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(71) Applicant: **AFFIBODY AB** [SE/SE]; Scheeles väg 2, 171 65 Solna (SE).

(72) Inventors: **EDVARDSSON, Anna**; Hallernavägen 30, 468 33 Vargön (SE). **GUDMUNDSDOTTER, Lindvi**; Domherrestigen 4, 141 40 Huddinge (SE). **KIJANI, Siavash**; Norra Stommen 217, 438 32 Landvetter (SE). **KLINT, Susanne**; Olof Dalins Väg 16, 112 52 Stockholm (SE).

(74) Agent: **AWA SWEDEN AB**; Box 45086, 104 30 Stockholm (SE).

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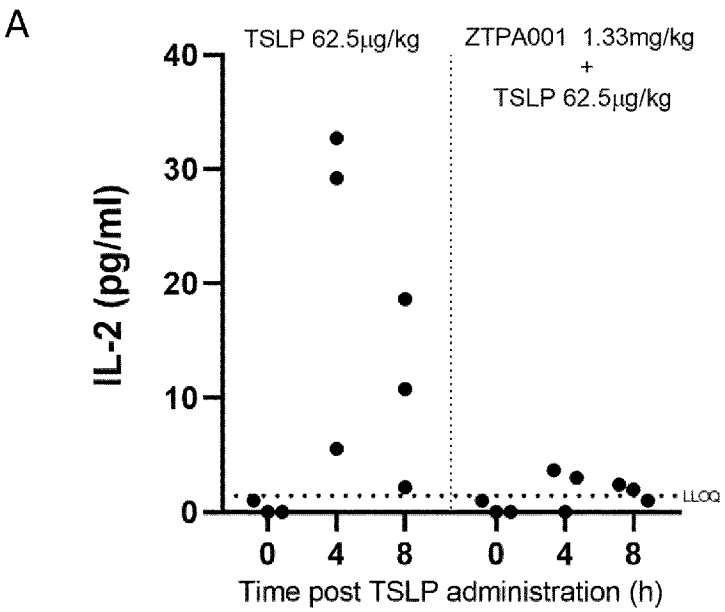

Figure 10A

(57) Abstract: The present disclosure relates to a class of engineered polypeptides having a binding affinity for thymic stromal lymphopoietin (TSLP), and provides a TSLP binding polypeptide comprising the sequence 5 $EAVX_4ALX_7EIW$ $X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LRD$ or a sequence having at least 93 % identity thereto. The present disclosure also relates to the use of such a TSLP binding polypeptide as a therapeutic, prognostic and/or diagnostic agent.

[Continued on next page]

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PROTEIN Z VARIANTS BINDING THYMIC STROMAL LYMPHOPOIETIN AND THEIR MEDICAL USE

Field of the invention

The present disclosure relates to a class of engineered polypeptides having a binding affinity for the protein thymic stromal lymphopoietin (in the following referred to as TSLP). The present disclosure also relates to the use of such a TSLP binding polypeptide as a therapeutic, prognostic and/or diagnostic agent.

Background

Thymic stromal lymphopoietin (TSLP) is an epithelial and mast cell derived cytokine that is implicated in the pathogenesis of several inflammatory diseases including initiation and persistence of airway inflammation. TSLP is a key regulator of many downstream inflammatory pathways. TSLP is a four-helix bundle cytokine that is closely related to IL-7, a member of the hematopoietin family of cytokines. TSLP was initially isolated from a mouse thymic stromal cell line and was found to be a growth factor for B cells. TSLP binds to its receptor (TSLP-R) to exert its biological activities. TSLP-R is a heterodimeric receptor that consists of the IL-7 receptor alpha-chain (IL-7Rα) and the TSLP receptor alpha chain 1 (TSLP-Rα, also known as CRL2, TSLP-R and CRLF2Y), which is closely related to the common receptor-γ chain (γc) that is found in IL-2, IL-4, IL-9 and IL-15 receptor complexes. The functional TSLP-R is mainly expressed in hematopoietic cells, dendritic cells (DC), T cells, B cells, natural killer (NK) cells, invariant natural killer T cells (iNKT), monocytes, basophils, mast cells, eosinophils, liver, brain, skeletal muscle, kidney, spleen and thymus (Markovic and Savvides, 2020, Front Immunol 11:1557).

