an IL-1R-I binding polypeptide and a half-life extending moiety have been produced and experimentally tested (Example 10). In one embodiment, said fusion protein or conjugate comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:1639-1657.

Half-life extension of the resulting fusion protein or conjugate may be accomplished by a second moiety having an inherently long *in vivo* half-life. A polypeptide that increases the hydrodynamic size of the fusion protein or conjugate may for example impose an increased half-life upon the fusion protein or conjugate. Alternatively, the second moiety may consist of a natural protein having a long *in vivo* half-life, such as serum albumin or the Fc portion of an antibody.

In one embodiment, said second moiety comprises one of the albumin binding domain of streptococcal protein G or a derivative thereof; albumin; an Fc portion of an antibody, and transferrin. In one embodiment, said fusion protein or conjugate comprises a sequence selected from the group consisting of SEQ ID NO:1639-1658.

In one embodiment, said second moiety is an Fc portion of an antibody, such as an IgG1 Fc (SEQ ID NO:1662) or an IgG4 Fc. An IgG4 Fc that may be used as a half-life extending moiety is the Fc part set out in Uniprot, accession number P01861, amino acid residues 99-327. In one embodiment, said fusion protein or conjugate comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:1646-1654.

In one embodiment, said Fc portion of an antibody is a mutated Fc which relative to an unmutated form of Fc displays improved affinity to FcRn and/or reduced effector response. Thus, said Fc portion is a variant of a naturally occurring Fc in which one or more mutations have been introduced. For example, said fusion protein or conjugate comprises a polypeptide having

an amino acid sequence selected from the group consisting of SEQ ID NO:1734-1737.

In another non-limiting example, wherein said second moiety has *in vivo* half-life increasing activity, said second moiety is transferrin. In particular
5 embodiments of the fusion protein or conjugate disclosed herein, there is provided a polypeptide selected from the group consisting of SEQ ID NO:1657.

In one particular embodiment, the *in vivo* half-life increasing activity is an albumin binding activity. Non-limiting examples of moieties providing such
10 an albumin binding activity are an albumin binding camelid Ig V_{HH} domain and an albumin binding engineered single Ig V_H domain.

In one embodiment, said albumin binding activity is provided by the albumin binding domain of the peptostreptococcal albumin binding (PAB) protein from *Finnegoldia magna*, or a derivative thereof.

15 In one embodiment, said albumin binding activity is provided by the albumin binding domain of streptococcal protein G, or a derivative thereof. Examples of such an albumin binding domain are polypeptides having an amino acid sequence as set out in SEQ ID NO:1659-1661. Preferably, the albumin binding domain is as set out in SEQ ID NO:1661. In one
20 embodiment, said fusion protein or conjugate comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:1639-1645.

In another non-limiting example, said *in vivo* half-life increasing activity is provided by a polyethylene glycol (PEG) or a hydroxyethyl starch (HES).

25 For example, said fusion protein or conjugate, comprising at least one further moiety, may comprise [IL-1R-I binding polypeptide] – [half-life increasing moiety] – [moiety with affinity for selected target]. It is to be understood that the three moieties in this example may be arranged in any order from the N- to the C-terminal of the polypeptide.

30 Another non-limiting example is a binding activity which allow the fusion protein to display a modified tissue distribution compared to the unfused IL-1R-I binding polypeptide. In one embodiment the binding activity is binding to the transferrin receptor. Such a binding activity could infer that the conjugate or fusion protein is transferred across the blood brain barrier and
35 into the brain tissue (Yu *et al*, 2011, Sci Transl Med 3(84):84ra44). In a preferred embodiment, such binding activity is provided by an antibody fragment.

Another non-limiting example of a binding activity is an activity which acts to block a biological activity.

In one embodiment, there is provided an IL-1R-I binding polypeptide, fusion protein or conjugate wherein the binding activity acts to block a
5 biological activity.

With regard to the description above of fusion proteins or conjugates incorporating an IL-1R-I binding polypeptide according to the disclosure, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between an IL-1R-I binding polypeptide or
10 polypeptides according to the invention on the one hand, and moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein or conjugate. Similarly, the designations first and second monomer units are made for clarity reasons to distinguish between
15 said units. Thus, for example, said first moiety (or monomer unit) may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein or conjugate.

The skilled person is aware that the construction of a fusion protein often involves the use of linkers between the functional moieties to be fused,
20 and there are different kinds of linkers with different properties, such as flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers. Linkers have been used to for example increase stability or improve folding of fusion proteins, to increase expression, improve biological activity, enable targeting and alter pharmacokinetics of fusion proteins. Thus, in one
25 embodiment, the polypeptide according to any aspect disclosed herein further comprises at least one linker, such as at least one linker selected from flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers.

In one embodiment, said linker is arranged between a first moiety consisting of an IL-1R-I binding polypeptide as defined herein and a second
30 moiety consisting of a polypeptide having a desired biological activity. In another embodiment, said linker is arranged within said first moiety. For example, one or more linker(s) may be arranged between monomeric units of the polypeptide as defined herein. In yet another embodiment, linkers may be arranged within said first moiety and between said first moiety and said
35 second moiety.

Flexible linkers are often used in the art when the joined domains require a certain degree of movement or interaction, and may be particularly

useful in some embodiments. Such linkers are generally composed of small, non-polar (for example G) or polar (for example S or T) amino acids. Some flexible linkers primarily consist of stretches of G and S residues, for example (GGGGS)_p. Adjusting the copy number "p" allows for optimization of linker in order to achieve appropriate separation between the functional moieties or to maintain necessary inter-moiety interaction. Apart from G and S linkers, other flexible linkers are known in the art, such as G and S linkers containing additional amino acid residues, such as T and A, to maintain flexibility, as well as polar amino acid residues to improve solubility. In one embodiment, said linker is a flexible linker comprising at least one amino acid residue(s) selected from the group consisting of glycine, serine and alanine. The skilled person is aware of other suitable linkers.

In one embodiment, said linker is selected from GS, VDSS, VDGS, VEGS, ASGS, (GGGGS)₂ (SEQ ID NO:1683), AS(GGGGS)₂ (SEQ ID NO:1684) and ((KEAAA)₃KELAA)₂ (SEQ ID NO:1685). In one particular embodiment, said linker is AS(GGGGS)₂ (SEQ ID NO:1684) or ((KEAAA)₃KELAA)₂ (SEQ ID NO:1685). In one embodiment, said fusion protein or conjugate comprising a linker comprises a polypeptide having an amino acid sequence as set out in SEQ ID NO:1639-1658. In particular, an exemplary fusion protein or conjugate comprising the linker AS(GGGGS)₂ is set out in the amino acid sequence of SEQ ID NO:1646-1658, such as SEQ ID NO:1648 and 1657.

In further aspects of the present disclosure, there is provided a polynucleotide encoding an IL-1R-I binding polypeptide or a fusion protein as described herein; an expression vector comprising said polynucleotide; and a host cell comprising said expression vector.

Also encompassed by this disclosure is a method of producing a polypeptide or fusion protein as described above, comprising culturing said host cell under conditions permissive of expression of said polypeptide from its expression vector, and isolating the polypeptide.

The IL-1R-I binding polypeptide of the present disclosure may alternatively be produced by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising

- step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide according to the first aspect having protected reactive side-chains,

- removal of the protecting groups from the reactive side-chains of the polypeptide, and
- folding of the polypeptide in aqueous solution.

5 It should be understood that the IL-1R-I binding polypeptide according to the present disclosure may be useful as a therapeutic, diagnostic or prognostic agent in its own right or as a means for targeting other therapeutic or diagnostic agents, with e.g. direct or indirect effects on IL-1R-I. A direct therapeutic effect may for example be accomplished by inhibiting IL-1R-I
10 signaling.

 Thus, in another aspect, there is provided a composition comprising an IL-1R-I binding polypeptide, fusion protein or conjugate as described herein and at least one pharmaceutically acceptable excipient or carrier. In one embodiment, said composition further comprises at least one additional active
15 agent, such as at least two additional active agents, such as at least three additional active agents. Non-limiting examples of additional active agents that may prove useful in such combination are immune response modifying agents and anti-cancer agents as described herein. In one embodiment, said composition comprises a fusion protein or conjugate wherein said desired
20 biological activity is an *in vivo* half-life increasing activity such that said second moiety increases *in vivo* half-life of the fusion protein or conjugate. Examples of moieties providing such half-life increasing activities are disclosed elsewhere herein.

 Hence, in another aspect of the present disclosure, there is provided
25 an IL-1R-I binding polypeptide, fusion protein, conjugate or composition as described herein for use as a medicament, a prognostic agent or a diagnostic agent. In one embodiment, said IL-1R-I binding polypeptide is provided for use as a medicament.

 In one embodiment, there is provided an IL-1R-I binding polypeptide,
30 fusion protein or conjugate or composition as described herein, for use as a medicament to modulate IL-1R-I function *in vivo*. As used herein, the term "modulate" refers to changing the activity, such as rendering IL-1R-I function hypomorph, partially inhibiting or fully inhibiting IL-1R-I function.

 In one embodiment, there is provided an IL-1R-I binding polypeptide,
35 fusion protein or conjugate or composition as described herein, for use, in the treatment, prognosis or diagnosis of an IL-1R-I related disorder.

As used herein, the term "IL-1R-I related disorder" refers to any disorder, disease or condition in which IL-1R-I plays a regulatory role in the signaling pathway. Thus, in one embodiment, there is provided an IL-1R-I binding polypeptide, fusion protein or conjugate or composition as described
 5 herein, for use in the treatment of an IL-1R-I related disorder, such as a disorder selected from the group consisting of inflammatory disease, auto-inflammatory syndrome, autoimmune disease, infectious disease, cardiovascular disease, ischaemic disease, cancer and diabetes. In one
 10 embodiment, said IL-1R-I related disorder is selected from the group consisting of inflammatory disease, autoinflammatory syndrome and autoimmune disease.

In one embodiment, said IL-1R-I related disorder is selected from the group consisting of familial Mediterranean fever (FMF); cryopyrin-associated periodic syndrome (CAPS); TNF receptor-associated periodic syndrome
 15 (TRAPS); hyper-IgD syndrome (HIDS); periodic fever; aphthous stomatitis; pharyngitis; adenitis (PFAPA); rheumatoid arthritis (RA), juvenile RA, juvenile idiopathic arthritis, systemic juvenile idiopathic arthritis,; adult-onset Still's disease; Schnitzler syndrome; Muckle Wells Syndrome; macrophage activation syndrome; Behçet's disease; uveitis; acne vulgaris; pyoderma
 20 gangrenosum; gout; type 2 diabetes, new-onset diabetes; dry eye syndrome; hidradenitis suppurativa; neutrophilic dermatoses, in particular cytophagic histiocytic panniculitis, Weber-Christian disease, and neutrophilic panniculitis; cardiovascular disease, myocardial infarction, stroke; liver failure, kidney failure; acute lung injury; pseudogout, calcium-pyrophosphate deposition
 25 disorder, chondrocalcinosis, deficiency of the IL-1 receptor antagonist (DIRA), deficiency of the IL-36 receptor antagonist (DITRA), ADAM2 deficiency (DADA2), pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, pyoderma gangrenosum, acne and suppurative hidradenitis (PASH) syndrome, PAPA and suppurative hidradenitis (PAPASH) syndrome,
 30 autoinflammatory syndrome with lymphedema (AISLE), Phospholipase C-gamma-2 mutation autoinflammatory syndrome, NALP12-associated periodic syndrome (NAPS12), mevalonate kinase deficiency (MKD), psoriatic arthritis, reactive arthritis, ankylosing spondylitis, haemochromatosis-related arthritis, periarticular calcinosis, osteoarthritis, inflammatory osteoarthritis, hand
 35 osteoarthritis, pustular psoriasis such as generalised pustular psoriasis (GPP), palmoplantar pustulosis (PPP), acrodermatitis continua of Hallopeau (ACH); Blau syndrome; Sweet syndrome; various vasculitides such as giant-

cell arteritis (GCA), polymyalgia rheumatica (PMR), Takayasu arteritis, Kawasaki disease, urticarial vasculitis, and Henoch-Schönlein purpura (HSP); neutrophilic urticaria and idiopathic cold urticaria; lichen planus; polymyositis, dermatomyositis, juvenile dermatomyositis and inclusion-body myositis;

5 various stages of myeloma such as smoldering myeloma (SMM), indolent myeloma, Waldenstrom's macroglobulinemia, and multiple myeloma; tumor-induced cachexia; solid tumor growth; neonatal disorders such as bronchopulmonary dysplasia (prophylaxis), necrotising enterocolitis (NEC), retinopathy of prematurity (ROP), cerebral palsy due to perinatal cerebral

10 ischemia, and infant respiratory distress syndrome (IRDS); Whipple's disease; traumatic brain injury; refractory epilepsy; systemic inflammatory response syndrome (SIRS); cutaneous lupus; Jessner-Kanof disease; amyotrophic lateral sclerosis; systemic sclerosis (scleroderma); septic shock; acute pancreatitis; chronic recurrent multifocal osteomyelitis, non-bacterial

15 osteitis (NBO), synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO) syndrome, and Majeeed syndrome; relapsing polychondritis; idiopathic recurrent pericarditis (IRP); myocarditis; Erdheim-Chester disease; juvenile xanthogranuloma; islet-cell transplantation; haemodialysis-induced systemic inflammation; graft-versus-host disease; ANCA-associated glomerulonephritis

20 and recurrent glomerulonephritis; Cogan syndrome; autoimmune inner-ear disease; chronic granulomatous disease (CGD); Castleman's disease; cardiac failure; diastolic cardiac failure; antisyntetase syndrome; acute ACL injury; acute haemorrhagic leukoencephalitis; AA amyloidosis; Di George syndrome; generalised fatigue; chronic fatigue syndrome (CFS); gulf-war

25 illness (GWI); and narcolepsy.

In another embodiment, said IL-1R-I related disorder is cancer, such as a cancer selected from the group consisting of colon cancer, breast cancer, lung cancer, head and neck cancer, melanoma and prostate cancer.

30 The skilled person will appreciate that said IL-1R-I binding polypeptide, fusion protein or conjugate or a composition comprising an anti-IL-1R-I binding polypeptide, fusion protein or conjugate as described herein may be administered to a subject using standard administration techniques, including oral, topical, intravenous, intraperitoneal, subcutaneous, pulmonary,

35 transdermal, intramuscular, intranasal, buccal, sublingual, duodenal or suppository administration. Thus, in one embodiment, there is provided an IL-1R-I binding polypeptide, fusion protein or conjugate or a composition as

described herein for oral, topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual, duodenal or suppository administration. In one embodiment, said administration is oral, intravenous, subcutaneous or duodenal administration
5 and in one particular embodiment, said administration is oral administration.

As used herein, the term "subject" refers to a mammalian subject, such as a human subject.

Non-limiting examples of indications where topical administration routes could be especially relevant in IL-1R-I mediated disease include ocular
10 diseases such as dry eye disease, uveitis, scleritis, and ulcerative keratitis; dermatological diseases such as ichthyosis and generalized pustular psoriasis; pulmonary diseases such as adult respiratory distress syndrome, bronchopulmonary dysplasia, chronic obstructive pulmonary disease, asbestosis, and silicosis.

15 In some embodiments it may be beneficial that the polypeptide, fusion protein, conjugate or composition for use as described above, is administered repeatedly within a period of time, for example within a 24 hour period from disease onset. In one particular embodiment wherein said IL-1R-I binding polypeptide, fusion protein, conjugate or composition is administered
20 repeatedly within a 24 hour period from disease onset, such as at least two times within a 24 hour period from disease onset, such as at least three times within a 24 hour period from disease onset, to a subject in need thereof. In yet a particular embodiment, said IL-1R-I binding polypeptide, fusion protein, conjugate or composition is administered continuously during a limited time
25 period such as during 24 hours from disease onset. Repeated or continuous administration may be preferred when distribution to the brain is required, such as in acute treatment of stroke.

In some embodiments wherein said fusion protein or conjugate comprises a second moiety having an *in vivo* half-life increasing activity, said
30 fusion protein or conjugate, or composition comprising such a fusion protein or conjugate, is administered once weekly to a subject in need thereof.

In a related aspect, there is provided a method of treatment of an IL-1R-I related disorder, comprising administering to a subject in need thereof an effective amount of an IL-1R-I binding polypeptide, fusion protein, conjugate
35 or a composition as described herein. In a more specific embodiment of said method, the IL-1R-I binding polypeptide, fusion protein, conjugate or composition as described herein modulates IL-1R-I function *in vivo*.

In one embodiment, said IL-1R-I related disorder is selected from the group as defined above in connection with the previous aspect.

In some embodiments of said method of treatment, it may be beneficial that the polypeptide, fusion protein, conjugate or composition as described
5 above, is administered repeatedly within a period of time, for example within a 24 hour period from disease onset. In one particular embodiment wherein said IL-1R-I binding polypeptide, fusion protein, conjugate or composition is administered repeatedly within a 24 hour period from disease onset, such as at least two times within a 24 hour period from disease onset, such as at least
10 three times within a 24 hour period from disease onset, to a subject in need thereof. In yet a particular embodiment, said IL-1R-I binding polypeptide, fusion protein, conjugate or composition is administered continuously during a limited time period, such as within 24 hours from disease onset. Repeated or continuous administration may be preferred when distribution to the central
15 nervous system is required, such as in acute treatment of stroke.

In other embodiments, of said method of treatment, said fusion protein or conjugate comprises a second moiety having an *in vivo* half-life increasing activity, or said composition comprises a fusion protein or conjugate comprising a second moiety having an *in vivo* half-life increasing activity. In
20 such methods, said fusion protein, conjugate or composition may be administered once weekly to a subject in need thereof.

It may be beneficial to administer a therapeutically effective amount of an IL-1R-I binding polypeptide, fusion protein or conjugate or composition as described herein and at least one second drug substance, such as an
25 immune response modulating agent as described above or an anti-cancer agent.

As used herein, the term "co-administration" encompasses both concomitant and sequential administration. Thus, in one embodiment there is provided a method as defined above further comprising co-administration of
30 an immune response modulating agent as described above. In another embodiment there is provided a method as defined above further comprising co-administration of an anti-cancer agent as described above.

In another aspect of the present disclosure, there is provided a method of detecting IL-1R-I, comprising providing a sample suspected to contain IL-
35 1R-I, contacting said sample with an IL-1R-I binding polypeptide, fusion protein, conjugate or a composition as described herein, and detecting the binding of the IL-1R-I binding polypeptide, fusion protein, conjugate or

composition to indicate the presence of IL-1R-I in the sample. In one embodiment, said method further comprises an intermediate washing step for removing non-bound polypeptide, fusion protein, conjugate or composition, after contacting the sample.

5

While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or molecule to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment contemplated, but that the invention will include all embodiments falling within the scope of the appended claims.

15

Brief description of the figures

Figure 1 is a listing of the amino acid sequences of examples of IL-1R-I binding polypeptides of the present disclosure (SEQ ID NO:1-1638 and 1667-1679), albumin binding polypeptides (SEQ ID NO:1659-1661); human IgG1 Fc (SEQ ID NO:1662), human albumin (SEQ ID NO:1663) and human transferrin (SEQ ID NO:1664), examples of IL-1R-1 binding fusion polypeptides comprising said polypeptides (SEQ ID NO:1639-1658 and 1734-1737) optionally including a linker, control polypeptides (GS-hIL-1 β , SEQ ID NO:1733) as well as the amino acid sequences of cynomolgus IL-1R-I-His₆ (SEQ ID NO:1665) and cynomolgus IL-1R-I-Fc (SEQ ID NO:1666) used for selection, screening and/or characterization for illustration of the invention. The deduced IL-1R-I binding motifs (BMs) of the IL-1R-I binding polypeptides disclosed herein extend from residue 8 to residue 36 in sequences with SEQ ID NO:1-1638 and 1667-1668 and 1670-1679. In SEQ ID NO:1669, said BM extends from residue 5 to residue 33. The amino acid sequences of the 49 amino acid residues long polypeptides (BMod) predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55, except for the N-terminally truncated Z variant with SEQ ID NO:1669 wherein it extends from residue 4 to residue 52.

Figure 2 is a diagram showing concentration-response curves for inhibition of IL-1 β induced IL-6 release in NHDF cells for six anti-IL-1R-I binding fusion proteins (PSI0536, SEQ ID NO:1648; PSI0537, SEQ ID

NO:1649; PSI0534, SEQ ID NO:1646; PSI0535, SEQ ID NO:1647; PSI0538, SEQ ID NO:1650; PSI0539, SEQ ID NO:1651). Anakinra (dotted line) was included as a reference.

Figure 3 is a diagram showing concentration of IL-1R-I binding Z variants at 50 % inhibition (IC_{50}) of IL-1 β induced IL-6 release in human whole blood from five to ten individual donors. The median IC_{50} was 0.31, 1.25 and 0.30 nM for PSI0536 (SEQ ID NO:1648), PSI537 (SEQ ID NO:1649) and anakinra, respectively.

10 Examples

Summary

The following Examples disclose the development of novel Z variant molecules targeted to interleukin 1 receptor I (IL-1R-I) based on phage display technology. The IL-1R-I binding polypeptides described herein were sequenced, and their amino acid sequences are listed in Figure 1 with the sequence identifiers SEQ ID NO:1-1632. Additional IL-1R-I binding polypeptides disclosed herein are listed in Figure 1 with sequence identifiers SEQ ID NO:1633-1638 and 1667-1679. The Examples further describe the characterization of IL-1R-I binding polypeptides and *in vitro* functionality of said polypeptides. As used herein, the term "IL-1R-I binding Z variants" refers to 58 amino acid long IL-1R-I binding polypeptides comprising an IL-1R-I binding motif as disclosed herein.

25 Example 1

Selection and screening of IL-1R-I binding Z variants

In this Example, human and cynomolgus IL-1R-I (hIL-1R-I and cIL-1R-I, respectively) were used as target proteins in phage display selections using a phage library of Z variants. The DNA of selected clones was sequenced, the Z variants were produced in *E. coli* as periplasmic fractions and assayed against IL-1R-I in ELISA (enzyme-linked immunosorbent assay).

Materials and methods

35 Biotinylation of target protein: Human IL-1R-I-Fc (hIL-1RI-Fc Creative BioMart cat. no. IL1RI-771H) and human IL-1R-I (hIL-1RI, RnD Systems cat. no. 269-1R/CF) were biotinylated according to the manufacturer's

recommendations at room temperature (RT) for 30 min using No-Weigh EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, cat. no. 21327) at a 10 x molar excess. Prior to the biotinylation, a buffer exchange to phosphate buffered saline (PBS, 10 mM phosphate, 137 mM NaCl, 2.68 mM KCl, pH 7.4) was performed for hIL-1R-I-Fc using a dialysis cassette (Slide-a-lyzer 3.5 K, 3500 MWCO, Thermo Scientific, cat. no. 66333), according to the manufacturer's instructions, and hIL-1R-I was dissolved in 100 mM phosphate buffer pH 6.5. After the biotinylation, a buffer exchange to PBS was performed for both proteins, as described above.

10 Expression of his-tagged target protein: Expression of cynomolgus IL-1R-I-His₆ (cIL-1R-I-His₆, SEQ ID NO:1665) was performed using the FreeStyle 293-F expression system (Thermo Fisher Scientific), essentially according to the manufacturer's protocol. Supernatants were harvested by centrifugation 5 days after transfection of expression vectors and stored at -70°C. The frozen supernatant from the FreeStyle 293-F cultures were thawed and filtrated (0.22 µm). The supernatant, containing the cIL-1R-I-His₆ was purified using affinity chromatography with an IMAC column. The purified protein was buffer exchanged to PBS. The purity of the protein was analyzed by SDS-PAGE stained with Coomassie Blue and the molecular weight was analyzed using mass spectrometry (HPLC/MS or MALDI-TOF/MS).

20 Phage display selection of IL-1R-I binding Z variants: A library of random variants of protein Z displayed on bacteriophage in fusion to a wild type albumin binding domain (abbreviated ABD001, SEQ ID NO:1660) was used to select IL-1R-I binding Z variants. The library, which is denoted Zlib006Naive.II, has a size of 1.5×10^{10} library members (Z variants). The construction in the phage library vector pAY02592 and the phage stock production was previously described in WO 2016/113246.

30 Selections against hIL-1R-I-Fc, biotinylated hIL-1R-I-Fc (b-hIL-1R-I-Fc), biotinylated hIL-1R-I (b-hIL-1R-I) and cIL-1R-I-His₆ (SEQ ID NO:1665) were performed in four selection cycles. In the first cycle, four selection tracks were run in parallel; 1-1 to 1-4. The selection tracks 1-1 and 1-4 were divided into two parts in cycle two, resulting in a total of six parallel tracks in round 2, 3 and 4; 2-1 to 2-9, 3-1 to 3-9 and 4-1 to 4-9, respectively, as presented in Table 2. The number of phage particles used for selections was more than 2000 times the number of eluted phage particles in the previous cycle, but a lower amount was used in selection tracks 4-1 to 4-5. Phage stock preparation, selection procedure and amplification of phage between

selection cycles were performed essentially as described in WO 2009/077175 with the following exceptions 1-8. Exception 1: PBS supplemented with 10 % fetal calf serum (FCS, Gibco, cat. no. 10108-165), 1.5 μ M human serum albumin (HSA, Novozymes, cat. no. 230-005) and 0.1 % Tween20 (Acros Organics, cat. no. 233362500) was used as selection buffer. Exception 2: pre-selection was performed in cycles 1-4 at >60 min at RT or overnight at 4 °C by incubation of phage stock with Dynabeads® M-280 Streptavidin (SA beads, Life technologies/Invitrogen, cat. no. 11206D), SA beads pre-coated with biotinylated human IgG1-Fc (b-hFc, Jackson Immuno Research cat. no. 009-060-008) (Fc/SA beads), SA beads pre-coated with b-hFc and (Z00000)₂-Cys-biotin, prepared essentially as described in WO 2009/077175 (Fc/Z00000/SA beads), protein A beads (Life technologies/Novex, cat. no. 10002D) pre-coated with b-hFc (Fc/protein A beads) or Dynabeads® His-Tag Isolation and Pulldown (IM beads, Life Technologies/Novex, cat. no. 10104D), respectively, as presented in Table 2. b-hFc was incubated with the beads (5, 8.5 or 10 μ g b-hFc per mg Z00000/SA beads, protein A beads or SA beads, respectively) for >1 h at RT, then the beads were washed with 2 x PBST (PBS supplemented with 0.1 % Tween20) before use in pre-selection. Exception 3: all tubes and beads used in the selections were pre-blocked with PBS supplemented with 3 % BSA (bovine serum albumin, Sigma cat. no. A3059) and 0.1 % Tween20. Exception 4: selections were performed in solution at RT and the time for selection was 140 min in the first cycle and 120 min in the following cycles. Exception 5: target-phage complexes were captured on beads using the following amount of target protein and beads: 3.2 μ g hIL-1R-I-Fc per mg Z00000/SA beads, 4 μ g hIL-1R-I-Fc per mg protein A beads, 2 μ g b-hIL-1R-I-Fc or 2 μ g b-hIL-1R-I per mg SA beads and 4.5 μ g cIL-1R-I-His₆ per mg IM beads, respectively. The bead type used for each selection track is the same as presented for pre-selection in Table 2. Exception 6: *E. coli* strain ER2738 cells (Lucigen, Middleton, WI, USA) grown in medium supplemented with 10 μ g/ml tetracycline were used for phage infection. Exception 7: a 5 x excess of M13K07 helper phage compared to bacteria was allowed to infect log phase bacteria. Exception 8: amplification of phage particles after round 2 and 3 was performed essentially as described in WO 2016/113246; Example 5, but using Tryptic Soy Broth + Yeast Extract (TSB-YE) medium supplemented with 100 μ M IPTG, 25 μ g/ml kanamycin, 100 μ g/ml ampicillin and 2 % glucose in the overnight culture.

An overview of the selection strategy, describing an increased stringency in the selection cycles obtained by using a lowered target concentration and an increased number of washes, is shown in Table 2.

5 Table 2: Overview of the phage display selection from a naive library.

Cycle	Selection track	Phage stock from library or selection track	Preselection beads	Target	Target concentration (nM)	Number of washes
1	1-1	Zlib006Naive.II	Fc/Z00000/SA	hIL-1R-I-Fc	100	2
1	1-2	Zlib006Naive.II	Fc/protein A	hIL-1R-I-Fc	100	2
1	1-3	Zlib006Naive.II	Fc/SA	b-hIL-1R-I-Fc	100	2
1	1-4	Zlib006Naive.II	SA	b-hIL-1R-I	100	2
2	2-1	1-1	Fc/Z00000/SA	hIL-1R-I-Fc	67	4
2	2-2	1-1	Fc/Z00000/SA	hIL-1R-I-Fc	33	4
2	2-3	1-2	Fc/protein A	hIL-1R-I-Fc	67	4
2	2-5	1-3	Fc/SA	b-hIL-1R-I-Fc	67	4
2	2-7	1-4	SA	b-hIL-1R-I	67	4
2	2-9	1-4	IM	cIL-1R-I-His ₆	67	4
3	3-1	2-1	Fc/Z00000/SA	hIL-1R-I-Fc	44	6
3	3-2	2-2	Fc/Z00000/SA	hIL-1R-I-Fc	11	6
3	3-3	2-3	Fc/protein A	hIL-1R-I-Fc	44	6
3	3-5	2-5	Fc/SA	b-hIL-1R-I-Fc	44	6
3	3-7	2-7	SA	b-hIL-1R-I	44	6
3	3-9	2-9	SA	b-hIL-1R-I	44	6
4	4-1	3-1	Fc/Z00000/SA	hIL-1R-I-Fc	30	8
4	4-2	3-2	Fc/Z00000/SA	hIL-1R-I-Fc	3.7	8
4	4-3	3-3	Fc/protein A	hIL-1R-I-Fc	30	8
4	4-5	3-5	Fc/SA	b-hIL-1R-I-Fc	30	8
4	4-7	3-7	SA	b-hIL-1R-I	30	8
4	4-9	3-9	IM	cIL-1R-I-His ₆	30	8

Washes were performed for 1 min using PBST 0.1 %, and elution was carried out as in the protocol for bead bound phage/target complexes eluted at pH 2 described in WO 2009/077175.

- 10 Production of Z variants for ELISA: The Z variants were produced essentially as described in WO 2016/113246 with the exception that the final volume of pellet dissolving from the 1 ml culture was 1000 µl PBST 0.05 % (PBS supplemented with 0.05 % Tween20).

- 15 The final supernatant of the periplasmic extract contained the Z variants as fusions to ABD (Z-ABD; ABD001, SEQ ID NO:1660), expressed as AQHDEALE-[Z#####]-VDYV-[ABD]-YVPG (Grönwall et al. (2007) J

Biotechnol, 128:162-183). Z##### refers to individual 58 amino acid residue Z variants.

ELISA screening of Z variants: The binding of Z variants to human IL-1R-I was analyzed in ELISA, essentially as described in WO 2016/113246, using 1 nM hIL-1R-I-Fc as target, and using a goat anti-human IgG-HRP (Southern Biotech, cat. no. 2040-05) diluted 1:10,000 for detection. As blank control, PBST 0.05 % was added instead of the Z-ABD periplasmic sample and as negative control, a periplasmic extract with ABD001 (SEQ ID NO:1660) was added instead of the Z-ABD periplasmic sample. Control plates were assayed in a similar setup using 100 nM b-hFc instead of target protein and streptavidin conjugated HRP (Thermo Scientific, cat. no. N100) diluted 1:30,000 for detection.

Sequencing: In parallel with the ELISA screening, the clones were picked for sequencing. PCR fragments, amplified from single colonies, were sequenced and analyzed essentially as described in WO 2009/077175.

EC₅₀ analysis of Z variants: A selection of IL-1R-I binding Z variants was subjected to an analysis of response against a dilution series of hIL-1R-I-Fc using ELISA as described above. The target protein hIL-1R-I-Fc was diluted stepwise 1:10 from 100 to 0.01 nM. As a background control, the Z variants were assayed with no target protein added. Obtained data was analyzed using GraphPad Prism 5 and non-linear regression, and the EC₅₀ values (the half maximal effective concentration) were calculated.

Results

Phage display selection of IL-1R-I binding Z variants: Individual clones were obtained after four cycles of phage display selections against human and cynomolgus IL-1R-I.

ELISA screening of Z variants: The clones obtained after four cycles of selection were produced individually in 96-well plates and were screened for hIL-1R-I-Fc binding activity in ELISA using 1 nM target protein. In parallel, an ELISA to exclude Fc binding was run. Clones positive to hIL-1R-I were identified and none of the IL-1R-I positive clones were positive to Fc solely. No response was obtained for the ABD negative control. 45 identified Z variants displayed an ELISA screening result that was more than 0,71 AU (8.5 x the control background).

Sequencing: In parallel with ELISA screening, sequencing was performed for clones obtained after four cycles of selection. Each variant was

given a unique identification number #####, and individual variants are referred to as Z#####. The amino acid sequences of a subset of the identified 58 amino acid residues long Z variants are listed in Figure 1 and in the sequence listing as SEQ ID NO:1-19. The deduced IL-1R-I binding motifs extend from residue 8 to residue 36 in each sequence. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55.

EC₅₀ analysis of Z variants: A subset of said Z variants (SEQ ID NO:1-19) was subjected to an ELISA target titration using hIL-1R-I-Fc. Obtained results were used for calculation of EC₅₀ values (Table 3).

Table 3: Calculated EC₅₀ values from ELISA titration analysis.

Z variant	SEQ ID NO	EC ₅₀ (M)	Z variant	SEQ ID NO	EC ₅₀ (M)
Z12884	13	6.4x10 ⁻¹⁰	Z12922	3	2.9x10 ⁻⁹
Z12891	19	2.2x10 ⁻⁹	Z12929	17	1.7x10 ⁻⁸
Z12895	12	2.3x10 ⁻¹⁰	Z12933	2	1.5x10 ⁻⁹
Z12905	7	7.3x10 ⁻⁹	Z12941	4	4.6x10 ⁻¹⁰
Z12910	11	2.3x10 ⁻¹⁰	Z12961	16	2.9x10 ⁻¹⁰
Z12911	18	3.2x10 ⁻¹⁰	Z12964	1	3.9x10 ⁻¹⁰
Z12912	10	3.9x10 ⁻¹⁰	Z12967	9	1.7x10 ⁻¹⁰
Z12915	5	4.3x10 ⁻⁹	Z12974	8	2.1x10 ⁻¹⁰
Z12917	15	3.3x10 ⁻¹⁰	Z12975	6	2.6x10 ⁻¹⁰
Z12920	14	6.6x10 ⁻⁹			

15

Example 2

Production and characterization of primary IL-1R-I binding Z variants

This Example describes the general procedure for subcloning and production of His-tagged Z variants originating from the primary phage selection, characterization of their target binding, target blocking and melting points.

20

Materials and methods

Subcloning of Z variants with a His₆ tag: The DNA of respective Z variant was amplified from the phage library vector pAY02592. A subcloning strategy for construction of monomeric Z variant molecules with N-terminal His₆ tag was applied using standard molecular biology techniques. The Z

25

gene fragments were subcloned into an expression vector resulting in the encoded sequence MGSSHHHHHHLQ-[Z#####]-VD.

Cultivation: *E. coli* T7E2 cells (GeneBridges) were transformed with plasmids containing the gene fragment of each respective IL-1R-I binding Z variant. The resulting recombinant strains were cultivated in media supplemented with 50 µg/ml kanamycin, either at 30 °C in 50 ml scale using the EnPresso protocol (BioSilta), or at 37 °C in 930 ml TSB-YE medium. In order to induce protein expression, IPTG was added to a final concentration of 0.2 mM at OD600 ≈ 10 (EnPresso) or 2 (TSB+YE). After induction, the cultivations were incubated for 16 h (EnPresso) or 5 h (TSB+YE). The cells were harvested by centrifugation.

Purification of IL-1R-I binding Z variants with a His₆ tag: Approximately 1-2 g of each cell pellet was resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) supplemented with Benzonase® (Merck). After cell disruption, cell debris was removed by centrifugation and each supernatant was applied on a 1 ml His GraviTrap IMAC column (GE Healthcare). Contaminants were removed by washing with wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH 7.4) and the Z variants were subsequently eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). After the IMAC purification, the buffer was exchanged to PBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4) using PD-10 desalting columns (GE Healthcare). All Z variants were subjected to a second purification step. Each Z variant was loaded on a 1 ml Resource 15RPC column (GE Healthcare), previously equilibrated with RPC solvent A (0.1 % TFA, 10 % ACN, 90 % water). After column wash with RPC solvent A, bound proteins were eluted with a linear gradient 0-50 % RPC solvent B (0.1 % TFA, 80 % ACN, 20 % water) for 20 ml. The buffer was then exchanged to PBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4) using PD-10 desalting columns (GE Healthcare).

Protein concentrations were determined by measuring the absorbance at 280 nm, using a NanoDrop® ND-1000 spectrophotometer (Saveen Werner AB) and the extinction coefficient of the respective protein. Samples with a concentration less than approximately 1 mg/ml were concentrated using Amicon Ultra - 4, Ultracel - 3K (MerckMillipore). The purity was analyzed by SDS-PAGE stained with Coomassie Blue and the identity of each purified Z variant was confirmed using LC/MS analysis.

Biacore affinity analysis: Affinities (K_D) for human and cynomolgus IL-1R-I were determined for His₆-tagged Z variants using a Biacore 2000 instrument (GE Healthcare). hIL-1R-I or cIL-1R-I-His₆ was immobilized on the carboxylated dextran layer in different flow cells of a CM5 chip (GE Healthcare, cat. no. BR100012). The immobilization was performed using amine coupling chemistry according to the manufacturer's protocol. Acetate pH 4.5 (GE Healthcare, cat. no. BR100350) was used for ligand dilution and HBS-EP (GE Healthcare, cat. no. BR100188) was used as running buffer. One flow cell surface on the chip was activated and deactivated for use as blank during analyte injections. In the kinetic experiment, HBS-EP was used as running buffer, the flow rate was 50 μ l/min and the temperature was 25 °C. The analytes, i.e. the Z variants, were each diluted in HBS-EP buffer to final concentrations of 40 nM and 10 nM, and were injected over the target chip surfaces for 2 min, followed by dissociation in running buffer for 8 min. Kinetic constants were calculated from the obtained sensorgrams using a 1:1 binding model, in the BiaEvaluation software 4.1 (GE Healthcare).

In vitro IL-1 β neutralization assay: The TF-1 cell line proliferates in response to many different cytokines including IL-1 β . This cell line was used to assess the ability of primary IL-1R1 binders to inhibit IL-1 β actions. TF-1 cells were maintained in RPMI1640 with L-glutamine (Lonza) supplemented with 10 % FCS (Gibco), Penicillin-Streptomycin (Lonza) and 2 ng/ml rhGM-CSF (R&D Systems). Prior to use, cells were washed twice in RPMI1640 without rhGM-CSF. Cells were then counted and dispensed into 96 well flat bottomed plates at a density of 3×10^4 cells per well. In separate plates, serial dilutions of the inhibitory binders; His₆-tagged IL-1R-I binding Z variants, with a concentration range of 400-0.1 nM, or the IL-1R-I binding protein Kineret (anakinra, Sobi), with a concentration range of 0.3-0.0003 nM, were incubated in the presence of 0.6 nM IL-1 β (PeproTech). The pre-mixed complexes of the Z variant polypeptides and IL-1 β were transferred to wells containing TF-1 cells. The cells were stimulated for 72 h at 37 °C in a humidified 5 % CO₂ atmosphere. During the last four hours of incubation, 19 μ l of 2 x diluted CCK-8 (Fluka, Sigma Aldrich), was added per well to determine the proliferative responses. The absorbance was measured at 450 nm using a microplate reader (Victor3, Perkin Elmer). The data on cell growth was assessed by non-linear regression to a four-parameter dose-response curve, and the half maximal inhibitory concentration (IC₅₀) was determined using GraphPadPrism program.

Circular dichroism (CD) spectroscopy analysis: Purified His₆-tagged Z variants were diluted to 0.5 mg/ml in PBS. For each diluted Z variant, a CD spectrum at 250-195 nm was obtained at 20 °C. In addition, a variable temperature measurement (VTM) was performed to determine the melting temperature (T_m). In the VTM, the absorbance was measured at 221 nm while the temperature was raised from 20 to 90 °C, with a temperature slope of 5 °C/min. A new CD spectrum was obtained at 20 °C after the heating procedure in order to study the refolding ability of the Z variants. The CD measurements were performed on a Jasco J-810 spectropolarimeter (Jasco Scandinavia AB) using a cell with an optical path length of 1 mm.

Results

Production of His₆-tagged Z variants: The IL-1R-I binding Z variants with a His₆ tag were expressed as soluble gene products in *E. coli*. The amount of purified protein from approximately 1-2 g bacterial pellet was determined spectrophotometrically by measuring the absorbance at 280 nm and ranged from approximately 3 to 25 mg for the different IL-1R-I binding Z variants before RPC purification. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the IL-1R-I binding Z variant. The correct identity and molecular weight of each Z variant were confirmed by HPLC-MS analysis.

Biacore affinity analysis: The interactions of His₆-tagged IL-1R-I-binding Z variants with human and cynomolgus IL-1R-I were analyzed in a Biacore instrument by injecting two concentrations of each Z variant over surfaces containing immobilized IL-1R-I. The calculated affinities (K_D) for human IL-1R-I are presented in Table 4. The strongest binder to human IL-1R-I (Z12967, SEQ ID NO:9) bound to human IL-1R-I with an affinity of 1.2×10^{-9} M and to cynomolgus IL-1R-I with an affinity of 3.5×10^{-8} M.

Table 4. Affinities for His₆-Z polypeptides binding to IL-1R-I.

Z variant	SEQ ID NO	Human IL-1R-I K _D (M)
Z12884	13	1.8x10 ⁻⁸
Z12895	12	5.5x10 ⁻⁹
Z12911	18	7.1x10 ⁻⁹
Z12917	15	8.0x10 ⁻⁹
Z12922	3	9.8x10 ⁻⁹
Z12967	9	1.2x10 ⁻⁹
Z12974	8	1.5x10 ⁻⁸
Z12975	6	8.9x10 ⁻⁸

In vitro IL-1 β neutralization assay: The IL-1 β inhibition ability of His₆-tagged IL-1R-I-binding Z variants was analyzed in a TF-1 cell assay. The resulting IC₅₀ values are presented in Table 5.

Table 5: IC₅₀ values for His₆-Z polypeptides from a TF-1 cell assay.

Z variant	SEQ ID NO	IC ₅₀ (nM)
Z12884	13	27
Z12895	12	7.1
Z12911	18	30
Z12917	15	11
Z12922	3	66
Z12967	9	1.1
Z12974	8	60
Z12975	6	35
Kineret	-	0.05

CD analysis: The CD spectra determined for the IL-1R-I binding Z variants with a His₆ tag showed that each had an α -helical structure at 20 °C. This result was also verified in the variable temperature measurements, wherein the melting temperatures were determined (Table 6). Reversible folding was seen for all the IL-1R-I binding Z variants when overlaying spectra measured before and after heating to 90 °C.

Table 6: Melting temperatures (T_m).

Z variant	SEQ ID NO	T_m (°C)	Z variant	SEQ ID NO	T_m (°C)
Z12911	18	64	Z12975	6	51
Z12967	9	58	Z12884	13	49
Z12922	3	47	Z12917	15	56
Z12974	8	49	Z12895	12	55

Example 3

Design and construction of a first matured library of IL-1R-I binding Z variants

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In this Example, a matured library was constructed. The library was used for selection of further IL-1R-I binding Z variants. Selections from matured libraries are usually expected to result in binders with increased affinity (Orlova *et al.*, (2006) Cancer Res 66(8):4339-48).

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Materials and methods

Library design: The library was primarily based on human IL-1R-I binding Z variants from the primary selection described in Example 1 and 2. Sequences of a subset of the Z variants derived from the primary selection are listed in Figure 1 and in the sequence listing as SEQ ID NO:1-19. In the new library, denoted Zlib006IL-1RI.I, ten variable positions in the Z molecule scaffold were biased towards certain amino acid residues, and three positions were kept constant. Two oligonucleotides, generated by the TRIM technology, which enables incorporation of randomized sets of trinucleotide building blocks, were ordered from Ella Biotech (Martinsried, Germany). The oligonucleotides represented helix 1 and helix 2 of the Z molecule, respectively, and contained an annealing region of 18 overlapping nucleotides. The assembled double stranded DNA sequence was: 5'-AA ATA AAT CTC GAG GTA GAT GCC AAA TAC GCC AAA GAA NNN NNN NNN GCG NNN NNN GAG ATC NNN NNN CTG CCT AAC CTC ACC NNN NNN CAA NNN NNN GCC TTC ATC NNN AAA TTA NNN GAT GAC CCA AGC CAG AGC TCA TTA TTT A-3' (SEQ ID NO:1680; designed codons are denoted NNN). The sequence encodes a partially randomized helix 1 and 2 of the Z variant amino acid sequence flanked by the restriction sites XhoI and SacI. The design for each amino acid residue of the new library, including ten variable amino acid positions (9, 10, 11, 13, 14, 17, 18, 25, 32 and 35) and three constant amino acid positions (24, 27 and 28) in the Z molecule

scaffold, are displayed in Table 7. The resulting theoretical library size was 9.7×10^8 variants. An even theoretical distribution of the different amino acids was applied within each amino acid position, except from position 9, 13, 17, 18, 32 and 35, where a higher portion of selected amino acids was applied.