TSLP exists in two isoforms in humans. The short form of TSLP is missing both the signal peptide and the first two alpha helices. It is constitutively expressed in all epithelial cell layers and skin epidermis, where it mediates homeostatic functions. The short form of TSLP has not been proven to exert any proinflammatory activities but is rather implicated as an antimicrobial peptide (Tsilingiri *et al.*, 2017, Cell Mol Gastroenterol Hepatol 3(2):174-182). The long isoform is expressed at a low/undetectable level at steady state and upregulated during inflammation in several tissues (hereinafter the long isoform will be referred to as “TSLP”, while the short isoform will be referred to as “the short form of TSLP” or “sfTSLP”).

Furthermore, posttranslational modifications, such as glycosylation and furine cleavage, may alter the functional activity of TSLP. Hence, it may be important for TSLP-targeting drugs to block both mature and cleaved active TSLP metabolites (Varricchi *et al.*, 2018, Front Immunol 9:1595).

TSLP is produced in response to proinflammatory stimuli, for example insults such as viral infections, mechanical stress, allergens and pollutants, triggering an inflammatory cascade. TSLP has been proven to promote inflammatory responses primarily through its activity on innate lymphoid cells (mainly ILC2), dendritic cells and mast cells. TSLP plays a crucial role in the induction and maintenance of allergic inflammatory Th2, ILC2 and mast cell responses by production of proallergenic cytokines, chemokines and costimulatory molecules that drive naïve T cells to become Th2 cells producing IL-4, IL-5 and IL-13. These are all critical mediators of allergic inflammation (Gauvreau *et al.*, 2020, Expert Opin Ther Targets 24(8):777-792).

Human TSLP expression has been reported to be increased in asthmatic airways and to correlate with disease severity. Chronic allergic asthma is often characterized by Th2-type inflammation, while non-allergic asthmatic inflammation is predominantly neutrophilic with a mixed Th1 and Th17 cytokine response. As TSLP is situated at the top of the signaling cascade, the inhibition of TSLP facilitates treatment of both allergic and non-allergic asthma. In addition to its fundamental role in asthma development, TSLP is known to be involved in the pathogenesis of other allergic conditions (Gauvreau *et al.*, *supra*). Increased levels of TSLP protein are found in skin lesions of patients with atopic dermatitis. Aberrant expression of TSLP has been observed in allergic diseases of the gastrointestinal tract, including Crohn's disease, eosinophilic eosophagitis and ulcerative colitis, and in cancer. Higher TSLP expression has also been observed in patients with chronic obstructive pulmonary disease (COPD) compared with healthy individuals (Cianferoni and Spergel, 2014, Expert Rev Clin Immunol 10(11):1463-74).

Published data provides clinical evidence that inhibition of TSLP with tezepelumab, an anti-TSLP monoclonal antibody, leads to a lower annualized rate of asthma exacerbations than placebo administration (Corren and Ziegler, 2019, Nat Immunol 20(12):1603-1609). The outcome is independent of baseline eosinophil count or other Th2 biomarkers. Among patients on treatment with long-acting beta-agonists and medium-to-high doses of inhaled

glucocorticoids, those who received tezepelumab had lower rates of clinically significant asthma exacerbations than those receiving placebo.

These findings highlight the potential advantages of targeting an upstream cytokine such as TSLP, which may affect disease activity more broadly than inhibition of a single downstream pathway. Furthermore, in a phase III setting, tezepelumab reduced exacerbations, irrespective of baseline blood eosinophil count, and improved lung function, asthma control and health-related quality of life in a broad population of patients with severe, uncontrolled asthma (Menzies-Gow *et al.*, 2021, N Engl J Med 384(19):1800-1809). Tezepelumab was recently approved for treatment of severe asthma and is sold under the brand name Tezpire®. It is currently being investigated in clinical trials for treatment of COPD, rhinosinusitis, eosinophilic esophagitis and chronic urticaria.

The prevalence of allergic disease such as asthma, allergic rhinitis, atopic dermatitis and food allergies appears to be increasing in recent years, particularly in developed countries, affecting an increasing percentage of the population (Rutowski *et al.*, 2014, Postepy Dermatol Alergol 31(2):77-83).