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Table 7: Design of first maturation library Zlib006IL-1RI.I.

Amino acid position in the Z variant molecule	Amino acid randomization (percentage when uneven distribution)	No. of different amino acids
9	A(30 %),E,F,H,I,K,L,Q,R,S,T,V,W,Y	14
10	A,D,E,F,H,I,K,L,Q,R,S,T,V,W,Y	15
11	A,D,E,F,H,I,K,L,N,Q,R,S,T,V,W,Y	16
13	A,F,H,I,L,Q(20 %),S,T,V,W,Y(20 %)	11
14	A,D,E,F,H,I,K,L,M,Q,R,S,T,V,W,Y	16
17	F,W(20 %),Y	3
18	A(11 %),D,E,F,G,H,I,K,L,N,Q,R,S,T,V,W,Y	17
24	R	1
25	H,K,R,S	4
27	Y	1
28	T	1
32	A,I,L,R(70 %)	4
35	F(80 %),L(20 %)	2

Library construction: The two oligonucleotides were assembled, amplified, cloned into vector pAY02592, and transformed into *E. coli* ER2738, essentially as described for matured libraries in WO 2015/189430. Clones from the Zlib006IL-1RI.I library were sequenced (as described in WO 2009/077175) in order to verify the content and to evaluate the outcome of the constructed library vis-à-vis the library design.

Preparation of phage stock: A phage stock containing the phagemid library was prepared in shake flasks in two batches. Cells from a glycerol stock containing the phagemid library were inoculated in 2 and 3 l, respectively, of TSB-YE medium, supplemented with 2 % glucose, 10 µg/ml tetracycline and 100 µg/ml ampicillin. The cultivations were grown at 37 °C until OD₆₀₀ reached log-phase 0.5-0.9. The cultivations were infected using a 4-10 x molar excess of M13K07 helper phage and were incubated for 30 minutes at 37 °C. Batch 1 cultivations were pelleted by centrifugation, dissolved in TSB-YE medium, supplemented with 100 µg/ml ampicillin, 25 µg/ml kanamycin and 0.1 mM IPTG and grown at 30 °C overnight. To batch 2

cultivation, 0.1 mM IPTG was added, followed by an incubation of 1 h at 37 °C. After addition of 25 µg/ml kanamycin, the cultures were grown at 30 °C overnight. For both batches, the overnight grown cells were pelleted by centrifugation at 4,000 g and the phage particles remaining in the medium were thereafter precipitated twice in PEG/NaCl, filtered and dissolved in PBS and glycerol as described in Grönwall *et al.*, *supra*. Phage stocks were stored at -80 °C until use in selection.

Results

Library construction: The new library Zlib006IL-1RI.I was designed based on a set of IL-1R-I binding Z variants with verified binding properties (Example 1 and 2). The theoretical size of the designed library was 9.7×10^8 Z variants. The actual size of the library, determined by titration after transformation to *E. coli* ER2738 cells, was 5.6×10^9 transformants. The library quality was tested by sequencing 192 transformants and by comparing their actual sequences with the theoretical design. The contents of the actual library compared to the designed library were shown to be satisfactory. A first matured library of potential binders to IL-1R-I was thus successfully constructed.

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Example 4

Selection and screening of Z variants from the first matured library

In this Example, human and cynomolgus IL-1R-I were used as target proteins in phage display selections using a matured phage library of Z variants. The DNA of selected clones was sequenced, the Z variants were produced in *E. coli* periplasmic fractions and assayed against IL-1R-I in ELISA and Biacore.

Materials and methods

Expression of target protein: Expression of Fc fused cynomolgus IL-1R-I (cIL-1R-I-Fc, SEQ ID NO:1666) was performed using the Expi293 expression system (Thermo Fisher Scientific), essentially according to the manufacturer's protocol. Supernatants were harvested by centrifugation 7 days after transfection of expression vectors and stored at -70 °C. The frozen supernatant from the Expi293 culture were thawed and filtrated (0.22 µm). The supernatant containing the cIL-1R-I-Fc was purified using affinity chromatography with a MabSelect SuRe column. The purified protein was

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buffer exchanged to PBS. The purity of the protein was analyzed by SDS-PAGE stained with Coomassie Blue and the molecular weight was analyzed using mass spectrometry (HPLC/MS or MALDI-TOF/MS).

Phage display selection of IL-1R-I binding Z variants: The biotinylated

- 5 human target proteins b-hIL-1R-I-Fc and b-hIL-1R-I, and the cynomolgus target proteins cIL-1R-I-Fc (SEQ ID NO:1666) and b-cIL-1R-I-Fc (cIL-1R-I-Fc biotinylated as hIL-1R-I-Fc in Example 1), were used in phage selections using the new library of Z variant molecules described in Example 3. An overview of the selection strategy, describing the parallel selection tracks and
- 10 an overall increased stringency in the selection cycles obtained by using a lowered target concentration and an increased number of washes, is shown in Table 8. The selections were performed in four to five cycles essentially as described in Example 1, with the following exceptions 1-6. Exception 1: pre-selection was performed for >60 min at RT by incubation of phage stocks with
- 15 Fc/SA beads (selection tracks 1-1, 1-2, 1-4, 1-6, 1-8, 2-14 and 2-16), SA beads (selection tracks 1-3, 1-7, 1-9, 2-15 and 2-17) or Fc/protein A beads (selection track 1-5), respectively. Exception 2: the time for selection was 30-100 min as presented for each track in Table 8 Exception 3: target-phage complexes were captured on beads using the following amount of target
- 20 protein and beads: 8 µg b-hIL-1R-I-Fc, 1 µg b-hIL-1R-I or 4 µg cIL-1R-I-Fc per mg SA beads, respectively, and 5 µg cIL-1R-I-Fc per mg protein A beads. In selection round 5, 2 mg SPHERO neutravidin beads (Spherotech cat. no. NVM-20-5) were used for target-phage complex capture. Exception 4: in the second final wash step of tracks 2-14, 2-15, 2-17, 3-14, 3-15 and 3-17,
- 25 respectively, hIL-1R-I was added to the wash buffer in 100 times higher concentration than the target concentration of the respective track and the wash was run for 15-20 min. Exception 5: the phage/target/bead complexes of selection tracks 4-1 to 4-13 were divided into two after the bead wash procedure. Half of the samples was submitted to elution and the other half,
- 30 denoted 4-1x to 4-13x, was submitted to an extra 66 h, wash step before elution. Exception 6: two selection rounds four tracks, 4-4x and 4-6x, were submitted to a fifth selection round, resulting in tracks 5-1 and 5-2, as presented in Table 8. *E. coli* XL-1 Blue cells (Agilent Technologies, cat. no. 200268), grown in medium supplemented with 10 µg/ml tetracycline, were
- 35 used for phage infection and a 10 x excess of M13K07 helper phage compared to bacteria was allowed to infect log phase bacteria. Amplification

of phage particles after round 1 to 3 was performed essentially as described for round 2 and 3 in Example 1.

Table 8: Overview of selections from the first matured library.

Cycle	Selection track	Phage stock from library or selection track	Target	Target concentration (nM)	Selection time (min)	Number of washes
1	1-1	Zlib006IL-1R-I	b-hIL-1R-I-Fc	50	70	4
1	1-2	Zlib006IL-1R-I	b-hIL-1R-I-Fc	10	70	4
1	1-3	Zlib006IL-1R-I	b-hIL-1R-I	50	70	4
1	1-4	Zlib006IL-1R-I	b-cIL-1R-I-Fc	100	70	2
1	1-5	Zlib006IL-1R-I	cIL-1R-I-Fc	100	70	2
1	1-6	Zlib006IL-1R-I	b-hIL-1R-I-Fc	10	45	5
1	1-7	Zlib006IL-1R-I	b-hIL-1R-I	10	45	5
1	1-8	Zlib006IL-1R-I	b-hIL-1R-I-Fc	10	45	5
1	1-9	Zlib006IL-1R-I	b-hIL-1R-I	10	45	5
2	2-1	1-1	b-hIL-1R-I-Fc	20	60	6
2	2-2	1-1	b-hIL-1R-I-Fc	5	60	6
2	2-3	1-2	b-hIL-1R-I-Fc	4	60	8
2	2-4	1-2	b-hIL-1R-I-Fc	4	60	12
2	2-5	1-3	b-hIL-1R-I	20	60	6
2	2-6	1-3	b-hIL-1R-I	10	60	6
2	2-7	1-3	b-hIL-1R-I	10	60	10
2	2-8	1-4	b-cIL-1R-I-Fc	50	60	4
2	2-9	1-5	cIL-1R-I-Fc	50	60	4
2	2-10	1-1	b-cIL-1R-I-Fc	100	60	2
2	2-11	1-1	b-cIL-1R-I-Fc	50	60	6
2	2-12	1-1	cIL-1R-I-Fc	100	60	2
2	2-13	1-1	cIL-1R-I-Fc	50	60	6
2	2-14	1-8	b-hIL-1R-I-Fc	2	30	10*
2	2-15	1-9	b-hIL-1R-I	2	30	10*
2	2-16	1-9	b-cIL-1R-I-Fc	25	30	3
2	2-17	1-6/1-7 pool	b-hIL-1R-I	2	30	10*
3	3-1	2-1	b-hIL-1R-I-Fc	5	60	8
3	3-2	2-2	b-hIL-1R-I-Fc	0.5	60	8
3	3-3	2-3	b-hIL-1R-I-Fc	1	60	12

3	3-4	2-4	b-hIL-1R-I-Fc	1	60	24
3	3-5	2-5	b-hIL-1R-I	5	60	8
3	3-6	2-6	b-hIL-1R-I	1	60	8
3	3-7	2-7	b-hIL-1R-I	2	60	20
3	3-8	2-8	b-cIL-1R-I-Fc	25	60	6
3	3-9	2-9	cIL-1R-I-Fc	25	60	6
3	3-10	2-10	b-hIL-1R-I-Fc	25	60	6
3	3-11	2-11	b-hIL-1R-I-Fc	5	60	8
3	3-12	2-12	b-hIL-1R-I-Fc	25	60	6
3	3-13	2-13	b-hIL-1R-I-Fc	5	60	8
3	3-14	2-14	b-hIL-1R-I-Fc	0.5	40	16*
3	3-15	2-15	b-hIL-1R-I	0.5	40	16*
3	3-16	2-16	b-hIL-1R-I	2	40	9
3	3-17	2-17	b-hIL-1R-I	0.4	40	16*
4	4-1	3-1	b-hIL-1R-I-Fc	1	40	10**
4	4-2	3-2	b-hIL-1R-I-Fc	0.05	40	10**
4	4-3	3-3	b-hIL-1R-I-Fc	0.2	40	16**
4	4-4	3-4	b-hIL-1R-I-Fc	0.2	40	36**
4	4-5	3-5	b-hIL-1R-I	1	40	10**
4	4-6	3-6	b-hIL-1R-I	0.1	40	10**
4	4-7	3-7	b-hIL-1R-I	0.4	40	30**
4	4-8	3-8	b-cIL-1R-I-Fc	12.5	40	8**
4	4-9	3-9	cIL-1R-I-Fc	50	40	8**
4	4-10	3-10	b-cIL-1R-I-Fc	50	40	6**
4	4-11	3-11	b-cIL-1R-I-Fc	10	40	12**
4	4-12	3-12	cIL-1R-I-Fc	50	40	6**
4	4-13	3-13	cIL-1R-I-Fc	10	40	12**
4	4-14	3-14	b-hIL-1R-I-Fc	0.1	40	20
4	4-15	3-15	b-hIL-1R-I	0.1	40	20
4	4-16	3-16	b-cIL-1R-I-Fc	5	40	16
4	4-17	3-17	b-hIL-1R-I	0.2	40	16
5	5-1	4-4x	b-hIL-1R-I-Fc	50	100	3
5	5-2	4-6x	b-hIL-1R-I	50	100	3

*Addition of hIL-1R-I in the second final wash step.

**Selection track divided into two after washes. One part submitted to an extra wash, over the weekend, before elution.

In the first selection cycle, nine selection tracks were run; 1-1 to 1-9. In cycle two, some tracks were divided into two, three or six tracks and two tracks were pooled into one track, resulting in a total of 17 parallel tracks in cycle 2, 3 and 4; 2-1 to 2-17, 3-1 to 3-17 and 4-1 to 4-17, respectively. In cycle 5, two tracks were run; 5-1 and 5-2. In selection tracks 1-1 to 1-5, batch 1 of Zlib006IL-1RI.I was used, and in 1-6 to 1-9, batch 2 was used, respectively. All selection tracks are presented in Table 8. The number of phage particles used for selections was typically more than 2,000 times the number of eluted phage particles in the previous cycle.

Sequencing: Individual clones from cycle 4 or 5 of the different selection tracks were picked for sequencing. Amplification and sequence analysis of gene fragments were performed essentially as described in Example 1.

Production of Z variants for ELISA and Biacore screening: Z variants were produced essentially as described in Example 1 with the exception that the culture volume was 1.2 ml and that the periplasmic extracts were clarified by filtration using 1.2 µm 96 well filter plates (MerckMillipore cat. no. MSANLY50) after the freeze-thawing procedure. Z variants screened in Biacore were diluted five times in HBS-EP buffer.

ELISA screening of Z variants: The binding of Z variants to human was analyzed in ELISA, essentially as described in Example 1, using 0.3 nM hIL-1R-I-Fc as target protein. As blank control, PBST 0.05 % was added instead of the Z-ABD periplasmic extract.

EC₅₀ analysis of Z variants: A selection of IL-1R-I binding Z variants was subjected to an analysis of response against a dilution series of hIL-1R-I-Fc using ELISA as described above. The target protein hIL-1R-I-Fc was diluted stepwise 1:10 from 100 to 0.01 nM. As a background control, the Z variants were assayed with no target protein added. Obtained data was analyzed using GraphPad Prism 5 and non-linear regression, and the EC₅₀ values (the half maximal effective concentration) were calculated. A periplasmic extract with Z12967 (SEQ ID NO:9) was analyzed in parallel for signal comparison.

Biacore screening of Z variants: The binding of Z variants to human IL-1R-I was analyzed in a kinetic screening using a Biacore T200 instrument. A

polyclonal goat anti-ABD antibody (goat anti-ABD) was immobilized on CM5 chip surfaces basically as described for other proteins in Example 2. For the kinetic screening, analytes were injected in two steps. First, a Z-ABD (ABD001, SEQ ID NO:1660) periplasmic extract was injected over the surface at 5 µl/min for 1 min. As a second step, 100 nM hIL-1R-I was injected at 30 µl/min for 2 min, followed by 2 min of dissociation in running buffer HBS-EP. Glycine-HCl pH 2.0 (cat. no. BR100355, GE Healthcare) was used for regeneration of the antibody surfaces between the cycles. The temperature of the assay was 25 °C. Before performing the kinetic analyses, the signal from 100 nM hIL-1R-I injected over a reference surface containing goat anti-ABD but no Z-ABD sample was subtracted from the sensorgram of Z-ABD binding to hIL-1R-I. Rough screening affinities (K_D) were calculated from the reference subtracted 100 nM hIL-1R-I response using a 1:1 binding model of the BiaEvaluation software 4.1 (GE Healthcare). A periplasmic extract of Z12967 was included in the screening analysis for comparison. Periplasmic extracts of four Z variants were also submitted to a single cycle kinetics (SCK) assay, using the same setup as above, but injecting five hIL-1R-I concentrations and evaluating the data with a 1:1 model for single cycle kinetics.

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Results

Phage display selection of IL-1R-I binding Z variants: Individual clones were obtained after four or five cycles of phage display selections against human and cynomolgus IL-1R-I.

25 Sequencing: Sequencing was performed for clones obtained after four or five cycles of selection. Each variant was given a unique identification number #####, and individual variants are referred to as Z#####. The amino acid sequences of the 58 amino acid residues long Z variants are listed in Figure 1 and in the sequence listing as SEQ ID NO:20-1209. The deduced IL-1R-I binding motifs extend from residue 8 to residue 36 in each sequence. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55. It was observed that in one sequenced Z variant (Z18557, SEQ ID NO:1205) a valine residue was present in position 12.

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ELISA screening of Z variants: Clones obtained after four or five cycles of selection were produced individually in 96-well plates and were screened

for human IL-1R-I binding activity in ELISA. 98 % of the assayed Z variants gave a positive signal of 2 x the blank control or higher against 0.3 nM hIL-1R-I-Fc.

- 5 EC₅₀ analysis of Z variants: A subset of 115 Z variants displaying results over 0.99 AU (16.5 x the blank control) was subjected to ELISA target titration using hIL-1R-I-Fc. Obtained results were used for calculation of EC₅₀ values (Table 9). The result for Z12967 (SEQ ID NO:9) was 2.4×10^{-10} M.

Table 9: Calculated EC₅₀ values from ELISA titration analysis.

Z variant	SEQ ID NO	EC ₅₀ (M)	Z variant	SEQ ID NO	EC ₅₀ (M)	Z variant	SEQ ID NO	EC ₅₀ (M)
Z15811	21	2.5×10^{-10}	Z16054	258	2.4×10^{-10}	Z16246	441	3.9×10^{-10}
Z15813	23	3.5×10^{-10}	Z16058	262	2.3×10^{-10}	Z16255	449	1.7×10^{-10}
Z15824	34	2.3×10^{-10}	Z16070	273	2.3×10^{-10}	Z16263	457	2.6×10^{-10}
Z15840	49	2.5×10^{-10}	Z16072	275	2.2×10^{-10}	Z16264	458	3.0×10^{-10}
Z15862	71	2.1×10^{-10}	Z16084	287	2.2×10^{-10}	Z16277	470	2.6×10^{-10}
Z15876	1206	4.2×10^{-10}	Z16089	291	2.4×10^{-10}	Z16301	494	2.2×10^{-10}
Z15886	94	2.6×10^{-10}	Z16092	293	2.4×10^{-10}	Z16302	495	2.8×10^{-10}
Z15897	105	2.5×10^{-10}	Z16093	294	2.1×10^{-10}	Z16305	498	2.2×10^{-10}
Z15923	131	2.4×10^{-10}	Z16097	298	2.0×10^{-10}	Z16312	504	2.4×10^{-10}
Z15927	135	2.2×10^{-10}	Z16102	302	2.8×10^{-10}	Z16319	511	3.0×10^{-10}
Z15929	137	2.4×10^{-10}	Z16106	306	2.3×10^{-10}	Z16321	513	2.7×10^{-10}
Z15932	1207	2.3×10^{-10}	Z16116	316	2.1×10^{-10}	Z16329	519	4.5×10^{-10}
Z15949	154	2.5×10^{-10}	Z16117	317	2.1×10^{-10}	Z16341	531	1.7×10^{-10}
Z15953	158	2.1×10^{-10}	Z16121	321	2.3×10^{-10}	Z16363	550	2.0×10^{-10}
Z15960	165	2.3×10^{-10}	Z16122	322	2.9×10^{-10}	Z16377	562	2.1×10^{-10}
Z15961	166	2.1×10^{-10}	Z16127	325	2.1×10^{-10}	Z16378	563	2.0×10^{-10}
Z15975	1209	2.4×10^{-10}	Z16135	333	2.5×10^{-10}	Z16388	573	2.9×10^{-10}
Z15978	182	4.8×10^{-10}	Z16141	339	2.8×10^{-10}	Z16405	590	3.6×10^{-10}
Z15987	191	2.3×10^{-10}	Z16143	341	2.1×10^{-10}	Z16678	853	2.4×10^{-10}
Z15996	200	2.5×10^{-10}	Z16155	351	2.9×10^{-10}	Z16681	856	2.3×10^{-10}
Z15997	201	2.5×10^{-10}	Z16156	352	2.4×10^{-10}	Z16688	863	2.7×10^{-10}
Z15999	203	2.4×10^{-10}	Z16161	357	2.5×10^{-10}	Z16749	921	2.7×10^{-10}
Z16000	204	2.4×10^{-10}	Z16162	358	2.7×10^{-10}	Z16771	943	2.4×10^{-10}
Z16003	207	2.8×10^{-10}	Z16163	359	1.7×10^{-10}	Z17244	1147	4.2×10^{-10}
Z16004	208	2.5×10^{-10}	Z16164	360	6.8×10^{-10}	Z17255	1158	2.5×10^{-10}
Z16007	211	2.2×10^{-10}	Z16165	361	2.2×10^{-10}	Z17257	1160	4.8×10^{-10}
Z16008	212	2.3×10^{-10}	Z16168	364	2.8×10^{-10}	Z17269	1171	2.7×10^{-10}
Z16011	215	2.2×10^{-10}	Z16169	365	1.8×10^{-10}	Z17270	1172	3.0×10^{-10}
Z16023	227	2.1×10^{-10}	Z16173	369	3.6×10^{-10}			

Z16034	238	2.3×10^{-10}	Z16178	374	2.4×10^{-10}	Z17274	1176	2.1×10^{-10}
Z16041	245	2.4×10^{-10}	Z16206	402	4.5×10^{-10}	Z17278	1180	2.7×10^{-10}
Z16042	246	2.4×10^{-10}	Z16207	403	2.2×10^{-10}	Z17279	1181	2.8×10^{-10}
Z16043	247	2.3×10^{-10}	Z16219	415	2.4×10^{-10}	Z17280	1182	2.2×10^{-10}
Z16046	250	2.7×10^{-10}	Z16234	429	5.4×10^{-10}	Z17288	1190	4.2×10^{-10}
Z16048	252	2.5×10^{-10}	Z16236	431	2.4×10^{-10}	Z17292	1194	2.0×10^{-10}
Z16049	253	2.4×10^{-10}	Z16241	436	4.9×10^{-10}	Z17296	1198	3.2×10^{-10}
Z16050	254	2.3×10^{-10}	Z16243	438	2.2×10^{-10}	Z17297	1199	2.4×10^{-10}
Z16051	255	2.5×10^{-10}	Z16245	440	2.2×10^{-10}	Z18557	1205	1.9×10^{-10}
Z16053	257	2.5×10^{-10}						

On average, the Z variants originating from this library (first matured library) displayed an improvement in EC_{50} values by a factor 10 compared with the average EC_{50} for the Z variants originating from the primary selection.

- 5 Biacore screening of Z variants: A selection of IL-1R-I binding Z variants was submitted to a Biacore kinetic screening. A single concentration of hIL-1R-I was injected over each Z-ABD captured from periplasmic extracts on a sensor chip surface containing an anti-ABD antibody. The calculated screening affinities are presented in Table 10. Z16062 (SEQ ID NO:266) got
- 10 the best K_D value (7.6×10^{-9} M) of the assayed binders. The K_D of Z12967 was 2.5×10^{-8} M. The four binders assayed in a SCK experiment got K_D values that deviated from their respective screening kinetics affinities by 0-14 %.

Table 10: Calculated K_D values from Biacore kinetic screening.

Z variant	SEQ ID NO	K_D (M)	Z variant	SEQ ID NO	K_D (M)	Z variant	SEQ ID NO	K_D (M)
Z15824	34	9.2×10^{-9}	Z16019	223	1.5×10^{-8}	Z16042	246	9.7×10^{-9}
Z15840	49	1.1×10^{-8}	Z16020	224	2.0×10^{-8}	Z16043	247	8.3×10^{-9}
Z15864	73	1.6×10^{-8}	Z16021	225	9.6×10^{-9}	Z16044	248	1.7×10^{-8}
Z15873	82	2.3×10^{-8}	Z16022	226	1.5×10^{-8}	Z16045	249	2.0×10^{-8}
Z15876	1206	8.3×10^{-9}	Z16023	227	9.2×10^{-9}	Z16046	250	1.8×10^{-8}
Z15882	90	9.0×10^{-9}	Z16024	228	1.4×10^{-8}	Z16047	251	1.9×10^{-8}
Z15889	97	1.5×10^{-8}	Z16025	229	1.9×10^{-8}	Z16048	252	1.1×10^{-8}
Z15897	105	1.2×10^{-8}	Z16026	230	1.6×10^{-8}	Z16049	253	1.4×10^{-8}
Z15908	116	1.7×10^{-8}	Z16027	231	1.4×10^{-8}	Z16050	254	1.3×10^{-8}
Z15947	152	1.1×10^{-8}	Z16028	232	1.9×10^{-8}	Z16051	255	1.5×10^{-8}
Z15953	158	1.1×10^{-8}	Z16029	233	2.1×10^{-8}	Z16052	256	1.2×10^{-8}
Z15972	177	1.2×10^{-8}	Z16030	234	1.7×10^{-8}	Z16053	257	1.1×10^{-8}
Z15986	190	9.5×10^{-9}	Z16031	235	2.0×10^{-8}	Z16054	258	2.9×10^{-8}

Z16009	213	2.0×10^{-8}	Z16032	236	2.6×10^{-8}	Z16055	259	1.2×10^{-8}
Z16010	214	1.2×10^{-8}	Z16033	237	1.9×10^{-8}	Z16056	260	1.3×10^{-8}
Z16011	215	1.1×10^{-8}	Z16034	238	9.1×10^{-9}	Z16057	261	3.3×10^{-8}
Z16012	216	1.2×10^{-8}	Z16035	239	1.7×10^{-8}	Z16058	262	1.4×10^{-8}
Z16013	217	1.8×10^{-8}	Z16036	240	1.1×10^{-8}	Z16059	263	1.6×10^{-8}
Z16014	218	1.1×10^{-8}	Z16037	241	1.4×10^{-8}	Z16060	264	1.9×10^{-8}
Z16015	219	1.5×10^{-8}	Z16038	242	8.9×10^{-9}	Z16061	265	1.2×10^{-8}
Z16016	220	1.2×10^{-8}	Z16039	243	1.4×10^{-8}	Z16062	266	7.6×10^{-9}
Z16017	221	1.8×10^{-8}	Z16040	244	2.0×10^{-8}	Z16063	267	1.2×10^{-8}
Z16018	220	1.6×10^{-8}	Z16041	245	1.0×10^{-8}	Z16065	268	7.8×10^{-9}

Example 5

Production and characterization of IL-1R-I binding Z variants from the first matured library

5

This Example describes the general procedure for subcloning and production of His₆-tagged and ABD-fused Z variants (originating from the first maturation library phage selection), characterization of their IL-1R-I binding, blocking and melting points.

10

Materials and methods

Subcloning of Z variants with a His₆ tag: The DNA of the respective Z variant was amplified from the phage library vector pAY02592 and was subcloned with an N-terminal His₆ tag using standard molecular biology

15 techniques essentially as described in Example 2.

Subcloning of Z variants in fusion with ABD: The N terminal sequence of respective Z variant was mutated, in position 1 and 2 to amino acid residues A and E respectively, using standard molecular biology techniques. The resulting new Z variants were subcloned into an expression vector

20 containing an ABD variant, giving the encoding sequence [Z#####]-ASGS-ABD, where the ABD variant was PP013 (SEQ ID NO:1661). Z##### refers to individual, 58 amino acid residue long Z variants.

Cultivation: *E. coli* T7E2 cells (GeneBridges) were transformed with plasmids containing the gene fragment of each respective IL-1R-I binding Z

25 variant. The resulting recombinant strains were cultivated in media supplemented with 50 µg/ml kanamycin at 30 °C in 50 ml scale using the EnPresso protocol (BioSilta). In order to induce protein expression, IPTG was

added to a final concentration of 0.2 mM at OD600 $\approx$ 10. After induction, the cultivations were incubated for 16 h. The cells were harvested by centrifugation.

Purification of IL-1R-I binding Z variants with a His₆ tag: Purification

5 was performed essentially as described in Example 2, but without the buffer exchange between the IMAC and RPC purification steps for the majority of the samples. During RPC purification, the RPC solvent A was changed to 0.1 % TFA in 100 % water and the linear gradient was 0-60 % RPC solvent B for 18 ml.

10 Purification of IL-1R-I binding Z variants with ABD fusion:

Approximately 2 g of each cell pellet was re-suspended in TST-buffer (25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.05 % Tween20, pH 8.0) supplemented with Benzonase® (Merck). After cell disruption and clarification by centrifugation, each supernatant was applied on a gravity flow column with 15 1 ml agarose immobilized with an anti-ABD ligand (produced in-house). After washing with TST-buffer and 5 mM NH₄Ac pH 5.5 buffer, the ABD fused Z variants were eluted with 0.1 M HAc. The buffer of the eluate was exchanged to PBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4) using PD-10 desalting columns (GE Healthcare).

20 Protein concentrations were determined by measuring the absorbance at 280 nm, using a NanoDrop® ND-1000 spectrophotometer (Saveen Werner AB) and the extinction coefficient of the respective protein. The purity was analyzed by SDS-PAGE stained with Coomassie Blue and the identity of each purified Z variant was confirmed using LC/MS analysis.

25 Biacore kinetic analysis: The kinetic constants (k_{on} and k_{off}) and affinities (K_D) for human and cynomolgus IL-1R-I were determined for His₆-tagged Z variants using a Biacore 2000 instrument (GE Healthcare). The experiment was run essentially as described in Example 2, using 100, 10 and 1 nM of the Z variants. The analyte injection time was 3 min followed by 15 30 min dissociation and 2 x 5 s glycine pH 3.0 (GE Healthcare, cat. no. BR100357), supplemented with 0.5 M NaCl, was used for regeneration of the surfaces between the cycles. Kinetic constants were calculated from the obtained sensorgrams of two or three concentrations of the respective Z variant, using a 1:1 binding model in the BiaEvaluation software 4.1 (GE 35 Healthcare). His₆-tagged Z12967 was included in the assay for comparison.

In vitro IL-1 β neutralization assay: His₆-tagged IL-1R-I specific Z variants were tested for their inhibitory capacity in the TF-1 cell assay. The

assay was run as described in Example 2. The data on cell growth was assessed by non-linear regression to a four-parameter dose-response curve, and IC₅₀ values were determined using GraphPadPrism program. His₆-tagged Z12967 was included for comparison.

- 5 Circular dichroism (CD) spectroscopy analysis: CD was analyzed for His₆-tagged Z variants, as described in Example 2.

Results

- 10 Subcloning with His₆ tag: Z variants were subcloned into constructs with an N-terminal His₆.

- 15 Production of His₆-tagged Z variants: The IL-1R-I binding Z variants with a His₆ tag were expressed as soluble gene products in *E. coli*. The amount of purified protein was determined spectrophotometrically by measuring the absorbance at 280 nm and ranged from approximately 1 to 7 mg protein per g pellet. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the IL-1R-I binding Z variant. The correct identity and molecular weight of each Z variant were confirmed by HPLC-MS analysis.

- 20 Biacore kinetic analysis: The interactions of His₆-tagged IL-1R-I-binding Z variants with human and cynomolgus IL-1R-I were analyzed in a Biacore instrument by injecting various concentrations of the Z variants over a surface containing immobilized IL-1R-I. A summary of the calculated kinetic parameters (K_D, k_{on} and k_{off}) for the human IL-1R-I binding is given in Table 11. The strongest binder to human IL-1R-I, Z18557 (SEQ ID NO:1205), got a K_D of 1.5 x 10⁻¹⁰ against human IL-1R-I and 7.6 x 10⁻⁹ M against cynomolgus IL-1R-I. The K_D of Z12967 to human IL-1R-I was 5.1 x 10⁻¹⁰ M.

Table 11. Kinetic parameters and affinities for His₆-Z polypeptides binding to human IL-1R-I.

Z variant	SEQ ID NO	k _{on} (1/Ms)	k _{off} (1/s)	K _D (M)
Z15862	71	2.8x10 ⁶	1.9x10 ⁻³	6.7x10 ⁻¹⁰
Z15876	1206	2.1x10 ⁶	1.3x10 ⁻³	6.2x10 ⁻¹⁰
Z15927	135	2.4x10 ⁶	1.2x10 ⁻³	5.1x10 ⁻¹⁰
Z15953	158	6.0x10 ⁶	1.5x10 ⁻³	2.4x10 ⁻¹⁰
Z15961	166	4.3x10 ⁶	2.0x10 ⁻³	4.6x10 ⁻¹⁰
Z16007	211	2.3x10 ⁶	2.1x10 ⁻³	9.2x10 ⁻¹⁰
Z16023	227	5.3x10 ⁶	1.5x10 ⁻³	2.9x10 ⁻¹⁰
Z16043	247	2.5x10 ⁶	1.1x10 ⁻³	4.4x10 ⁻¹⁰

Z16062	266	2.6x10 ⁶	1.1x10 ⁻³	4.4x10 ⁻¹⁰
Z16065	268	6.9x10 ⁶	1.3x10 ⁻³	1.9x10 ⁻¹⁰
Z16072	275	4.6x10 ⁶	2.2x10 ⁻³	4.7x10 ⁻¹⁰
Z16093	294	5.6x10 ⁶	1.4x10 ⁻³	2.5x10 ⁻¹⁰
Z16097	298	3.1x10 ⁶	1.5x10 ⁻³	4.8x10 ⁻¹⁰
Z16116	316	2.6x10 ⁶	2.3x10 ⁻³	8.8x10 ⁻¹⁰
Z16117	317	4.0x10 ⁶	2.7x10 ⁻³	6.9x10 ⁻¹⁰
Z16127	325	6.7x10 ⁶	1.9x10 ⁻³	2.9x10 ⁻¹⁰
Z16143	341	3.1x10 ⁶	1.6x10 ⁻³	5.3x10 ⁻¹⁰
Z16163	359	3.5x10 ⁶	2.0x10 ⁻³	5.5x10 ⁻¹⁰
Z16165	361	2.1x10 ⁶	3.6x10 ⁻³	1.7x10 ⁻⁹
Z16169	365	2.6x10 ⁶	1.0x10 ⁻³	3.9x10 ⁻¹⁰
Z16219	415	2.5x10 ⁶	5.6x10 ⁻³	2.2x10 ⁻⁹
Z16245	440	2.7x10 ⁶	3.1x10 ⁻³	1.2x10 ⁻⁹
Z16255	449	3.0x10 ⁶	3.1x10 ⁻³	1.0x10 ⁻⁹
Z16301	494	3.1x10 ⁶	2.1x10 ⁻³	6.6x10 ⁻¹⁰
Z16341	531	3.0x10 ⁶	1.6x10 ⁻³	5.2x10 ⁻¹⁰
Z16363	550	3.2x10 ⁶	1.8x10 ⁻³	5.7x10 ⁻¹⁰
Z16377	562	2.1x10 ⁶	1.1x10 ⁻³	5.4x10 ⁻¹⁰
Z16378	563	6.6x10 ⁶	3.6x10 ⁻³	5.5x10 ⁻¹⁰
Z16681	856	3.6x10 ⁶	5.0x10 ⁻³	1.4x10 ⁻⁹
Z17274	1176	1.1x10 ⁷	2.4x10 ⁻³	2.2x10 ⁻¹⁰
Z17280	1182	2.4x10 ⁶	5.3x10 ⁻³	2.2x10 ⁻⁹
Z17292	1194	1.3x10 ⁷	5.0x10 ⁻³	3.8x10 ⁻¹⁰
Z18557	1205	4.4x10 ⁶	6.7x10 ⁻⁴	1.5x10 ⁻¹⁰

In vitro IL-1 β neutralization assay: The IL-1 β inhibition ability of His₆-tagged IL-1R-I-binding Z variants was analyzed in a TF-1 cell assay. The resulting IC₅₀ values are presented in Table 12. Z18557 (SEQ ID NO:1205) displayed the best inhibition capacity with an IC₅₀ value below 0.5 nM. The IC₅₀ of Z12967 was 5.2 nM.

Table 12: IC₅₀ values for His₆-Z polypeptides from a TF-1 cell assay.

Z variant	SEQ ID NO	IC ₅₀ (nM)	Z variant	SEQ ID NO	IC ₅₀ (nM)
Z15862	71	6.6	Z16163	359	1.8
Z15876	1206	2.4	Z16165	361	11.5
Z15927	135	2.5	Z16169	365	0.76
Z15953	158	3.7	Z16219	415	24.2
Z15961	166	2.9	Z16245	440	8.2
Z16007	211	5.9	Z16255	449	8.5

Z16023	227	3.8	Z16301	494	4.5
Z16043	247	2.5	Z16341	531	3.9
Z16062	266	1.8	Z16363	550	8.1
Z16065	268	1.7	Z16377	562	3.1
Z16072	275	4.3	Z16378	586	32.7
Z16093	294	3.3	Z16681	856	20.1
Z16097	298	3.4	Z17274	1176	4.6
Z16116	316	4.1	Z17280	1182	5.4
Z16117	317	8.5	Z17292	1194	13.2
Z16127	325	4.1	Z18557	1205	<0.5
Z16143	341	4.8			

- CD analysis: The CD spectra determined for the IL-1R-I binding Z variants with a His₆ tag showed that each had an α -helical structure at 20 °C. This result was also verified in the variable temperature measurements, wherein melting temperatures were determined (Table 13). Reversible folding was seen for all the IL-1R-I binding Z variants when overlaying spectra measured before and after heating to 90 °C.

Table 13: Melting temperatures (T_m).

Z variant	SEQ ID NO	T _m (°C)	Z variant	SEQ ID NO	T _m (°C)
Z15876	1206	55	Z16117	317	63
Z15927	135	56	Z16127	325	59
Z15953	158	56	Z16163	359	57
Z15961	166	59	Z16169	365	48
Z16023	227	60	Z16245	440	63
Z16043	247	54	Z16341	531	59
Z16062	266	54	Z16377	562	52
Z16065	268	59	Z16681	856	68
Z16093	294	52	Z17280	1182	62
Z16097	298	56	Z18557	1205	60
Z16116	316	59			

10

Example 6

Design and construction of two second matured libraries of IL-1R-I binding Z variants

- 15 In this Example, two second matured libraries were constructed. The libraries were used for selections of additional IL-1R-I binding Z variants.

Materials and methods

Library design: The libraries were primarily based on sequences of human IL-1R-I binding Z variants described in Example 4 and 5. In the new libraries, denoted Zlib006IL-1RI.II and Zlib006IL-1RI.III, 10 variable positions
 5 in the Z molecule scaffold were biased towards certain amino acid residues and three positions were kept constant, respectively, according to a strategy mainly based on Z variants (SEQ ID NO:19-1209) from the selection from the first matured library. In addition, in both libraries, a new amino acid position was included for variation; position 12 of the 58 aa Z variant sequence,
 10 resulting in 11 variable positions, in total. Position 12 of Z18557 (SEQ ID NO:1205), characterized in Example 5, was valine. The library design allowed for the amino acids A, V and I in position 12.

For Zlib006IL-1RI.II, a DNA linker was generated using split-pool synthesis containing the following sequence ordered from DNA 2.0 (Menlo
 15 Park, CA, USA): 5'-AA ATA AAT CTC GAG GTA GAT GCC AAA TAC GCC AAA GAA NNN NNN NNN NNN NNN NNN GAG ATC NNN NNN CTG CCT AAC CTC ACC NNN NNN CAA NNN NNN GCC TTC ATC NNN AAA TTA NNN GAT GAC CCA AGC CAG AGC TCA TTA TTT A-3' (SEQ ID NO:1681, designed codons are denoted NNN). The design for each amino acid residue
 20 of the new library, including eleven variable amino acid positions (9, 10, 11, 12, 13, 14, 17, 18, 25, 28 and 35) and three constant amino acid positions (24, 27 and 32) in the Z molecule scaffold, are displayed in Table 14. The resulting theoretical library size was 2.2×10^7 variants. An even theoretical distribution of the different amino acids was applied within each amino acid
 25 position, except from position 10, 11, 12, 13, 17, 28 and 35, where a higher portion of selected amino acids was applied.

For Zlib006IL-1RI.III, the library was constructed similarly as Zlib006IL-1RI.I, described in Example 3, using the TRIM technology. The DNA
 sequence, 5'-AA ATA AAT CTC GAG GTA GAT GCC AAA TAC GCC AAA
 30 GAA NNN NNN NNN NNN NNN NNN GAG ATC NNN NNN CTG CCT AAC CTC ACC NNN NNN CAA NNN NNN GCC TTC ATC NNN AAA TTA NNN GAT GAC CCA AGC CAG AGC TCA TTA TTT A-3 (SEQ ID NO:1682, designed codons are denoted NNN) was ordered from Ella Biotech (Martinsried, Germany). The design for each amino acid residue of the new
 35 library, including eleven variable amino acid positions (9, 10, 11, 12, 13, 14, 17, 18, 25, 32 and 35) and three constant amino acid positions (24, 27 and 28) in the Z molecule scaffold, are displayed in Table 15. The resulting

theoretical library size was 1.0×10^8 variants. An even theoretical distribution of the different amino acids was applied within each amino acid position, except from position 10, 11, 12, 32 and 35, where a higher portion of selected amino acids was applied.

5

Table 14: Zlib006IL-1RI.II library design, second maturation.

Amino acid position in the Z variant molecule	Amino acid randomization (percentage when uneven distribution)	No. of different amino acids
9	A,E,I,L,T,V	6
10	E(50 %),I,L,R,V,Y	6
11	A,E(27 %),I,K,Q,R,T,V,Y	9
12	A(50 %),I,V	3
13	A,I,L,Q(50 %),V,Y	6
14	F,M,Q,W,Y	5
17	F(70 %),Y(30 %)	2
18	A,D,E,F,G,H,I,K,L,Q,R,S,T,V,W,Y	16
24	R	1
25	K,R	2
27	Y	1
28	I,T(71.4 %),V	3
32	R	1
35	F(25 %),I(12.5 %),L(50 %),Y(12.5 %)	4

Table 15: Zlib006IL-1RI.III library design, second maturation.

Amino acid position in the Z variant molecule	Amino acid randomization (percentage when uneven distribution)	No. of different amino acids
9	A,E,I,L,T,V	6
10	E(60 %),I,V	3
11	A,D,E(15 %),F,H,I,K,L,Q,R,S,T,V,W,Y	15
12	A(50 %),I(20 %), V(30 %)	3
13	A,E,F,H,I,K,L,Q,R,S,T,V,W,Y	14
14	A,E,F,H,I,K,L,Q,R,S,T,V,W,Y	14
17	F,I,L,W,Y	5
18	A,D,E,F,G,H,I,K,L,Q,R,S,T,V,W,Y	16
24	R	1
25	K,R	2
27	Y	1
28	T	1
32	I(10 %),R(90 %)	2
35	F(30 %),L(70 %)	2

Library construction and phage stock preparation: Zlib006IL-1RI.II and Zlib006IL-1RI.III were constructed as described in WO 2016/113246 (Example 4) and in Example 3, respectively. For both libraries, *E. coli* XL-1 Blue was used for library transformation. Clones from the libraries of Z variants were sequenced (as described in Example 3) in order to verify the content and to evaluate the outcome of the constructed library vis-à-vis the library design.

Phage stocks containing the phagemid libraries were prepared in shake flasks. Cells from glycerol stocks containing the phagemid library were inoculated in 0.5 or 1 l, respectively, of TSB-YE medium, essentially as described for Zlib006IL-1RI.I batch 2 in Example 3. The cultivations were infected using a 10-20 x molar excess of M13K07 helper phage at an OD₆₀₀ of 0.8-0.9. Phage stocks were prepared as described in Example 3.

Results

Library construction: The new libraries were designed based on a set of IL-1R-I binding Z variants, selected from Zlib006IL-1RI.I, with verified binding properties (Examples 4 and 5). The theoretical sizes of Zlib006IL-1RI.II and Zlib006IL-1RI.III were 2.2×10^7 and 1.0×10^8 Z variants, respectively. The actual sizes of the libraries, determined by titration after transformation to *E. coli* XL-1 Blue cells, were 1.2×10^9 and 7.0×10^9 transformants, respectively.

The library qualities were tested by sequencing of 192 and 96 transformants from Zlib006IL-1RI.II and Zlib006IL-1RI.III, respectively, and by comparing their actual sequences with the theoretical design. The contents of the actual library compared to the designed library were shown to be satisfactory. Two matured libraries of potential binders to IL-1R-I were thus successfully constructed.

Example 7

Selection of Z variants from the second matured libraries

In this Example, human and cynomolgus IL-1R-I were used as target proteins in phage display selections using two second matured phage libraries of Z variants.

Materials and methods

Phage display selection of IL-1R-I binding Z variants: The biotinylated human target proteins b-hIL-1R-I-Fc and b-hIL-1R-I, and the cynomolgus target protein b-cIL-1R-I-Fc, were used in phage selections using the new libraries of Z variant molecules described in Example 6. An overview of the selection strategy, describing the parallel selection tracks and an overall increased stringency in the selection cycles obtained by using a lowered target concentration and an increased number of washes, is shown in Table 16.

10

Table 16: Overview of selections from the two second matured libraries.