For many inflammatory diseases, the means of diagnosis and monitoring of treatment is clinical evaluation and analysis of general biomarkers. Traditional reliance upon clinical assessments for diagnosing inflammatory diseases and monitoring treatment can be associated with sub-optimal patient outcomes. There is a need for reliable methods for diagnosing inflammatory conditions, assessing disease status, and monitoring response to treatment. In addition, rational design and application of new therapeutics for inflammatory diseases require the discovery, validation and implementation of informative indicators of biological processes or pharmacological responses to therapeutic intervention. This may be facilitated by the development of proper companion diagnostic tools that enable a personalized medicine approach with medical decisions, practices, interventions and/or products being tailored to the individual patient based on their predicted response or risk of disease. Studies have shown that upregulation of TSLP in bronchoalveolar lavage fluid (BALF) is closely correlated to disease severity in asthma. Levels of TSLP in BALF has been shown to correlate inversely with lung function which suggest that it may serve as useful diagnostic marker (Li *et al.*, 2018, J Immunol 200(7):2253-2262).

Since tissue penetration rate is negatively associated with the size of the molecule, a relatively large antibody molecule inherently has poor tissue distribution and penetration capacity and may not be suitable for local lung delivery, e.g. to treat respiratory diseases like asthma and COPD. Moreover, although antibodies are widely used in a variety of routine contexts owing to high affinity and specificity to a multitude of possible antigens, such as for analytical, purification, diagnostic and therapeutic purposes, they still suffer from several drawbacks. Such drawbacks include aggregation tendencies, limited stability and limited solubility, which make antibodies less suitable for alternative administration routes such as inhalation.

Thus, the use of monoclonal antibodies is not always optimal for therapy, and there is continued need for provision of agents with a high affinity for TSLP. Of great interest is also the provision of uses of such molecules in the treatment, diagnosis and prognosis of disease.

Summary of the invention

It is an object of the present disclosure to provide new TSLP 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 of for example various forms of inflammatory disease, while alleviating the abovementioned and other drawbacks of current therapies.

It is furthermore an object of the present disclosure to provide a molecule suitable for prognostic and diagnostic applications, for example prognostic and diagnostic application in relation to various forms of inflammatory disease.

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

Thus, in a first aspect of the disclosure, there is provided a TSLP binding polypeptide, comprising a TSLP binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EAVX_4ALX_7EIW\ X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}\ X_{21}AFIX_{25}X_{26}LRD$ (SEQ ID NO:1081)

wherein, independently of each other,

X_4 is selected from D, E, and H;
X_7 is selected from I, L, M and V;
X_{11} is selected from A, D, E, K, N, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, T, W and Y;
X_{21} is selected from D, E, G, H, K, M, N, Q and R;
X_{25} is selected from A, H, I, K, L, Q, R, V and Y; and
X_{26} is selected from K and S;

and

ii) an amino acid sequence which has at least 93 % identity to the sequence defined in i).

In one embodiment, there is provided a TSLP binding polypeptide, wherein, in sequence i),

X_4 is selected from D, E and H;
X_7 is selected from I, L, M and V;
X_{11} is selected from A, D, E, K, N, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, T, W and Y;
X_{21} is selected from D, E, G, H, K, M, N, Q and R;
X_{25} is selected from A, H, I, K, L, Q, R, V and Y; and
X_{26} is selected from K and S.

In another embodiment, there is provided a TSLP binding polypeptide, wherein, in sequence i),

X_4 is selected from E and H;
X_7 is selected from I, L and V;

X_{11} is selected from A, D, E, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, G, H, K, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, W and Y;
X_{21} is selected from D, E, H, K, M, N, Q and R;
X_{25} is selected from H, I, K, L, Q, R, V and Y; and
X_{26} is selected from K and S.

In yet another embodiment, there is provided a TSLP binding polypeptide, wherein, in sequence i),
X_4 is selected from E and H;
X_7 is selected from I, L and V;
X_{11} is selected from A, D, E, Q, S and T;
X_{16} is T;
X_{17} is selected from D, E, G, H, N, Q, R, S, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, L, N, Q, R, S, T, W and Y;
X_{20} is selected from H, W and Y;
X_{21} is selected from D, E, H, N and Q;
X_{25} is selected from I, L, R, V and Y; and
X_{26} is K.