Cycle	Selection track	Phage stock from library or selection track	Target	Target concentration (nM)	Selection time (min)	Number of washes
1	1-1	Zlib006IL-1RI.II	b-hIL-1R-I-Fc	10	45	5
1	1-2	Zlib006IL-1RI.II	b-hIL-1R-I	10	45	5
1	1-3	Zlib006IL-1RI.II	b-cIL-1R-I-Fc	25	45	3
1	1-4	Zlib006IL-1RI.III	b-hIL-1R-I-Fc	20	45	5
1	1-5	Zlib006IL-1RI.III	b-hIL-1R-I	20	45	5
1	1-6	Zlib006IL-1RI.III	b-cIL-1R-I-Fc	50	45	3
1	1-7	Zlib006IL-1RI.II	b-hIL-1R-I-Fc	10	45	5
1	1-8	Zlib006IL-1RI.II	b-hIL-1R-I	10	45	5
1	1-9	Zlib006IL-1RI.II	b-cIL-1R-I-Fc	25	45	3
1	1-10	Zlib006IL-1RI.III	b-hIL-1R-I-Fc	20	45	5
1	1-11	Zlib006IL-1RI.III	b-hIL-1R-I	20	45	5
1	1-12	Zlib006IL-1RI.III	b-cIL-1R-I-Fc	50	45	3
2	2-1	1-1	b-hIL-1R-I-Fc	1	30	10*
2	2-2	1-2	b-hIL-1R-I	2	30	10
2	2-3	1-2	b-hIL-1R-I	1	30	10
2	2-4	1-2	b-hIL-1R-I	1	30	10*
2	2-5	1-3	b-hIL-1R-I	10	30	8
2	2-6	1-4	b-hIL-1R-I-Fc	4	30	8
2	2-7	1-5	b-hIL-1R-I	4	30	8
2	2-8	1-5	b-hIL-1R-I	2	30	10
2	2-9	1-5	b-hIL-1R-I	2	30	10*
2	2-10	1-6	b-hIL-1R-I	20	30	6
2	2-11	1-7/1-8/1-9 pool	b-hIL-1R-I	1	30	10*

2	2-12	1-10/1-11/1-12 pool	b-hIL-1R-I	2	30	10*
3	3-1	2-1	b-hIL-1R-I-Fc	0.4	40	16*
3	3-2	2-2	b-hIL-1R-I	0.4	40	15
3	3-3	2-3	b-hIL-1R-I	0.1	40	20
3	3-4	2-4	b-hIL-1R-I	0.1	40	16*
3	3-5	2-5	b-cIL-1R-I-Fc	5	40	12
3	3-6	2-6	b-hIL-1R-I-Fc	0.8	40	12
3	3-7	2-7	b-hIL-1R-I	0.8	40	12
3	3-8	2-8	b-hIL-1R-I	0.2	40	20
3	3-9	2-9	b-hIL-1R-I	0.2	40	16*
3	3-10	2-10	b-cIL-1R-I-Fc	10	40	9
3	3-11	2-11	b-hIL-1R-I	0.4	40	16*
3	3-12	2-12	b-hIL-1R-I	0.4	40	16*
4	4-1	3-1	b-hIL-1R-I-Fc	0.125	40	20**
4	4-2	3-2	b-hIL-1R-I	0.125	40	20
4	4-3	3-3	b-hIL-1R-I	0.05	40	30
4	4-4	3-4	b-hIL-1R-I	0.05	40	20**
4	4-5	3-5	b-hIL-1R-I	1	40	16
4	4-6	3-6	b-hIL-1R-I-Fc	0.16	40	16**
4	4-7	3-7	b-hIL-1R-I	0.16	40	16
4	4-8	3-8	b-hIL-1R-I	0.1	40	30
4	4-9	3-9	b-hIL-1R-I	0.1	40	20**
4	4-10	3-10	b-hIL-1R-I	2	40	16
4	4-11	3-11	b-hIL-1R-I	0.2	40	16
4	4-12	3-12	b-hIL-1R-I	0.2	40	16

*Addition of hIL-1R-I in the second final wash step.

**Selection track divided into two before second final wash step. One part submitted to an over-night wash in the presence of hIL-1R-I and one part submitted to a 1 h wash in PBST.

5

The selections were performed in four cycles essentially as described in Example 4, with the following exceptions 1-3. Exception 1: pre-selection was performed in selection round 1 and 2 for >60 min at RT by incubation of phage stocks with SA beads (b-hIL-1R-I tracks) or Fc/SA beads (b-IL-1RI-Fc tracks). Exception 2: in the second final wash step of tracks 2-1, 2-4, 2-9, 2-11, 2-12, 3-1, 3-4, 3-9, 3-11 and 3-12, respectively, hIL-1R-I was added to the wash buffer in 100 times higher concentration than the target concentration of the respective track and the wash was run for 15-20 min. Exception 3: In the fourth selection round, the tracks 4-1, 4-4 and 4-6 were divided into two before the second final wash step. For one part, hIL-1R-I was added to the wash buffer in 100 times higher concentration than the target concentration of

the respective track and the second final wash was run overnight. For the other part, a 1 h wash in PBST was applied for the second final wash.

In the first selection cycle, twelve selection tracks were run; 1-1 to 1-12. In cycle two, two tracks were divided into three, and six tracks were pooled into two tracks, resulting in a total of 12 parallel tracks in cycle 2, 3 and 4; 2-1 to 2-12, 3-1 to 3-12 and 4-1 to 4-12, respectively. All selection tracks are presented in Table 16. The number of phage particles used for selections was typically more than 2000 times the number of eluted phage particles in the previous cycle.

10

Results

Phage display selection of IL-1R-I binding Z variants: Individual clones were obtained after four cycles of phage display selections against human and cynomolgus IL-1R-I.

15

Example 8

Screening of Z variants from the first and second matured libraries

In this Example, DNA of selected clones from Example 4 and Example 7 was sequenced, the Z variants were produced in *E. coli* periplasmic fractions and assayed against IL-1R-I in ELISA and Biacore.

20

Materials and methods

Sequencing of potential binders: Individual clones from cycle 4 of selections using the three matured libraries Zlib006IL-1RI.I, Zlib006IL-1RI.II and Zlib006IL-1RI.III were picked for sequencing. Amplification and sequence analysis of gene fragments were performed essentially as described in Example 1.

25

Production of Z variants for ELISA and Biacore screening: Z variants were produced essentially as described in Example 4 with the exception that the periplasmic extracts were prepared by heat-treatment (82 °C, 20 min) followed by clarification by filtration. Z variants screened in Biacore were diluted five times in HBS-EP buffer.

30

ELISA screening of Z variants: The binding of Z variants to human IL-1R-I was analyzed in ELISA, essentially as described in Example 1, using 0.2 nM hIL-1R-I-Fc as target protein. As negative control, a periplasmic extract of ABD001 was used.

35

EC₅₀ analysis of Z variants: A selection of IL-1R-I binding was subjected to an analysis of response against a dilution series of hIL-1R-I-Fc using ELISA as described above. The target protein hIL-1R-I-Fc was diluted stepwise 1:10 from 20 to 0.002 nM. As a background control, the Z variants
5 were assayed with no target protein added. Obtained data was analyzed using GraphPad Prism 5 and non-linear regression, and the EC₅₀ values (the half maximal effective concentration) were calculated. Periplasmic extracts with Z12967, Z16065 and Z16218 (corresponding to SEQ ID:9, 268 and 414, respectively) were analyzed in parallel for signal comparison. As negative
10 control, a periplasmic extract of ABD001 was used.

Biacore screening of Z variants: The binding of Z variants to human IL-1R-I was analyzed in a kinetic screening, using a Biacore 2000 instrument, essentially as described in Example 4, but with the exception that Z-ABD (ABD001, SEQ ID NO:1660) periplasmic extracts were injected during 2 min.
15 Periplasmic extracts of Z12967, Z16065 and Z16218 were included in the screening analysis in duplicates for comparison. A periplasmic extract with ABD (ABD001, SEQ ID NO:1660) was included as negative control.

Results

20 Sequencing: Sequencing was performed for clones obtained after four cycles of selection. Each variant was given a unique identification number #####, and individual variants are referred to as Z#####. The amino acid sequences of the 58 amino acid residues long Z variants are listed in Figure 1 and in the sequence listing as SEQ ID NO: 1210-1632. The deduced IL-1R-I
25 binding motifs extend from residue 8 to residue 36 in each sequence. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55.

ELISA screening of Z variants: Clones obtained after four cycles of
30 selection were produced individually in 96-well plates and were screened for human IL-1R-I binding activity in ELISA. 99.5 % of the assayed Z variants gave a positive signal of 2 x the blank control or higher against 0.2 nM hIL-1R-I-Fc.

EC₅₀ analysis of Z variants: A subset of 102 Z variants displaying
35 results over 0.74 AU (8.2 x the blank control) was subjected to ELISA target titration using hIL-1R-I-Fc. Obtained results were used for calculation of EC₅₀

values (Table 17). The results for Z12967, Z16065 and Z16218 were 2.6×10^{-10} , 2.1×10^{-10} and 2.2×10^{-10} M, respectively.

Table 17: Calculated EC_{50} values from ELISA titration analysis.

Z variant	SEQ ID NO	EC_{50} (M)	Z variant	SEQ ID NO	EC_{50} (M)	Z variant	SEQ ID NO	EC_{50} (M)
Z15840	49	2.3×10^{-10}	Z18811	1405	2.8×10^{-10}	Z18920	1270	2.4×10^{-10}
Z15862	71	2.5×10^{-10}	Z18813	1375	2.0×10^{-10}	Z18966	1272	2.5×10^{-10}
Z15876	1206	1.9×10^{-10}	Z18814	1368	2.7×10^{-10}	Z18967	1388	3.1×10^{-10}
Z15934	1208	1.7×10^{-10}	Z18819	1276	2.9×10^{-10}	Z18968	1472	2.0×10^{-10}
Z15945	150	2.3×10^{-10}	Z18820	1417	3.3×10^{-10}	Z18971	1559	3.5×10^{-10}
Z15975	1209	1.9×10^{-10}	Z18824	1264	2.8×10^{-10}	Z18982	1553	2.5×10^{-10}
Z16062	266	2.1×10^{-10}	Z18825	1275	3.5×10^{-10}	Z19012	1588	2.4×10^{-10}
Z16183	379	2.3×10^{-10}	Z18826	1356	2.4×10^{-10}	Z19053	1526	2.4×10^{-10}
Z16208	404	2.2×10^{-10}	Z18827	1318	2.4×10^{-10}	Z19054	1528	2.5×10^{-10}
Z16603	782	2.2×10^{-10}	Z18831	1308	2.9×10^{-10}	Z19056	1586	2.2×10^{-10}
Z16606	785	2.3×10^{-10}	Z18832	1340	1.5×10^{-10}	Z19057	1558	2.2×10^{-10}
Z18754	1252	3.5×10^{-10}	Z18834	1361	2.5×10^{-10}	Z19059	1240	3.5×10^{-10}
Z18757	1321	3.0×10^{-10}	Z18836	1430	2.3×10^{-10}	Z19060	1228	2.2×10^{-10}
Z18758	1281	2.4×10^{-10}	Z18838	1337	2.4×10^{-10}	Z19072	1554	1.6×10^{-10}
Z18759	1314	2.4×10^{-10}	Z18843	1309	2.8×10^{-10}	Z19075	1618	1.5×10^{-10}
Z18760	1435	1.7×10^{-10}	Z18846	1331	1.9×10^{-10}	Z19078	1248	2.2×10^{-10}
Z18763	1431	2.6×10^{-10}	Z18866	1324	3.1×10^{-10}	Z19085	1564	2.1×10^{-10}
Z18766	1274	3.3×10^{-10}	Z18874	1421	2.2×10^{-10}	Z19090	1540	2.0×10^{-10}
Z18769	1339	2.9×10^{-10}	Z18875	1420	2.9×10^{-10}	Z19115	1489	2.3×10^{-10}
Z18770	1284	2.8×10^{-10}	Z18877	1301	3.5×10^{-10}	Z19117	1631	2.1×10^{-10}
Z18771	1341	2.5×10^{-10}	Z18878	1302	3.7×10^{-10}	Z19118	1579	2.3×10^{-10}
Z18774	1389	2.6×10^{-10}	Z18879	1390	3.4×10^{-10}	Z19123	1241	2.6×10^{-10}
Z18776	1418	2.2×10^{-10}	Z18881	1355	2.8×10^{-10}	Z19151	1594	2.3×10^{-10}
Z18777	1326	2.6×10^{-10}	Z18882	1311	3.1×10^{-10}	Z19158	1571	1.5×10^{-10}
Z18785	1323	2.5×10^{-10}	Z18884	1403	2.3×10^{-10}	Z19159	1364	2.1×10^{-10}
Z18786	1320	2.4×10^{-10}	Z18886	1432	2.9×10^{-10}	Z19171	1593	2.3×10^{-10}
Z18790	1439	2.6×10^{-10}	Z18887	1386	2.1×10^{-10}	Z19172	1551	1.6×10^{-10}
Z18799	1423	2.3×10^{-10}	Z18888	1422	2.3×10^{-10}	Z19180	1393	2.0×10^{-10}
Z18803	1327	2.5×10^{-10}	Z18889	1344	2.4×10^{-10}	Z19181	1549	1.8×10^{-10}
Z18804	1329	2.7×10^{-10}	Z18890	1352	2.5×10^{-10}	Z19182	1628	1.7×10^{-10}
Z18805	1437	2.7×10^{-10}	Z18904	1587	3.8×10^{-10}	Z19183	1235	2.2×10^{-10}
Z18806	1271	2.7×10^{-10}	Z18908	1591	2.4×10^{-10}	Z19302	1419	2.6×10^{-10}
Z18808	1342	4.0×10^{-10}	Z18912	1251	1.4×10^{-10}	Z19304	1273	2.7×10^{-10}
Z18810	1278	2.9×10^{-10}	Z18917	1509	2.2×10^{-10}	Z19305	1261	2.6×10^{-10}

Biacore screening of Z variants: A subset of 188 IL-1R-I binding Z variants was submitted to a Biacore kinetic screening. A single concentration of hIL-1R-I was injected over each Z-ABD captured from periplasmic extracts on a sensor chip surface containing an anti-ABD antibody. The calculated screening affinities are presented in Table 18. The K_D values of Z12967, Z16065 and Z16218 were 2.7×10^{-8} , 7.5×10^{-9} and 1.3×10^{-8} M (average of duplicates), respectively.

Z18814, which has an amino acid sequence that only differs from Z16065 in position X_5 of the binding motif BM, has three times higher affinity than Z16065. Z16065 has the highest binding affinity (K_D) among the IL-1R-I binding Z variants (Table 11) with an alanine residue in position X_5 . Z18814 has an isoleucine residue in position X_5 . Several Z variants with valine or isoleucine in position X_5 were found to have high affinity to IL-1R-I.

15 Table 18: Calculated K_D values from Biacore kinetic screening.

Z variant	SEQ ID NO	Biacore screening K_D (M)	Z variant	SEQ ID NO	Biacore screening K_D (M)	Z variant	SEQ ID NO	Biacore screening K_D (M)
Z15840	49	6.9×10^{-9}	Z18830	1295	2.9×10^{-9}	Z18938	1370	4.5×10^{-9}
Z15862	71	1.3×10^{-8}	Z18831	1308	3.0×10^{-9}	Z18941	1409	3.3×10^{-9}
Z15876	1206	5.7×10^{-9}	Z18832	1340	1.6×10^{-9}	Z18942	1483	1.5×10^{-8}
Z15932	1207	5.5×10^{-9}	Z18833	1297	3.8×10^{-9}	Z18948	1414	4.3×10^{-9}
Z15934	1208	9.4×10^{-9}	Z18834	1361	2.4×10^{-9}	Z18952	1290	3.0×10^{-9}
Z15945	150	9.6×10^{-9}	Z18836	1430	2.9×10^{-9}	Z18956	1625	1.4×10^{-8}
Z15975	1209	8.9×10^{-9}	Z18838	1337	3.3×10^{-9}	Z18958	1283	2.8×10^{-9}
Z16062	266	6.4×10^{-9}	Z18839	1440	3.1×10^{-9}	Z18959	1357	2.9×10^{-9}
Z16183	379	6.5×10^{-9}	Z18841	1359	2.5×10^{-9}	Z18966	1272	2.9×10^{-9}
Z16191	387	6.3×10^{-9}	Z18843	1309	3.1×10^{-9}	Z18967	1388	3.2×10^{-9}
Z16208	404	1.1×10^{-8}	Z18844	1349	4.0×10^{-9}	Z18968	1472	2.4×10^{-9}
Z16397	582	1.3×10^{-8}	Z18846	1331	2.1×10^{-9}	Z18971	1559	5.5×10^{-9}
Z16440	624	1.3×10^{-8}	Z18847	1363	3.0×10^{-9}	Z18982	1553	5.2×10^{-9}
Z16603	782	9.1×10^{-9}	Z18848	1304	3.0×10^{-9}	Z18989	1592	4.5×10^{-9}
Z16606	785	9.6×10^{-9}	Z18849	1299	3.0×10^{-9}	Z19012	1588	4.5×10^{-9}
Z16737	910	1.2×10^{-8}	Z18850	1367	2.6×10^{-9}	Z19031	1600	3.1×10^{-9}
Z18754	1252	2.5×10^{-9}	Z18851	1291	3.6×10^{-9}	Z19043	1505	3.4×10^{-9}
Z18756	1366	2.9×10^{-9}	Z18853	1298	2.3×10^{-9}	Z19053	1526	8.7×10^{-9}
Z18757	1321	3.0×10^{-9}	Z18856	1330	2.9×10^{-9}	Z19054	1528	7.0×10^{-9}
Z18758	1281	4.8×10^{-9}	Z18860	1265	6.2×10^{-9}	Z19056	1586	6.9×10^{-9}
Z18759	1314	3.4×10^{-9}	Z18861	1369	3.3×10^{-9}	Z19057	1558	4.2×10^{-9}
Z18760	1435	3.0×10^{-9}	Z18863	1362	2.4×10^{-9}	Z19059	1240	9.2×10^{-9}
Z18762	1415	2.1×10^{-9}	Z18866	1324	2.7×10^{-9}	Z19060	1228	1.0×10^{-8}

Z18763	1431	5.2×10^{-9}	Z18868	1391	2.7×10^{-9}	Z19072	1554	5.2×10^{-9}
Z18766	1274	3.1×10^{-9}	Z18870	1428	3.0×10^{-9}	Z19075	1618	4.8×10^{-9}
Z18767	12353	5.1×10^{-9}	Z18871	1332	3.5×10^{-9}	Z19078	1248	9.5×10^{-9}
Z18769	1339	2.7×10^{-9}	Z18872	1410	2.6×10^{-9}	Z19079	1236	1.4×10^{-8}
Z18770	1284	2.6×10^{-9}	Z18873	1306	3.1×10^{-9}	Z19085	1564	4.9×10^{-9}
Z18771	1341	3.0×10^{-9}	Z18874	1421	2.3×10^{-9}	Z19090	1540	3.0×10^{-9}
Z18774	1389	3.0×10^{-9}	Z18875	1420	2.7×10^{-9}	Z19094	1231	1.8×10^{-8}
Z18776	1418	2.8×10^{-9}	Z18876	1286	3.7×10^{-9}	Z19099	1548	3.8×10^{-9}
Z18777	1326	3.8×10^{-9}	Z18877	1301	2.7×10^{-9}	Z19107	1566	6.7×10^{-9}
Z18782	1333	2.7×10^{-9}	Z18878	1302	3.8×10^{-9}	Z19115	1489	6.1×10^{-9}
Z18783	1257	3.4×10^{-9}	Z18879	1390	6.2×10^{-9}	Z19117	1631	4.2×10^{-9}
Z18784	1433	3.7×10^{-9}	Z18881	1355	3.2×10^{-9}	Z19118	1579	8.4×10^{-9}
Z18785	1323	2.7×10^{-9}	Z18882	1311	2.6×10^{-9}	Z19123	1241	1.1×10^{-8}
Z18786	1320	4.8×10^{-9}	Z18884	1403	2.7×10^{-9}	Z19127	1213	9.8×10^{-9}
Z18790	1439	3.0×10^{-9}	Z18885	1294	3.9×10^{-9}	Z19135	1247	1.6×10^{-8}
Z18794	1424	3.7×10^{-9}	Z18886	1432	3.9×10^{-9}	Z19141	1506	1.7×10^{-8}
Z18796	1352	3.6×10^{-9}	Z18887	1386	2.9×10^{-9}	Z19143	1471	1.4×10^{-9}
Z18798	1268	3.2×10^{-9}	Z18888	1422	2.6×10^{-9}	Z19144	1374	3.1×10^{-9}
Z18799	1423	2.6×10^{-9}	Z18889	1344	3.4×10^{-9}	Z19146	1434	2.9×10^{-9}
Z18800	1328	1.9×10^{-9}	Z18890	1352	3.1×10^{-9}	Z19147	1307	2.4×10^{-9}
Z18803	1327	3.1×10^{-9}	Z18893	1353	2.1×10^{-9}	Z19151	1594	2.7×10^{-9}
Z18804	1329	3.3×10^{-9}	Z18894	1312	2.5×10^{-9}	Z19153	1310	3.1×10^{-9}
Z18805	1437	3.7×10^{-9}	Z18895	1411	2.6×10^{-9}	Z19158	1571	1.5×10^{-9}
Z18806	1271	4.1×10^{-9}	Z18896	1436	3.5×10^{-9}	Z19159	1364	1.9×10^{-9}
Z18807	1400	3.8×10^{-9}	Z18898	1285	2.5×10^{-9}	Z19160	1379	3.8×10^{-9}
Z18808	1342	4.2×10^{-9}	Z18904	1587	3.4×10^{-9}	Z19166	1305	3.5×10^{-9}
Z18810	1278	3.1×10^{-9}	Z18908	1591	3.6×10^{-9}	Z19168	1500	4.6×10^{-9}
Z18811	1405	4.5×10^{-9}	Z18909	1303	2.7×10^{-9}	Z19169	1402	2.7×10^{-9}
Z18813	1375	2.0×10^{-9}	Z18910	1416	3.1×10^{-9}	Z19170	1334	2.0×10^{-9}
Z18814	1368	2.4×10^{-9}	Z18911	1441	2.8×10^{-9}	Z19171	1593	3.0×10^{-9}
Z18817	1343	3.5×10^{-9}	Z18912	1251	3.6×10^{-9}	Z19172	1551	4.5×10^{-9}
Z18819	1276	3.0×10^{-9}	Z18916	1536	4.1×10^{-9}	Z19180	1393	7.9×10^{-10}
Z18820	1417	3.3×10^{-9}	Z18917	1509	7.1×10^{-9}	Z19181	1549	9.7×10^{-9}
Z18821	1269	3.5×10^{-9}	Z18919	1350	2.8×10^{-9}	Z19182	1628	2.8×10^{-9}
Z18823	1282	2.4×10^{-9}	Z18920	1270	3.2×10^{-9}	Z19183	1235	1.2×10^{-8}
Z18824	1264	3.3×10^{-9}	Z18922	1533	4.9×10^{-9}	Z19302	1419	3.2×10^{-9}
Z18825	1275	4.9×10^{-9}	Z18923	1482	9.0×10^{-9}	Z19303	1262	4.0×10^{-9}
Z18826	1356	3.5×10^{-9}	Z18925	1479	1.1×10^{-8}	Z19304	1273	4.5×10^{-9}
Z18827	1318	4.3×10^{-9}	Z18927	1372	3.4×10^{-9}	Z19305	1261	3.8×10^{-9}
Z18828	1296	2.6×10^{-9}						

Example 9

Production and characterization of Z variants from the first and second matured libraries

5 This Example describes the general procedure for subcloning and production of His₆-tagged Z variants originating from the first and second maturation library phage selections, characterization of their target binding, target blocking and melting points.

10 *Materials and methods*

Subcloning of Z variants with a His₆ tag: A subset of Z variants screened in Example 8 was chosen for subcloning. The DNA of the respective Z variant was amplified from the phage library vector pAY02592 and was subcloned with an N-terminal His₆ tag using standard molecular biology techniques essentially as described in Example 2.

15 Cultivation: *E. coli* T7E2 cells (GeneBridges) were transformed with plasmids containing the gene fragment of each respective IL-1R-I binding Z variant. The resulting recombinant strains were cultivated in media supplemented with 50 µg/ml kanamycin at 30 °C in 50 ml scale using the EnPresso protocol (BioSilta). In order to induce protein expression, IPTG was added to a final concentration of 0.2 mM at OD₆₀₀ ≈ 10. After induction, the cultivations were incubated for 16 h. The cells were harvested by centrifugation.

20 Purification of IL-1R-I binding Z variants with a His₆ tag: Approximately 2/3 of the IL-1R-I binding Z variants were purified as described in Example 2 but without the buffer exchange between the IMAC and RPC purification steps. The linear gradient during RPC purification was also changed to 0-60 % RPC solvent B for 18 ml. The remaining 1/3 of the IL-1R-I binding Z variants were only IMAC purified before the final buffer exchange to PBS.

30 Purification was essentially performed as described in the first part of Example 2. Approximately 1-2 g of each cell pellet was used.

Biacore kinetic analysis: The kinetic constants (k_{on} and k_{off}) and affinities (K_D) for human and cynomolgus IL-1R-I were determined for His₆-tagged Z variants using a Biacore 2000 instrument (GE Healthcare). The experiment was run essentially as described in Example 5, using 48, 12, 3 and 0.75 nM of the Z variants. The analyte injection time was 3 min followed by 6 min dissociation and 2 x 5 s glycine pH 3.0, supplemented with 0.5 M

NaCl, was used for regeneration of the surfaces between the cycles. Kinetic constants were calculated from the obtained sensorgrams of three or four concentrations of the respective Z variant, using a 1:1 binding model in the BiaEvaluation software 4.1 (GE Healthcare). His₆-tagged Z12967 (SEQ ID NO:9), Z16065 (SEQ ID NO:268), Z16163 (SEQ ID NO:359) and Z18557 (SEQ ID NO:1205) were included in the assay for comparison.

In vitro IL-1 β neutralization assay: His₆-tagged IL-1R-I specific Z variants were tested for their inhibitory capacity in the TF-1 cell assay. The assay was run as described in Example 2. The data on cell growth was assessed by non-linear regression to a four-parameter dose-response curve, and IC₅₀ values were determined using GraphPadPrism program. His₆-tagged Z12967, Z16065, Z16163 and Z18557 were included for comparison.

Circular dichroism (CD) spectroscopy analysis: CD was analyzed for His₆-tagged Z variants, as described in Example 2.

Results

Production of His₆-tagged Z variants: The IL-1R-I binding Z variants with a His₆ tag were expressed as soluble gene products in *E. coli*. The amount of purified protein was determined spectrophotometrically by measuring the absorbance at 280 nm and ranged from approximately 0.4 to 7 mg protein per g pellet for IL-1R-I binding Z variants which were both IMAC and RPC purified. The amount of purified protein ranged from approximately 4 to 15 mg protein per g pellet for IL-1R-I binding Z variants which were only IMAC purified. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the IL-1R-I binding Z variant. The correct identity and molecular weight of each Z variant were confirmed by HPLC-MS analysis.

Biacore kinetic analysis: The interactions of His₆-tagged IL-1R-I binding Z variants with human and cynomolgus IL-1R-I were analyzed in a Biacore instrument by injecting various concentrations of the Z variants over a surface containing immobilized IL-1R-I. A summary of the calculated kinetic parameters (K_D , k_{on} and k_{off}) for the human IL-1R-I binding is given in Table 19. The K_D values of Z12967, Z16065, Z16163 and Z18557 were 8.2×10^{-10} , 5.1×10^{-10} , 7.6×10^{-10} and 1.4×10^{-10} M, respectively. The K_D values of cynomolgus IL-1R-I binding were 2.0×10^{-9} , 6.7×10^{-9} and 3.3×10^{-9} M for Z18754, Z18760 and Z18800, respectively.

Table 19. Kinetic parameters and affinities for His₆-Z polypeptides binding to human IL-1R-I.

Z variant	SEQ ID NO	k _{on} (1/Ms)	k _{off} (1/s)	K _D (M)
Z15876	1206	5.2x10 ⁶	4.1x10 ⁻³	8.0x10 ⁻¹⁰
Z15932	1207	1.8x10 ⁷	1.1x10 ⁻²	6.4x10 ⁻¹⁰
Z15934	1208	1.4x10 ⁷	1.1x10 ⁻²	7.5x10 ⁻¹⁰
Z15975	1209	4.1x10 ⁷	2.9x10 ⁻²	7.1x10 ⁻¹⁰
Z18754	1252	7.0x10 ⁷	6.0x10 ⁻³	8.6x10 ⁻¹¹
Z18760	1435	1.3x10 ⁷	2.7x10 ⁻³	2.1x10 ⁻¹⁰
Z18762	1415	4.2x10 ⁷	5.2x10 ⁻³	1.3x10 ⁻¹⁰
Z18769	1339	1.9x10 ⁷	3.1x10 ⁻³	1.6x10 ⁻¹⁰
Z18770	1284	3.0x10 ⁷	5.0x10 ⁻³	1.7x10 ⁻¹⁰
Z18799	1423	2.6x10 ⁷	4.5x10 ⁻³	1.8x10 ⁻¹⁰
Z18800	1328	1.8x10 ⁷	2.5x10 ⁻³	1.4x10 ⁻¹⁰
Z18813	1375	2.0x10 ⁷	3.9x10 ⁻³	2.0x10 ⁻¹⁰
Z18814	1368	3.0x10 ⁷	4.4x10 ⁻³	1.5x10 ⁻¹⁰
Z18817	1343	2.8x10 ⁷	4.8x10 ⁻³	1.7x10 ⁻¹⁰
Z18823	1283	4.7x10 ⁶	1.8x10 ⁻³	3.8x10 ⁻¹⁰
Z18831	1308	4.0x10 ⁷	5.3x10 ⁻³	1.3x10 ⁻¹⁰
Z18832	1340	5.9x10 ⁷	7.0x10 ⁻³	1.2x10 ⁻¹⁰
Z18834	1361	5.8x10 ⁷	8.0x10 ⁻³	1.4x10 ⁻¹⁰
Z18846	1331	4.7x10 ⁷	5.1x10 ⁻³	1.1x10 ⁻¹⁰
Z18850	1367	1.3x10 ⁷	2.3x10 ⁻³	1.7x10 ⁻¹⁰
Z18853	1298	2.2x10 ⁷	3.0x10 ⁻³	1.4x10 ⁻¹⁰
Z18856	1330	4.6x10 ⁷	5.7x10 ⁻³	1.2x10 ⁻¹⁰
Z18863	1362	2.2x10 ⁷	4.8x10 ⁻³	2.2x10 ⁻¹⁰
Z18866	1324	3.6x10 ⁷	4.2x10 ⁻³	1.2x10 ⁻¹⁰
Z18874	1421	4.9x10 ⁷	5.2x10 ⁻³	1.1x10 ⁻¹⁰
Z18875	1420	2.4x10 ⁷	2.8x10 ⁻³	1.2x10 ⁻¹⁰
Z18882	1311	1.2x10 ⁷	3.0x10 ⁻³	2.5x10 ⁻¹⁰
Z18888	1422	1.8x10 ⁷	2.9x10 ⁻³	1.6x10 ⁻¹⁰
Z18893	1353	8.6x10 ⁶	2.4x10 ⁻³	2.7x10 ⁻¹⁰
Z18898	1285	6.7x10 ⁷	4.4x10 ⁻³	6.6x10 ⁻¹¹
Z18912	1251	9.2x10 ⁷	1.9x10 ⁻²	2.1x10 ⁻¹⁰
Z18920	1270	3.6x10 ⁷	5.5x10 ⁻³	1.5x10 ⁻¹⁰
Z18968	1472	3.0x10 ⁷	8.4x10 ⁻³	2.8x10 ⁻¹⁰
Z19072	1554	4.3x10 ⁷	1.8x10 ⁻²	4.1x10 ⁻¹⁰
Z19075	1618	1.4x10 ⁷	6.4x10 ⁻³	4.5x10 ⁻¹⁰
Z19143	1471	1.7x10 ⁷	3.9x10 ⁻³	2.2x10 ⁻¹⁰
Z19147	1307	2.8x10 ⁷	4.3x10 ⁻³	1.5x10 ⁻¹⁰
Z19151	1594	2.8x10 ⁷	7.0x10 ⁻³	2.5x10 ⁻¹⁰
Z19158	1571	2.7x10 ⁷	4.4x10 ⁻³	1.6x10 ⁻¹⁰

Z19159	1364	2.7×10^7	5.0×10^{-3}	1.9×10^{-10}
Z19170	1334	2.1×10^7	4.3×10^{-3}	2.0×10^{-10}
Z19172	1551	4.9×10^7	2.3×10^{-2}	4.6×10^{-10}
Z19180	1393	2.2×10^7	3.1×10^{-3}	1.4×10^{-10}
Z19181	1549	2.0×10^7	1.6×10^{-2}	8.2×10^{-10}
Z19182	1628	2.1×10^7	5.5×10^{-3}	2.6×10^{-10}

In vitro IL-1 β neutralization assay: The IL-1 β inhibition ability of His₆-tagged IL-1R-I-binding Z variants was analyzed in a TF-1 cell assay. The resulting IC₅₀ values are presented in Table 20. The IC₅₀ values of Z12967, Z16065, Z16163 and Z18557 were 7.4, 3.3, 3.3 and 0.6 nM, respectively.

Table 20: IC₅₀ values for His₆-Z polypeptides from a TF-1 cell assay.

Z variant	SEQ ID NO	IC ₅₀ (nM)
Z15934	1208	6.4
Z15975	1209	3.3
Z18754	1252	0.6
Z18760	1435	0.9
Z18813	1375	1.2
Z18814	1368	0.4
Z18831	1308	0.4
Z18832	1340	0.5
Z18834	1361	0.5
Z18846	1331	0.2
Z18874	1421	0.3
Z18912	1251	1
Z18968	1472	1.4
Z19072	1554	2.4
Z19159	1364	1.2
Z19172	1551	2
Z19181	1549	4.6
Z19182	1628	1.5

CD analysis: The CD spectra determined for the IL-1R-I binding Z variants with a His₆ tag showed that each had an α -helical structure at 20 °C. This result was also verified in the variable temperature measurements, wherein melting temperatures were determined (Table 21). Reversible folding was seen for all the IL-1R-I binding Z variants when overlaying spectra measured before and after heating to 90 °C.

The thermo-stability and the α -helical content of the IL-1R-I binding Z variants having a valine or isoleucine residue in position X₅ of the BM (Z18912, Z18920, Z18968, Z19072, Z19075, Z19143, Z19147, Z19151, Z19158, Z19159, Z19170, Z19172, Z19180, Z19181 and Z19182) were not
 5 substantially different from the Z variants having an alanine residue in position X₅.

Table 21: Melting temperatures (T_m).

Z variant	SEQ ID NO	T _m (°C)	Z variant	SEQ ID NO	T _m (°C)
Z15876	1206	55	Z18866	1324	61
Z15932	1207	60	Z18874	1421	61
Z15934	1208	62	Z18875	1420	61
Z15975	1209	59	Z18882	1311	53
Z18754	1252	63	Z18888	1422	61
Z18760	435	57	Z18893	1353	60
Z18762	1415	62	Z18898	1285	59
Z18769	1339	59	Z18912	1251	53
Z18770	1284	59	Z18920	1270	56
Z18799	1423	60	Z18968	1472	62
Z18800	1328	67	Z19072	1554	63
Z18813	1375	61	Z19075	1618	56
Z18814	1368	61	Z19143	1471	56
Z18817	1343	60	Z19147	1307	57
Z18823	1283	52	Z19151	1594	59
Z18831	1308	58	Z19158	1571	55
Z18832	1340	58	Z19159	1364	54
Z18834	1361	58	Z19170	1334	52
Z18846	1331	60	Z19172	1551	59
Z18850	1367	62	Z19180	1393	58
Z18853	1298	59	Z19181	1549	61
Z18856	1330	61	Z19182	1628	62
Z18863	1362	57			

10

Example 10

Production and characterization of IL-1R-I binding fusion proteins

This Example describes the common steps for DNA construction and
 15 production of different IL-1R-I binding proteins variants fused to different *in vivo* half-life extending fusion partners.

Materials and methods

DNA construction: DNA encoding a set of IL-1R-I binding Z variants (see below) fused to human IgG1 Fc (SEQ ID NO:1662), an albumin binding polypeptide variant (PP013; SEQ ID NO:1661), human albumin (SEQ ID NO:1663) or human transferrin (SEQ ID NO:1664), optionally via different linkers (Table 22), were codon optimized for expression in *E. coli* or for expression in Chinese hamster ovary (CHO) cells and synthesized by the Invitrogen GeneArt Gene Synthesis service at Thermo Fisher Scientific. The genes were cloned in expression vectors for subsequent expression in *E. coli* or in CHO cells.

The fusion proteins were based on the binding motifs of the IL-1R-I binding Z variants a) Z12967 (SEQ ID NO:9), b) Z12895 (SEQ ID NO:12), c) Z18831 (SEQ ID NO:1308), d) Z18846 (SEQ ID NO:1331), e) Z18754 (SEQ ID NO:1252), f) Z19151 (SEQ ID NO:1594), g) Z18874 (SEQ ID NO:1421), h) Z18760 (SEQ ID NO:1435), i) Z18800 (SEQ ID NO:1328), j) Z18898 (SEQ ID NO:1285) and k) Z19147 (SEQ ID NO:1307) and the different fusions partners described above. The IL-1R-I binding Z variants a)-k) contained different scaffold mutations, (positions 1, 2, 23, 52 and/or 53) or a deletion combined with mutations (deletion at positions 1-3; Δ V1D2A3, mutations in positions 52 and 53), see Table 22 below. The mutated Z variants are listed in Figure 1 as SEQ ID NO:1667-1668 and 1670-1679. These obtained fusion polypeptides were PSI0405 (SEQ ID NO:1639), PSI0407 (SEQ ID NO:1640), PSI0411 (SEQ ID NO:1641), PSI0412 (SEQ ID NO:1642), PSI0415 (SEQ ID NO:1643), PSI0416 (SEQ ID NO:1644), PSI0417 (SEQ ID NO:1645), PSI0534 (SEQ ID NO:1646), PSI0535 (SEQ ID NO:1647), PSI0536 (SEQ ID NO:1648), PSI0537 (SEQ ID NO:1649), PSI0538 (SEQ ID NO:1650), PSI0539 (SEQ ID NO:1651), PSI0574 (SEQ ID NO:1652), PSI0575 (SEQ ID NO:1653), PSI0576 (SEQ ID NO:1654), PSI0580 (SEQ ID NO:1655), PSI0581 (SEQ ID NO:1656), PSI0582 (SEQ ID NO:1657), see Table 22.

Table 22: Fusion proteins and expression systems

SEQ ID NO	Fusion proteins	Construct	Expression system
1639	PSI0405	Z12967[V1A;D2E;N52S;D53E]-VEGS-ABD	<i>E. coli</i>
1640	PSI0407	Z12895[V1A;D2E;N52S;D53E]-VEGS-ABD	<i>E. coli</i>
1641	PSI0411	AS-ABD-GS-Z12967[V1A;D2E;N52S;D53E]	<i>E. coli</i>
1642	PSI0412	AS-ABD-(G ₄ S) ₂ -Z12967[V1A;D2E;N52S;D53E]	<i>E. coli</i>
1643	PSI0415	AS-ABD-Z12967[V1A;D2E;N52S;D53E]	<i>E. coli</i>
1644	PSI0416	AS-ABD-Z12967[ΔV1D2A3;N52S;D53E]	<i>E. coli</i>
1645	PSI0417	AS-ABD-((KEAAA) ₃ KELAA) ₂ -Z12967[V1A;D2E;N52S;D53E]	<i>E. coli</i>
1646	PSI0534	Z18831[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1647	PSI0535	Z18846[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1648	PSI0536	Z18754[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1649	PSI0537	Z19151[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1650	PSI0538	Z18874[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1651	PSI0539	Z18760[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1652	PSI0574	Z18800[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1653	PSI0575	Z18898[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1654	PSI0576	Z19147[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -IgG1 Fc	<i>E. coli</i>
1655	PSI0580	Z18754[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -Albumin	CHO
1656	PSI0581	Z18754[V1A;D2E;T23N;N52S;D53E]-AS(G ₄ S) ₂ -Albumin	CHO
1657	PSI0582	Z18754[V1A;D2E;N52S;D53E]-AS(G ₄ S) ₂ -Transferrin	CHO
1658	PSI0589	PSI0536[amino acids 1-86], dimeric	

- Cultivation and purification of fusion proteins: *E. coli* cells were transformed with expression vectors containing the gene fragments corresponding to the IL-1R-I binding fusion proteins, see Table 22, and then cultivated in bioreactors using fed-batch techniques or in shake flasks, followed by protein expression and harvest of cells by centrifugation. Cell pellets were stored at -20 °C. Expression of IL-1R-I binding Z variants, see Table 22, were also performed using the ExpiCHO expression system (Thermo Fisher Scientific), essentially according to the manufacturer's

protocol. Supernatants were harvested by centrifugation 12 days after transfection of expression vectors and stored at -70 °C.

Frozen *E. coli* cell pellets were resuspended and then disrupted by sonication and the cell debris subsequently removed by centrifugation followed by filtration (0.22 µm). The frozen supernatants from the ExpiCHO cultures were thawed and filtrated (0.22 µm). Each supernatant, containing the IL-1R-I binding Z variants was purified using conventional chromatography methods, such as affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography. Z variants for use in animal studies were also subjected to an endotoxin removal purification using Detoxi-Gel Endotoxin Removing Columns (Pierce, cat.no. 20344). Purified Z variants were buffer exchanged to PBS and, unless otherwise stated, PBS was also the formulation buffer used in subsequent experiments. The purity of the IL-1R-I binding Z variants was analyzed by SDS-PAGE stained with Coomassie Blue and the molecular weight of each purified Z variant was analyzed using mass spectrometry (HPLC/MS or MALDI-TOF/MS).

The IL-1R-I binding fusion protein PSI0589 (SEQ ID NO:1658), a dimeric Z18754 variant linked in the hinge region by disulfides, was obtained by removing the IgG1 Fc part from PSI0536, see Table 22. This was achieved by incubation of purified PSI0536 with the IdeS protease (FabRICATOR, Genovis AB). The cleavage products were then purified using conventional chromatography methods such as ion exchange chromatography and affinity chromatography. The purified dimeric Z18754 variant was subjected to buffer exchange into PBS, which was also the formulation buffer used in subsequent experiments. The purity was analyzed by SDS-PAGE stained with Coomassie Blue and the molecular weight was analyzed using mass spectrometry (HPLC/MS or MALDI-TOF/MS).

Biolayer Interferometry (BLI) kinetic analysis: Binding of four IL-1R-I binding fusion proteins to human and cynomolgus IL-1R-I was analyzed using BLI. The Fc fused IL-1R-I binding Z variants denoted PSI0536, PSI0537, PSI0534 and PSI0535 (SEQ ID NO:1648, 1649, 1646, 1647) were loaded onto a Protein A sensor (Pall, ForteBio cat. no. 18-5010) at 0.5 µg/ml for 150 s in each cycle using an OctetRED96 instrument (Pall, ForteBio). Subsequently, these sensors were exposed to different and increasing concentrations of human IL-1R-I (R&D systems cat. no. 269-1R/CF) ranging from 0.15 to 5 nM or cynomolgus IL-1R-I (SEQ ID NO:1665) ranging from 2.5

to 80 nM. Association was recorded for 300 s and dissociation for 300 s (human) or 90 s (cynomolgus) and spectrograms were recorded and analyzed according to a 1:1 Langmuir model using global fit (Octet System Data Analysis 8.2 software, Pall, ForteBio). Each sensor was referenced to a sensor with the same ligand loaded onto it but exposed to buffer only. Following each association/dissociation cycle, all sensors were regenerated by three pulses of 10 mM glycine at pH 2.5 for 10 s each. All steps were carried out at 25 °C at a shaking speed of 1000 rpm and all dilutions of fusion proteins (Z-Fc fusion proteins) and analytes (human or cynomolgus IL-1R-I) were in 1 x kinetics buffer (Pall, ForteBio).

Results

Cultivation and purification: All the IL-1R-I binding fusion proteins (Table 22) were expressed to high levels in *E. coli* or CHO cells as soluble proteins. Purification resulted in protein preparations with high purity, which was analyzed by SDS-PAGE stained with Coomassie Blue. The correct identity and molecular weight of each Z variant were confirmed by mass spectrometry analysis.

BLI kinetic analysis: Four Z-Fc fusion protein variants (SEQ ID NO: 1648, 1649, 1646, 1647) were analyzed for the ability to bind human and cynomolgus IL-1R-I loaded onto protein A sensor using BLI technology on an OctetRED96 instrument. All four variants bound IL-1R-I according to a 1:1 model with B-max values ranging from 0.46 to 0.72 nm (human) or 0.31 to 0.37 nm (cynomolgus). The kinetic data; association rate constant (k_{on}), dissociation rate constant (k_{off}) and dissociation constant K_D for the Z-Fc fusion protein variants are presented in Table 23.