In yet another embodiment, there is provided a TSLP binding polypeptide, wherein, in sequence i),
X_4 is E;
X_7 is selected from I and V;
X_{11} is selected from A, D, E, Q, S and T;
X_{16} is T;
X_{17} is selected from D, E, G, H, N, Q, R and Y;
X_{18} is selected from A, D, E, F, G, I, L, N, Q, R, S, T and Y;
X_{20} is selected from H, W and Y;
X_{21} is selected from D, E, H, N and Q;
X_{25} is selected from I, L, V and Y; and
X_{26} is K.

In yet another embodiment, there is provided a TSLP binding polypeptide according to any preceding claim, wherein, in sequence i),

X_4 is E;

X_7 is V;

X_{11} is selected from A and T;

X_{16} is T;

X_{17} is R;

X_{18} is selected from D and E;

X_{20} is W;

X_{21} is Q;

X_{25} is Y; and

X_{26} is K.

As used herein, "X_n" and "X_m" are used to indicate amino acids in 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_4 and X_7 indicate the amino acid in position four 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 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 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 X_4 in sequence i) is selected from D, E and H, and in another embodiment according to the first aspect, there is provided a polypeptide wherein X_4 in sequence i) is selected from D and E. 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 X_4 is selected from E and H, while X_7 is selected from I, L and V, and X_{25} is selected from H, I, K, L, Q, R, V and Y and so on.

Table 1

X_n	Possible residues
X_4	D, E and H
X_4	D and E
X_4	D and H
X_4	E and H
X_4	D
X_4	E
X_4	H
X_7	I, L, M and V
X_7	I, M and V
X_7	I, L and V
X_7	I and L
X_7	I and M
X_7	I and V
X_7	L, M and V
X_7	L and M
X_7	L and V
X_7	M and V
X_7	I
X_7	L
X_7	M
X_7	V
X_{11}	A, D, E, K, N, Q, R, S and T
X_{11}	A, D, E, K, Q, R, S and T
X_{11}	A, D, E, K, N, Q, S and T
X_{11}	A, D, E, N, Q, R, S and T
X_{11}	A, D, E, Q, R, S and T
X_{11}	A, D, E, Q, K, S and T
X_{11}	A, D, E, N, Q, S and T
X_{11}	A, D, E, Q, S and T
X_{11}	A, D, E, Q and T
X_{11}	A, D, E and T
X_{11}	A, E, Q and T
X_{11}	A and T
X_{11}	E and T
X_{11}	D, E, K, N, Q, R, S and T
X_{11}	D, E, Q and T
X_{11}	E, K, N, Q, R, S and T
X_{11}	E, Q and T
X_{11}	Q and T
X_{11}	A
X_{11}	D
X_{11}	E
X_{11}	K
X_{11}	N
X_{11}	Q
X_{11}	R
X_{11}	T
X_{16}	N and T
X_{16}	N
X_{16}	T
X_{17}	A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, G, H, I, K, L, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, F, G, H, K, L, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, F, G, H, I, K, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, F, G, H, I, L, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, F, G, H, K, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, G, H, K, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, G, H, N, Q, R, S, T, V, W and Y
X_{17}	A, D, E, G, H, N, Q, R, S, T, V and W
X_{17}	A, D, E, G, H, N, Q, R, S, T and V
X_{17}	D, E, G, H, N, Q, R, S, W and Y
X_{17}	D, E, G, H, N, Q, R, S and T
X_{17}	D, E, G, H, N, Q, R and Y
X_{17}	D, E, G, H, N, Q and R
X_{17}	D, E, G, H, N and R
X_{17}	D, E, G, H and R
X_{17}	D, E, H and R
X_{17}	D, H, N, Q and R
X_{17}	D, H and Q