Table 23: Kinetic parameters for IL-1R-I binding fusion proteins

Fusion proteins and SEQ ID NO	Human IL-1R-I			Cynomolgus IL-1R-I		
	K_D (M)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)	k_{on} (1/Ms)	k_{off} (1/s)
PSI0536 (SEQ ID NO:1648)	7.5×10^{-10}	8.99×10^5	6.79×10^{-4}	1.4×10^{-8}	1.70×10^6	2.34×10^{-2}
PSI0537 (SEQ ID NO:1649)	1.2×10^{-9}	8.41×10^5	1.02×10^{-3}	3.1×10^{-8}	1.78×10^6	5.48×10^{-2}
PSI0534 (SEQ ID NO:1646)	1.6×10^{-9}	4.04×10^5	6.42×10^{-4}	2.8×10^{-8}	1.08×10^6	2.99×10^{-2}
PSI0535 (SEQ ID NO:1647)	7.2×10^{-10}	5.83×10^5	4.21×10^{-4}	1.1×10^{-8}	1.49×10^6	1.66×10^{-2}

Example 11:In vitro pharmacological activity analysis using a cell-based assay*Materials and methods*

5 The inhibitory effect of IL-1R-I binding fusion proteins on IL-1 β induced IL-6 production in Normal Human Dermal Fibroblasts (NHDF) cells was monitored.

Cells were seeded three days prior to treatment with proteins. Proteins (IL-1R-I binding fusion proteins or anakinra) were diluted to a starting
10 concentration of 100 nM and subsequently serially 1:4 nine times resulting in a concentration range of 100 nM to 0.38 pM in serum-free growth medium in the presence of 9 μ M recombinant human serum albumin (rHSA). The IL-1R-I binding fusion proteins or anakinra were tested in presence of a challenge dose of 3.4 pM IL-1 β and the cells were incubated for 22 hours with proteins
15 at 37 °C, followed by harvesting of medium. Harvested medium was diluted 41 X before IL-6 content was analyzed using a human IL-6 ELISA kit (R&D Systems) according to manufacturer's recommendations. Data was analyzed using XLfit and IC₅₀ values were calculated from concentration-response curves.

20 The inhibitory effect of His₆-tagged IL-1R-I binding Z variants, produced as described in Example 9, on IL-1 β induced IL-6 production in NHDF cells was similarly monitored and IC₅₀ values calculated.

Chemically synthesized protein: A chemically synthesized version of SEQ ID NO:1672, denoted PSI0558, was ordered from BACHEM AG. The
25 activity of PSI0558 was assessed according to the method set out above.

Results

 The IL-1 β induced IL-6 release from NHDF cells was reduced in a concentration-dependent manner by the IL-1R-I binding fusion proteins as
30 well as by anakinra. Each IL-1R-I binding fusion protein was tested twice and the data from both experiments along with historical average of anakinra (n > 20) in this assay are presented in Table 24 and one of the two experiments is also presented in Figure 2.

Table 24: *In vitro* IC₅₀ values

Denotation	SEQ ID NO	IC ₅₀ (pM)
PSI0536	1648	66;45
PSI0537	1649	260;200
PSI0534	1646	140;110
PSI0535	1647	80;50
PSI0538	1650	87;70
PSI0539	1651	240;110
anakinra		Average 70

Some IL-1R-I binding fusion proteins were tested in the same manner but only one time. The results from this experiment are presented in Table 25.

5

Table 25: *In vitro* IC₅₀ values

Denotation	SEQ ID NO	IC ₅₀ (pM)
PSI0589	1658	1.8
PSI0582	1657	2.0
PSI0580	1655	1500
PSI0581	1656	1800

The IL-1 β inhibition ability of His₆-tagged IL-1R-I-binding Z variants, previously demonstrated in a TF-1 cell assay (Example 9, Table 20), was confirmed in this NHDF assay (results not shown).

10

The IC₅₀ value of the chemically synthesized PSI0558 was 690 pM.

Example 12

In vitro pharmacological activity analysis using a whole blood assay

15

The inhibitory effect of IL-1R-I binding fusion proteins on IL-1 β induced IL-6 production in whole blood from individual donors was measured.

Materials and methods

20

Blood from healthy donors was collected 1-3 hours prior to treatment with the IL-1R-I binding fusion proteins (SEQ ID NO:1648-1649). Proteins (IL-1R-I binding fusion proteins or anakinra) were diluted to starting concentration of 500 nM in serum free RPMI (ThermoFisher Scientific, RPMI 1640 Medium, GlutaMAX™ Supplement, Cat. No.: 61870010) and subsequently serially diluted 1:3 in a ten-step dilution series resulting in a concentration range of 500 nM to 25 pM. The IL-1R-I binding fusion polypeptides or anakinra were tested in presence of a challenge dose of 100 pM IL-1 β . The blood was incubated for 21-23 hours with proteins, followed by harvesting of plasma.

25

Harvested plasma was diluted 2 x and IL-6 content was analyzed by using a human IL-6 kit (V-PLEX Human IL-6 Kit Human interleukin-6, MSD) according to manufacturer's recommendations.

- 5 IL-6 concentrations (pg/ml) were calculated using a standard curve (provided by the kit) and data was analyzed using XLfit and IC₅₀ values were calculated from the concentration-response curves.

Results

- 10 The IL-1 β induced IL-6 release in human whole blood was reduced in a concentration-dependent manner by the IL-1R-I binding fusion proteins as well as by anakinra. IL-1R-I binding fusion proteins were tested in blood from five to six individual healthy donors and compared to anakinra data from ten individual blood donors (Figure 3). The median IC₅₀ values were 0.32, 1.25 and 0.31 nM for SEQ ID NO:1648 (PSI0536), SEQ ID NO:1649 (PSI0537) and anakinra, respectively.

Example 13

In vivo pharmacokinetics of IL-1R-1 binding fusion proteins in rats

- 20 In this Example, the pharmacokinetics of IL-1R-1-binding fusion proteins (SEQ ID NO:1646-1651) were evaluated in a single dose study in male rats.

Material and methods

- 25 Fusion proteins: The IL-1R-I binding fusion proteins PSI0534, PSI0535, PSI0536, PSI0537, PSI0538 and PSI0539 (see Table 22, SEQ ID NO:1646-1651) were evaluated in this study. All six test items were constituted as a solution in 25 mM sodium phosphate and 125 mM sodium chloride, pH 7.0.

- 30 In-life phase: The pharmacokinetic properties of the six fusion proteins were investigated in male Sprague-Dawley rats. For each test item, three rats were given an i.v. single dose of 15 mg/kg (2.5 ml/kg) injected in the lateral tail vein and another three rats were given a s.c. single dose of 30 mg/kg (5 ml/kg) injected in the neck region. Blood samples for serum preparation were collected pre dosing and at 5 and 20 min, 1, 4, 8, 24, 48, 72 and 96 hrs post i.v. dosing, or 20 min, 1, 4, 8, 24, 48, 72, 96 and 120 hrs post s.c. dosing.

- 35 Quantitative ELISA: Determination of fusion protein levels in rat serum samples was performed by enzyme-linked immunosorbent assay (ELISA).

The ELISA assay employed the quantitative sandwich enzyme immunoassay technique.

In brief; a goat polyclonal anti-Z antibody recognizing the Z variant domain of the IL-1R-I binding fusion protein (produced in house, Affibody AB) was coated onto a microplate. Unbound polyclonal antibody was washed away and casein was added as blocking agent to reduce unspecific binding to the plastic surface. After removal of unbound casein by a second wash step, standards and samples were pipetted to the wells and any fusion protein present was bound to the immobilized antibody. After washing away any unbound substances, a HRP labeled Swine anti-rabbit antibody (Dako, cat. no. P0399) was added. Following a wash to remove any unbound anti-rabbit HRP reagent, a substrate solution was added to the wells and color developed in proportion to the amount of fusion protein bound in the initial step. The color development was stopped and the intensity of the color was measured.

Pharmacokinetic analysis: The pharmacokinetic analysis was based on median serum concentration versus time data from each dose group. The observed maximum concentration (C_{max}) and the time to maximum serum concentration (t_{max}) were taken directly from the bioanalytical data. Other pharmacokinetic parameters, i.e. clearance (CL), apparent clearance following s.c. administration (CL/F), apparent volume of distribution at steady-state (V_{ss}), mean residence time (MRT) and terminal half-life ($t_{1/2z}$), were estimated by non-compartmental analysis using Phoenix WinNonlin software version 6.3 (Pharsight Corp., USA). Estimation of the terminal slope following i.v. dosing was based on four data points, from 24 to 96 hrs. Estimation of the terminal slope following s.c. dosing was based on five data points, from 24 to 120 hrs. Calculation of the subcutaneous bioavailability (F) was performed using Microsoft Excel.

Results

There was no significant difference in the pharmacokinetics between the six tested fusion proteins. The pharmacokinetics following i.v. administration was characterized by a clearance in the order of 2.4 ml/h·kg and a volume of distribution of about 140 ml/kg, translating into a mean residence time of ca 60 hrs (Table 26).

Table 26: Pharmacokinetic parameter estimates following an intravenous single dose administration, 15 mg/kg, to male rats

Parameter	PSI0534	PSI0535	PSI0536	PSI0537	PSI0538	PSI0539
CL (ml/h·kg)	2.83	2.40	2.68	2.06	2.36	2.03
V _{ss} (ml/kg)	159	149	143	136	146	113
MRT (hrs)	56.4	62.1	53.5	65.9	61.6	55.5
t _{1/2z} (hrs)	48.7	52.1	44.7	55.1	50.4	48.3

- 5 The subcutaneous bioavailability was on average 50 % and peak levels were observed at 24 hrs after dosing. The serum levels then declined with a half-life ranging from 45 to 59 hrs (Table 27).

Table 27: Pharmacokinetic parameter estimates following a subcutaneous single dose administration, 30 mg/kg, to male rats

Parameter	PSI0534	PSI0535	PSI0536	PSI0537	PSI0538	PSI0539
F (%)	41.5	49.6	46.7	60.3	47.2	52.2
C _{max} (nM)	828	866	948	1115	796	886
t _{max} (hrs)	24	24	24	24	24	24
CL/F (ml/h·kg)	6.81	5.70	6.05	4.69	5.98	5.42
V _z /F (ml/kg)	445	457	403	372	509	424
t _{1/2z} (hrs)	45.3	55.6	46.1	55.1	59.0	54.3
MRT (hrs)	72.4	87.3	74.5	87.4	91.7	86.2

Example 14

In vivo pharmacokinetics of an IL-1R-I binding fusion protein in monkeys

15 *Material and methods*

Fusion proteins: The IL-1R-I binding fusion protein denoted PSI0536 (SEQ ID NO:1648) was constituted as a solution in 25 mM sodium phosphate and 125 mM sodium chloride, pH 7.0.

- 20 In-life phase: The pharmacokinetic properties of PSI0536 were investigated in naïve male Cynomolgus monkeys. Three monkeys were given an i.v. single dose of 5 mg/kg (1 ml/kg) injected in the tail vein and another

three monkeys were given a s.c. single dose of 10 mg/kg (1 ml/kg) injected in the dorsal region. Blood samples for serum preparation were collected pre dosing and at 5 and 20 min, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 240 and 504 hrs post i.v. dosing, or 20 min, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 240 and 504 hrs post s.c. dosing.

Quantitative ELISA: Quantification of PSI0536 in serum from monkeys was performed according to the ELISA described in Example 13.

Pharmacokinetic analysis: Pharmacokinetic parameters were based on individual serum concentration *versus* time data and determined using WinNonlin software as described in Example 13. Estimation of the terminal slope following i.v. dosing was based on seven data points, from 72 to 504 hrs. Estimation of the terminal slope following s.c. dosing was based on eight data points, from 48 to 504 hrs. Calculation of the subcutaneous bioavailability (F) was performed using Microsoft Excel.

Results

The pharmacokinetics of PSI0536 (SEQ ID NO:1648) following i.v. administration was characterized by a low clearance (1.07 ml/h·kg) and a small volume of distribution (117 ml/kg), translating into a mean residence time of 109 hrs (Table 28).

Table 28: Mean ($\pm$ SD) pharmacokinetic parameter estimates of PSI0536 following an intravenous single dose of 5.04 ($\pm$ 0.01) mg/kg to three male monkeys

Parameter	Estimate
CL (ml/h·kg)	1.07 $\pm$ 0.04
V _{ss} (ml/kg)	117 $\pm$ 3
MRT (hrs)	109 $\pm$ 1
t _{1/2z} (hrs)	92.7 $\pm$ 3.7

The mean subcutaneous bioavailability of PSI0536 was 73.9%. Peak levels were observed at an average of 18.7 hrs after dosing. The serum levels then declined with a half-life of 77.3 hrs (Table 29).

Table 29: Mean ($\pm$ SD) pharmacokinetic parameter estimates of PSI0536 following a subcutaneous single dose of 10.1 ($\pm$ 0.1) mg/kg to three male monkeys

Parameter	Estimate
F (%)	73.9 $\pm$ 5.5
C _{max} (nM)	779 $\pm$ 159
t _{max} (hrs)	18.7 $\pm$ 7.5
CL/F (ml/h·kg)	1.46 $\pm$ 0.10
V _z /F (ml/kg)	163 $\pm$ 23
t _{1/2z} (hrs)	77.3 $\pm$ 6.3
MRT (hrs)	115 $\pm$ 14

5

Conclusion

Assuming a minimum target serum concentration of 100 nM or even 200 nM, the high subcutaneous bioavailability in combination with the low clearance facilitates a once weekly subcutaneous dose regimen of 10 mg/kg PSI0536 in monkey.

10

Example 15

Production and characterization of IL-1-R-I binding fusion proteins with mutations in the Fc portion

15

Four IL-1-R-I binding fusion proteins of the IL-1R-I binding polypeptide variant Z18754 (SEQ ID NO:1252) with mutated variants of the Fc portion of IgG1 or IgG4 were produced. In the IgG1 Fc portion, one mutation [N297A] was introduced in order to abolish effector functions through interaction with FC γ R3A. Fc-containing polypeptides produced in mammalian cells might otherwise cause such effector functions. In addition, in one fusion protein (SEQ ID NO:1735) two other residues were introduced, denoted LS (WO2009/086320), to increase affinity to FcRn, and in another fusion protein (SEQ ID NO:1736) three mutations denoted YTE (WO02060919) were introduced to increase affinity to FcRn. One fusion protein of Z18754 (SEQ ID NO:1252) with an IgG4 Fc a subtype, which is known to elicit low effector responses, was produced (SEQ ID NO:1737). One mutation, known as

20

25

S228P (van der Neut Kolfchoten M. et al., 2007, Science 317(5844):1554-7), was furthermore introduced in the hinge region of this fusion protein. This mutation abolishes the arm exchange that may take place for the IgG4 subclass of antibodies.

5

Material and methods

The fusion proteins PSI0653-PSI0656 (SEQ ID NO:1734-1737), see Table 30 for specifications, were produced in CHO cells according to Example 10.

10 The inhibitory effect of IL-1R-I binding fusion proteins on IL-1 β induced IL-6 production in Normal Human Dermal Fibroblasts (NHDF) cells was monitored as set out in Example 11.

The affinity towards FC γ R3A was analysed with Biolayer Interferometry essentially as disclosed in Example 10, with the exception that biotinylated
15 FC γ R3A (Sino Biological) was immobilized on a Streptavidin biosensor for kinetics (Pall/Fortebio) and the proteins were tested using concentrations of 100 and 1000 nM. In addition Human IgG (GammaNorm, Octapharma) was used as control in the measurements.

The affinity towards FcRn was assessed using Biacore essentially as
20 described in Example 9 with the exception that human biotinylated FcRn (Immunitrack ApS) was immobilized on a Biotin CAPture chip (GE Healthcare) and the analytes were tested in five concentrations, ranging from 2.5 - 200 nM.

25 *Table 30: Fusion proteins and expression systems*

SEQ ID NO	Fusion proteins	Construct	Expression system
1734	PSI0653	Z18754[N297A]-IgG1 Fc	CHO
1735	PSI0654	Z18754[N297A]-LS-IgG1 Fc	CHO
1736	PSI0655	Z18754[N297A]-YTE-IgG1 Fc	CHO
1737	PSI0656	Z18754[S228P]-IgG4 Fc	CHO

Results

30 Cultivation and purification: All the IL-1-R-I binding fusion proteins with mutated Fc portions (Table 30) were expressed at high levels in CHO cells as soluble proteins. Purification resulted in protein preparations with high purity, which were analyzed by SDS-PAGE stained with Coomassie Blue. The

correct identity and molecular weight of each Z variant were confirmed by mass spectrometry analysis.

- 5 *In vitro* pharmacological activity: The IL-1 β induced IL-6 release from NHDF cells was reduced in a concentration-dependent manner by the IL-1R-I binding fusion proteins. The IL-1R-I binding fusion proteins were tested one time. The results from this experiment are accounted for in Table 31.

Table 31: In vitro IC₅₀ values

SEQ ID NO:	Fusion proteins	IC ₅₀ (pM)
1734	PSI0653	4.3
1735	PSI0654	2.9
1736	PSI0655	5.9
1737	PSI0656	8.4

- 10 FC γ R3A affinity analysis: Human IgG bound to FC γ R3A with an affinity of 57 nM at 1000 nM concentration. The fusion proteins PSI0653-PSI0655 (SEQ ID NO 1734-1736) showed no binding at either concentration. PSI0656 (SEQ ID NO 1737) showed a weak binding at 1000 nM but no binding at 100 nM.

- 15 Biacore FcRn affinity analysis: The affinity towards FcRn were in the order SEQ ID NO 1735 > SEQ ID NO 1736 > SEQ ID NO 1734 = SEQ ID NO 1737.

- 20 Conclusion: The mutated Fc variants of the fusion proteins (SEQ ID NO:1734:1737) were equal to the previously produced proteins in terms of quality and activity (see Example 10). The removal of glycan attachment site removed the effector function of the IgG1 Fc portion in SEQ ID NO:1734-1736 as binding to FC γ R3A was abolished. The IgG4 Fc containing SEQ ID NO:1737 had low affinity towards FC γ R3A as expected for an Fc of that subclass. The FcRn affinity enhancing mutations in SEQ ID NO:1735 and
25 1736 displayed increased affinity, with SEQ ID NO:1735 showing the greatest increase.

Itemized listing of embodiments

1. IL-1R-I binding polypeptide, comprising an IL-1R-I binding motif *BM*, which motif consists of an amino acid sequence selected from:

5

- i) $EX_2X_3X_4X_5X_6X_7EIX_{10}X_{11}LPNLX_{16}RX_{18}QYX_{21}AFIX_{25}X_{26}LX_{28}D$
(SEQ ID NO:1686)

wherein, independently from each other,

10

X_2 is selected from A, D, E, F, H, I, L, Q, S, T and V;

X_3 is selected from A, D, E, F, H, I, K, L, N, Q, R, S, T, V, W and Y;

X_4 is selected from A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y;

X_5 is selected from A, I and V;

15

X_6 is selected from F, H, I, Q, R, T, V and Y;

X_7 is selected from A, D, E, F, G, H, I, L, M, Q, S, T, V, W and Y;

X_{10} is selected from F and Y;

X_{11} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;

20

X_{16} is selected from N and T;

X_{18} is selected from K, R and S;

X_{21} is selected from Q, T and V;

X_{25} is selected from I, M, R, V and Y;

X_{26} is selected from K and S, and

25

X_{28} is selected from F, I, L and M,

and

- ii) an amino acid sequence which has at least 96 % identity to the sequence defined in i) provided that X_5 is I or V.

30

2. IL-1R-I binding polypeptide, wherein said IL-1R-I binding motif consists of an amino acid sequence selected from a sequence wherein in i)

X_2 is selected from A, I, L, T and V;

X_3 is selected from E and Y;

35

X_4 is selected from A, E, I, K, Q, R, T, V and Y;

X_5 is selected from I and V;

X_6 is selected from Q and Y;

- X₇ is selected from F and M;
 X₁₀ is selected from F and Y;
 X₁₁ is selected from A, D, E, F, G, H, K, L, Q, R, S, T, V and Y;
 X₁₆ is selected from N and T;
 5 X₁₈ is selected from K and R;
 X₂₁ is selected from T and V;
 X₂₅ is selected from I and R;
 X₂₆ is selected from K and S, and
 X₂₈ is selected from F and L,
 10 and an amino acid sequence which has at least 93 % identity to the
 sequence defined in i).
3. IL-1R-I binding polypeptide according to any one of the preceding
 items, wherein sequence i) fulfills at least five of the ten conditions I-X:
- I. X₃ is E;
 15 II. X₅ is selected from I and V;
 III. X₆ is selected from Q and Y;
 IV. X₇ is selected from F and M;
 V. X₁₀ is selected from F and Y;
 VI. X₁₈ is selected from K and R;
 20 VII. X₂₁ is T;
 VIII. X₂₅ is R;
 IX. X₂₆ is selected from K and S; and
 X. X₂₈ is selected from F and L.
4. IL-1R-I binding polypeptide according to item 3, wherein sequence i)
 25 fulfills at least six of the ten conditions I-X.
5. IL-1R-I binding polypeptide according to item 4, wherein sequence i)
 fulfills at least seven of the ten conditions I-X.
6. IL-1R-I binding polypeptide according to item 5, wherein sequence i)
 fulfills at least eight of the ten conditions I-X.
- 30 7. IL-1R-I binding polypeptide according to item 6, wherein sequence i)
 fulfills at least nine of the ten conditions I-X.
8. IL-1R-I binding polypeptide according to item 7, wherein sequence i)
 fulfills all of the ten conditions I-X.
9. IL-1R-I binding polypeptide according to any one of items 1-8,
 35 wherein X₂X₃X₆ is VEQ or VEY.
10. IL-1R-I binding polypeptide according to any one of items 1-8,
 wherein X₂X₃X₆ is IEQ or VEQ.

11. IL-1R-I binding polypeptide according to any one of items 1-8, wherein X_6X_{10} is selected from the group consisting of QF, QY, YF and YY.
12. IL-1R-I binding polypeptide according to any one of items 1-8, wherein X_6X_{10} is QF.
- 5 13. IL-1R-I binding polypeptide according to any one of items 1-8, wherein $X_{10}X_{18}$ is selected from the group consisting of FK, FR, YK and YR.
14. IL-1R-I binding polypeptide according to any one of items 1-8, wherein $X_{10}X_{18}$ is FK or FR.
15. IL-1R-I binding polypeptide according to any one of items 1-8, wherein $X_{18}X_{25}X_{28}$ is KRL or RRL.
- 10 16. IL-1R-I binding polypeptide according to any one of items 1-8, wherein X_5X_7 is IM or VM.
17. IL-1R-I binding polypeptide according to any preceding item, wherein sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1-1632, and 1679, such as the group consisting of SEQ ID NO:20-1632, and 1679.
- 15 18. IL-1R-I binding polypeptide according to any preceding item, wherein sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1206-1632 and 1679, such as the group consisting of SEQ ID NO:1210-1632 and 1679.
- 20 19. IL-1R-I binding polypeptide according to any preceding item, wherein sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1679, such as the group consisting of SEQ ID NO:1252, 1328, 1435 and 1679.
- 25 20. IL-1R-I binding polypeptide according to item 19, wherein sequence i) corresponds to the sequence from position 8 to position 36 in SEQ ID NO:1252.
- 30 21. IL-1R-I binding polypeptide according to item 19, wherein sequence i) corresponds to the sequence from position 8 to position 36 in SEQ ID NO:1328.
22. IL-1R-I binding polypeptide according to item 19, wherein sequence i) corresponds to the sequence from position 8 to position 36 in SEQ ID NO:1435.
- 35

23. IL-1R-I binding polypeptide according to any preceding item, wherein said IL-1R-I binding motif forms part of a three-helix bundle protein domain.

24. IL-1R-I binding polypeptide according to item 23, wherein said IL-1R-I binding motif essentially forms part of two helices with an interconnecting loop, within said three-helix bundle protein domain.

25. IL-1R-I binding polypeptide according to item 24, wherein said three-helix bundle protein domain is selected from bacterial receptor domains.

26. IL-1R-I binding polypeptide according to item 25, wherein said three-helix bundle protein domain is selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof.

27. IL-1R-I binding polypeptide according to any preceding item, which comprises a binding module *BMod*, the amino acid sequence of which is selected from:

iii) K-[BM]-DPSQSX_aX_bLLX_cEAKKLX_dX_eX_fQ;
(SEQ ID NO:1687)

wherein

[BM] is an IL-1R-I binding motif as defined in any one of items 1-22;
X_a is selected from A and S;
X_b is selected from N and E;
X_c is selected from A, S and C;
X_d is selected from E, N and S;
X_e is selected from D, E and S;
X_f is selected from A and S; and

iv) an amino acid sequence which has at least 91 % identity to a sequence defined by iii).

28. IL-1R-I binding polypeptide according to any preceding item, wherein sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-1638, 1667-1668 and 1670-1679; such as the group consisting of SEQ ID NO:20-1638 and 1670-1679.

29. IL-1R-I binding polypeptide according to item 28, wherein sequence iii) corresponds to the sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1206-1632 and

1670-1679, such as the group consisting of SEQ ID NO:1210-1632 and 1670-1679.

30. IL-1R-I binding polypeptide according to item 28, wherein sequence iii) corresponds to the sequence from position 7 to position 55 in a
5 sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1670-1679, such as the group consisting of SEQ ID NO:1252, 1328, 1435, 1672, 1675-1676 and 1679.

31. IL-1R-I binding polypeptide according to item 30, wherein
10 sequence iii) corresponds to the sequence from position 7 to position 55 in SEQ ID NO:1672.

32. IL-1R-I binding polypeptide according to item 30, wherein sequence iii) corresponds to the sequence from position 7 to position 55 in SEQ ID NO:1675.

15 33. IL-1R-I binding polypeptide according to item 30, wherein sequence iii) corresponds to the sequence from position 7 to position 55 in SEQ ID NO:1676.

34. IL-1R-I binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

20	ADNNFNK-[BM]-DPSQSANLLSEAKKLNESQAPK	SEQ ID NO:1695;
	ADNKFNK-[BM]-DPSQSANLLAEAKKLNDASQAPK	SEQ ID NO:1696;
	ADNKFNK-[BM]-DPSVSKEILAEAKKLNDASQAPK	SEQ ID NO:1697;
	ADAQQNNFNK-[BM]-DPSQSTNVLGEAKKLNESQAPK	SEQ ID NO:1698;
	AQHDE-[BM]-DPSQSANVLGEAQLNDSQAPK	SEQ ID NO:1699;
25	VDNKFNK-[BM]-DPSQSANLLAEAKKLNDASQAPK	SEQ ID NO:1700;
	AEAKYAK-[BM]-DPSESSELLSEAKKLNSQAPK	SEQ ID NO:1701;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLNDASQAPK	SEQ ID NO:1702;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLNDSQAPK	SEQ ID NO:1703;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLNDSQAPK	SEQ ID NO:1704;
30	AEAKYAK-[BM]-DPSQSSELLSEAKKLNDSQAP	SEQ ID NO:1705;
	AEAKFAK-[BM]-DPSQSSELLSEAKKLNDSQAPK	SEQ ID NO:1706;
	AEAKFAK-[BM]-DPSQSSELLSEAKKLNDSQAP	SEQ ID NO:1707;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLNDASQAPK	SEQ ID NO:1708;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLSESQAPK	SEQ ID NO:1709;
35	AEAKYAK-[BM]-DPSQSSELLSEAKKLSESQAP	SEQ ID NO:1710;
	AEAKFAK-[BM]-DPSQSSELLSEAKKLSESQAPK	SEQ ID NO:1711;
	AEAKFAK-[BM]-DPSQSSELLSEAKKLSESQAP	SEQ ID NO:1712;

	AEAKYAK-[BM]-DPSQSSELLAEAKKLSEAQAPK	SEQ ID NO:1713;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLESSQAPK	SEQ ID NO:1714;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLESSQAP	SEQ ID NO:1715;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLESAQAPK	SEQ ID NO:1716;
5	AEAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAPK	SEQ ID NO:1717;
	AEAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAP	SEQ ID NO:1718;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLSDSQAPK	SEQ ID NO:1719;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLSDAQAPK	SEQ ID NO:1720;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLNSQAPK	SEQ ID NO:1721;
10	VDAKYAK-[BM]-DPSQSSELLAEAKKLNSAQAPK	SEQ ID NO:1722;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLSESQAPK	SEQ ID NO:1723;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSEAQAPK	SEQ ID NO:1724;
	VDAKYAK-[BM]-DPSQSSELLSEAKKLESSQAPK	SEQ ID NO:1725;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLESAQAPK	SEQ ID NO:1726;
15	VDAKYAK-[BM]-DPSQSSELLSEAKKLSDSQAPK	SEQ ID NO:1727;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSDSQAPK	SEQ ID NO:1728;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLSDAQAPK	SEQ ID NO:1729;
	VDAKYAK-[BM]-DPSQSSELLAEAKKLNSAQAPK	SEQ ID NO:1730;
	AEAKYAK-[BM]-DPSQSSELLAEAKKLNSAQAPK	SEQ ID NO:1731,
20	and	
	ADAKYAK-[BM]-DPSQSSELLSEAKKLNSQAPK	SEQ ID NO:1732,

wherein [BM] is an IL-1R-I binding motif as defined in any one of items 1-22.

35. IL-1R-I binding polypeptide according to any one of items 1-34,
- 25 which comprises an amino acid sequence selected from:
- xix) VDAKYAK-[BM]-DPSQSSELLSEAKKLNSQAPK (SEQ ID NO:1721)
- wherein [BM] is an IL-1R-I binding motif as defined in any one of items 1-22; and
- 30 style="padding-left: 40px;">xx) an amino acid sequence which in the sequences flanking the BM has at least 89 % identity to the sequence defined in xix).
36. IL-1R-I binding polypeptide according to any one of items 1-34,
- which comprises an amino acid sequence selected from:
- xxi) AEAKEYAK-[BM]-DPSQSSELLSEAKKLSESQAPK (SEQ ID
- 35 NO:1709);
- wherein [BM] is an IL-1R-I binding motif as defined in any one of items 1-22; and

xxii) an amino acid sequence which in the sequences flanking the BM has at least 89 % identity to the sequence defined in xxi).

37. IL-1R-I binding polypeptide according to item 35 or 36, wherein sequence xix) or xxi) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1-1632, 1667-1668 and 1670-1679; such as the group consisting of SEQ ID NO:20-1632, 1667-1668 and 1670-1679.

38. IL-1R-I binding polypeptide according to item 37 wherein xix) or xxi) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting SEQ ID NO:1206-1632, 1667-1668 and 1670-1679, such as the group consisting of SEQ ID NO:1210-1632, 1667-1668 and 1670-1679.

39. IL-1R-I binding polypeptide according to item 38, wherein sequence xix) or xxi) corresponds to the sequence from position 1 to position 58 in a sequence selected from the group consisting of SEQ ID NO:1252, 1285, 1307, 1308, 1328, 1331, 1415, 1421, 1435, 1594 and 1670-1679, such as the group consisting of SEQ ID NO:1252, 1328, 1435, 1672, 1675-1676 and 1679.

40. IL-1R-I binding polypeptide according to item 39, wherein sequence xxi) corresponds to the sequence from position 1 to position 58 in SEQ ID NO:1672.

41. IL-1R-I binding polypeptide according to item 39, wherein sequence xxi) corresponds to the sequence from position 1 to position 58 in SEQ ID NO:1675.

42. IL-1R-I binding polypeptide according to item 39, wherein sequence xxi) corresponds to the sequence from position 1 to position 58 in SEQ ID NO:1676.

43. IL-1R-I binding polypeptide according to any preceding item, which is capable of blocking IL-1R-I dependent signaling.

44. IL-1R-I binding polypeptide according to item 43, wherein the half maximal inhibitory concentration (IC_{50}) of the blocking is at most 1×10^{-7} M, such as at most, 1×10^{-8} M, such as at most 1×10^{-9} M, such as at most 5×10^{-10} M.

45. IL-1R-I binding polypeptide according to item 43 or 44, which is capable of blocking the interaction of IL-1R-I with IL-1 cytokines, such as the interaction of IL-1R-I with IL-1 α and/or IL-1 β .

46. IL-1R-I binding polypeptide according to any preceding item which is capable of binding to IL-1R-I such that the EC_{50} value of the interaction is at most 1×10^{-7} M, such as at most 1×10^{-8} M, such as at most 5×10^{-9} M, such as at most 1×10^{-10} M such as at most 5×10^{-10} M, such as at most 2×10^{-10} M.

47. IL-1R-I binding polypeptide according to any preceding item which is capable of binding to IL-1R-I such that the K_D value of the interaction is at most 1×10^{-6} M, such as at most 1×10^{-7} M, such as at most 1×10^{-8} M, such as at most 1×10^{-9} M, such as at most 9×10^{-10} .

48. IL-1R-I binding polypeptide according to any one of items 43-47, wherein said IL-1R-I is human IL-1R-I or cynomolgus IL-1R-I, such as human IL-1R-I.

49. IL-1R-I binding polypeptide according to any preceding item which comprises additional amino acids at the C-terminal and/or N-terminal end.

50. IL-1R-I binding polypeptide according to item 49, wherein said additional amino acid(s) improve(s) production, purification, stabilization *in vivo* or *in vitro*, coupling or detection of the polypeptide.

51. IL-1R-I binding polypeptide according to any preceding item in multimeric form, comprising at least two IL-1R-I binding polypeptide monomer units, the amino acid sequences of which are the same or different.

52. IL-1R-I binding polypeptide according to item 51, wherein said IL-1R-I binding polypeptide monomer units are covalently coupled together.

53. IL-1R-I binding polypeptide according to item 52, wherein the IL-1R-I binding polypeptide monomer units are expressed as a fusion protein.

54. IL-1R-I binding polypeptide according to any one of items 51-53, in dimeric form.

55. Fusion protein or conjugate comprising

- a first moiety consisting of an IL-1R-I binding polypeptide according to any preceding item; and
- a second moiety consisting of a polypeptide having a desired biological activity.

56. Fusion protein or conjugate according to item 55, wherein said desired biological activity is a therapeutic activity.

57. Fusion protein or conjugate according to item 55, wherein said desired biological activity is a binding activity.

58. Fusion protein or conjugate according to item 55, wherein said desired biological activity is a binding activity modifying tissue distribution of said fusion protein or conjugate.

59. Fusion protein or conjugate according to item 57, wherein said
5 desired biological activity is an *in vivo* half-life increasing activity such that said second moiety increases *in vivo* half-life of the fusion protein or conjugate.

60. Fusion protein or conjugate according to item 59, said *in vivo* half-life increasing activity is an albumin binding activity.

10 61. Fusion protein or conjugate according to item 60, wherein said albumin binding activity is provided by the albumin binding domain of streptococcal protein G or a derivative thereof.

62. Fusion protein or conjugate according to item 61, wherein said second moiety comprises a polypeptide having an amino acid sequence as
15 set out in SEQ ID NO:1659-1661, such as SEQ ID NO:1661.

63. Fusion protein or conjugate according to item 59, wherein said second moiety is serum albumin.

64. Fusion protein or conjugate according to item 59, wherein said second moiety is an Fc portion of an antibody, such as an IgG1 Fc or an IgG4
20 Fc.

65. Fusion protein or conjugate according to item 64, wherein said Fc is a mutated Fc which relative to an unmutated form of Fc displays improved affinity to FcRn and/or reduced effector response.

66. Fusion protein or conjugate according to item 58 or 59, wherein
25 said second moiety is transferrin.

67. Fusion protein or conjugate according to any one of items 59-65, comprising a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:1639-1658 and 1734-1737, such as from the group consisting of SEQ ID NO:1646-1654 and 1734-1737.

30 68. Fusion protein or conjugate according to item 57, wherein said binding activity acts to block a biological activity.

69. Fusion protein or conjugate according to any one of items 55-68, further comprising at least one linker, said linker optionally being selected from the group consisting of flexible amino acid linkers, rigid amino acid
35 linkers and cleavable amino acid linkers.

70. Fusion protein or conjugate according item 69, wherein said linker is arranged between said first moiety and said second moiety.

71. Fusion protein or conjugate according item 69, wherein said linker is arranged within said first moiety.

72. Fusion protein or conjugate according item 69 or 70, wherein said linker is a flexible linker comprising at least one amino acid residue(s)
5 selected from the group consisting of glycine, serine and alanine.

73. Fusion protein or conjugate according to item 72, wherein said linker is selected from the group consisting of GS, VDSS, VDGS, VEGS, ASGS, (GGGGS)₂ (SEQ ID NO:1683), AS(GGGGS)₂ (SEQ ID NO:1684) and ((KEAAA)₃KELAA)₂ (SEQ ID NO:1685).

10 74. Fusion protein or conjugate according to item 73, wherein said linker is selected from a AS(GGGGS)₂ (SEQ ID NO:1684) and ((KEAAA)₃KELAA)₂ (SEQ ID NO:1685).

75. A polynucleotide encoding a polypeptide or a fusion protein according to any one of items 1-74.

15 76. Expression vector comprising a polynucleotide according to item 75.

77. Host cell comprising an expression vector according to item 76.

78. Method of producing a polypeptide according to any one of items 1-77, comprising

20 - culturing a host cell according to item 77 under conditions permissive of expression of said polypeptide from said expression vector, and
- isolating said polypeptide.

79. Composition comprising an IL-1R-I binding polypeptide, fusion protein, or conjugate according to any one of items 1-74 and at least one
25 pharmaceutically acceptable excipient or carrier.

80. Composition according to item 79, further comprising at least one additional active agent, such as an agent selected from an immune response modifying agent and an anti-cancer agent.

81. Composition according to item 79 or 80, wherein said fusion protein
30 is a fusion protein according to any one of claims 59-67.

82. IL-1R-I binding polypeptide, fusion protein, or conjugate according to any one of items 1-74 or a composition according to any one of items 79-81 for use as a medicament, a diagnostic agent and/or a prognostic agent.

83. IL-1R-I binding polypeptide, fusion protein, conjugate or
35 composition according to item 82 for use as a medicament.

84. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to item 83, wherein said polypeptide, fusion protein, conjugate or composition modulates IL-1R-I function *in vivo*.

85. IL-1R-I binding polypeptide, fusion protein, conjugate or
5 composition according to item 82 for use as a diagnostic agent and/or a prognostic agent.

86. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 82-85, in the treatment, prognosis or diagnosis of an IL-1R-I related disorder.

10 87. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to item 86, wherein said IL-1R-I related disorder is selected from the group consisting of inflammatory disease, auto-inflammatory syndromes, autoimmune disease, infectious disease, cardiovascular disease, ischaemic disease, cancer and diabetes.

15 88. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to item 86 or 87, wherein said IL-1R-I related disorder is selected from the group consisting of familial Mediterranean fever (FMF); cryopyrin-associated periodic syndrome (CAPS); TNF receptor-associated periodic syndrome (TRAPS); hyper-IgD syndrome (HIDS);
20 periodic fever; aphthous stomatitis; pharyngitis; adenitis (PFAPA); rheumatoid arthritis (RA), juvenile RA, juvenile idiopathic arthritis, systemic juvenile idiopathic arthritis; adult-onset Still's disease; Schnitzler syndrome; Muckle Wells Syndrome; macrophage activation syndrome; Behçet's disease; uveitis; acne vulgaris; pyoderma gangrenosum; gout; type 2 diabetes, new-onset
25 diabetes; dry eye syndrome; hidradenitis suppurativa; neutrophilic dermatoses, in particular cytophagic histiocytic panniculitis, Weber-Christian disease, and neutrophilic panniculitis; cardiovascular disease, myocardial infarction, stroke; liver failure, kidney failure; acute lung injury; pseudogout, calcium-pyrophosphate deposition disorder, chondrocalcinosis, deficiency of
30 the IL-1 receptor antagonist (DIRA), deficiency of the IL-36 receptor antagonist (DITRA), ADAM2 deficiency (DADA2), pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, pyoderma gangrenosum, acne and suppurative hidradenitis (PASH) syndrome, PAPA and suppurative hidradenitis (PAPASH) syndrome, autoinflammatory
35 syndrome with lymphedema (AISLE), Phospholipase C-gamma-2 mutation autoinflammatory syndrome, NALP12-associated periodic syndrome (NAPS12), mevalonate kinase deficiency (MKD), psoriatic arthritis, reactive

- arthritis, ankylosing spondylitis, haemochromatosis-related arthritis, periarticular calcinosis, osteoarthritis, inflammatory osteoarthritis, hand osteoarthritis, pustular psoriasis such as generalised pustular psoriasis (GPP), palmoplantar pustulosis (PPP), acrodermatitis continua of Hallopeau (ACH); Blau syndrome; Sweet syndrome; various vasculitides such as giant-cell arteritis (GCA), polymyalgia rheumatica (PMR), Takayasu arteritis, Kawasaki disease, urticarial vasculitis, and Henoch-Schönlein purpura (HSP); neutrophilic urticaria and idiopathic cold urticaria; lichen planus; polymyositis, dermatomyositis, juvenile dermatomyositis and inclusion-body myositis;
- 10 various stages of myeloma such as smoldering myeloma (SMM), indolent myeloma, Waldenstrom's macroglobulinemia, and multiple myeloma; tumor-induced cachexia; solid tumor growth; neonatal disorders such as bronchopulmonary dysplasia (prophylaxis), necrotising enterocolitis (NEC), retinopathy of prematurity (ROP), cerebral palsy due to perinatal cerebral
- 15 ischemia, and infant respiratory distress syndrome (IRDS); Whipple's disease; traumatic brain injury; refractory epilepsy; systemic inflammatory response syndrome (SIRS); cutaneous lupus; Jessner-Kanof disease; amyotrophic lateral sclerosis; systemic sclerosis (scleroderma); septic shock; acute pancreatitis; chronic recurrent multifocal osteomyelitis, non-bacterial
- 20 osteitis (NBO), synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO) syndrome, and Majeed syndrome; relapsing polychondritis; idiopathic recurrent pericarditis (IRP); myocarditis; Erdheim-Chester disease; juvenile xanthogranuloma; islet-cell transplantation; haemodialysis-induced systemic inflammation; graft-versus-host disease; ANCA-associated glomerulonephritis
- 25 and recurrent glomerulonephritis; Cogan syndrome; autoimmune inner-ear disease; chronic granulomatous disease (CGD); Castleman's disease; cardiac failure; diastolic cardiac failure; antisyndetase syndrome; acute ACL injury; acute haemorrhagic leukoencephalitis; AA amyloidosis; Di George syndrome; generalised fatigue; chronic fatigue syndrome (CFS); gulf-war
- 30 illness (GWI); and narcolepsy.

89. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to item 87, wherein said IL-1R-I related disorder is cancer, such as a cancer selected from the group consisting of multiple myeloma, colon cancer, breast cancer, lung cancer, head and neck
- 35 cancer, melanoma and prostate cancer.

90. IL-1R-I binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 82-89, wherein said IL-1R-I

binding polypeptide, fusion protein, conjugate or composition is administered repeatedly within a 24 hour period from disease onset, such as at least two times within a 24 hour period from disease onset, such as at least three times within a 24 hour period from disease onset, such as continuously during 24
5 hours from disease onset, to a subject in need thereof.

91. Fusion protein, conjugate or composition for use according to any one of items 82-84, 86-89, wherein said fusion protein or conjugate is a fusion protein or conjugate according to any one of items 58-67 and said composition is a composition according to item 81.

10 92. Fusion protein, conjugate or composition for use according to item 91, wherein said fusion protein, conjugate or composition is administered once weekly to a subject in need thereof.

93. Method of treatment of an IL-1R-I related disorder, comprising administering to a subject in need thereof an effective amount of an IL-1R-I
15 binding polypeptide, fusion protein or conjugate according to any one of items 1-74 or a composition according to any one of items 79-81.

94. Method according to item 93, wherein said IL-1R-I related disorder is selected from the group consisting inflammatory disease, auto-inflammatory syndrome, autoimmune disease, infectious disease,
20 cardiovascular disease, ischaemic disease, cancer and diabetes.

95. Method according to item 93 or 94, wherein said IL-1R-I related disorder is selected from the group as defined in item 88.

96. Method according to item 94, wherein said IL-1R-I related disorder is cancer, such as a cancer selected from the group consisting of selected
25 from the group consisting of multiple myeloma, colon cancer, breast cancer, lung cancer, head and neck cancer, melanoma and prostate cancer.

97. Method according to any one of items 93-96, wherein said IL-1R-I binding polypeptide, fusion protein or conjugate or composition is administered repeatedly within a 24 h period from disease onset, such as at
30 least two times within a 24 hour period from disease onset, such as at least three times within a 24 hour period from disease onset, such as continuously during 24 hours from disease onset, to a subject in need thereof.

98. Method according to any one of claims 93-96, wherein said fusion protein or conjugate is a fusion protein or conjugate according to any one of
35 items 59-67 and said composition is a composition according to item 81.

99. Method according to item 97, wherein said fusion protein, conjugate or composition is administered once weekly to a subject in need thereof.