X_n	Possible residues
X_{17}	D, H and R
X_{17}	D and H
X_{17}	D and R
X_{17}	H and R
X_{17}	R
X_{18}	A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y
X_{18}	A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, W and Y
X_{18}	A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V and Y
X_{18}	A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V and W
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R, S, T, W and Y
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R, S, T and Y
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R, S, T and W
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R, S and T
X_{18}	A, D, E, F, G, H, I, L, N, Q, R, S and T
X_{18}	A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T and V
X_{18}	A, D, E, F, G, H, I, L, N, Q, R, S, T, W and Y
X_{18}	A, D, E, F, G, H, I, L, N, Q, R, S, T and Y
X_{18}	A, D, E, F, G, H, I, L, N, Q, R, S, T and W
X_{18}	A, D, E, F, G, H, I, L, N, Q, R, S and T
X_{18}	A, D, E, F, G, I, L, N, Q, R, S and T
X_{18}	A, D, E, F, G, H, I, L, N, Q, R and S
X_{18}	A, D, E, F, H, I, L, N, Q, R, S and T
X_{18}	A, D, E, F, G, H, I, L, N, Q, and R
X_{18}	A, D, E, G, H, I, L, N, Q, and R
X_{18}	A, D, E, F, G, I, L, N, Q, R, S, T and Y

X_n	Possible residues
X_{18}	A, D, E, F, G, H, I, N, Q, R, S, T and V
X_{18}	A, D, F, G, I, N, Q, R and V
X_{18}	A, D, E, F, G, H, I, K, L, N, Q, R and T
X_{18}	A, D, E, H, I, K, L, N and Q
X_{18}	A, D, E, I, K, L, N and Q
X_{18}	A, D, E, I, L, N and Q
X_{18}	A, D, E, I, L and Q
X_{18}	A, D, I, K, L, N and Q
X_{18}	A, D, K, L, N and Q
X_{18}	D, E and I
X_{18}	D and E
X_{20}	H, N, Q, T, W and Y
X_{20}	H, N, Q, W and Y
X_{20}	H, N, W and Y
X_{20}	H, N and Y
X_{20}	H, W and Y
X_{20}	N, W and Y
X_{20}	N, W and Y
X_{20}	W and Y
X_{20}	H and Y
X_{20}	H and N
X_{20}	H and W
X_{20}	H
X_{20}	N
X_{20}	Q
X_{20}	T
X_{20}	W
X_{20}	Y
X_{21}	D, E, G, H, K, M, N, Q and R
X_{21}	D, E, G, H, K, M, N and Q
X_{21}	D, E, H, K, M, N, Q and R
X_{21}	D, E, G, H, M, N, Q and R
X_{21}	D, E, G, H, K, N, Q and R
X_{21}	D, E, G, H, M, N and Q
X_{21}	D, E, G, H, K, N and Q
X_{21}	D, E, H, K, M, N and Q
X_{21}	D, E, H, K, N, Q and R
X_{21}	D, E, H, M, N and Q
X_{21}	D, E, H, M and N
X_{21}	D, E, H, N and Q
X_{21}	D, E and Q
X_{21}	E and Q
X_{21}	D

X_n	Possible residues
X_{21}	E
X_{21}	H
X_{21}	M
X_{21}	N
X_{21}	Q
X_{25}	A, H, I, K, L, Q, R, V and Y
X_{25}	H, I, K, L, Q, R, V and Y
X_{25}	I, K, L, Q, R, V and Y
X_{25}	H, I, L, Q, R, V and Y
X_{25}	I, L, Q, R, V and Y
X_{25}	I, L, Q, V and Y

X_n	Possible residues
X_{25}	I, L, R, V and Y
X_{25}	I, K, L, V and Y
X_{25}	I, L, V and Y
X_{25}	I, L and Y
X_{25}	I, L and Y
X_{25}	L and Y
X_{25}	Y
X_{26}	K and S
X_{26}	K
X_{26}	S

In a more specific embodiment defining a sub-class of TSLP binding polypeptides, sequence i) fulfills at least four of the eight conditions I-VIII:

I. X_4 is E or H;
II. X_7 is selected from I, L and V;
III. X_{11} is selected from A, D, E, Q, S and T;
IV. X_{16} is T;
V. X_{20} is selected from H, W and Y;
VI. X_{21} is selected from D, E, H, N and Q;
VII. X_{25} is selected from I, L, R, V and Y; and
VIII. X_{26} is K.

In some examples of a TSLP binding polypeptide according to the first aspect, sequence i) fulfills at least five of the eight conditions I-VIII. More specifically, sequence i) may fulfill at least six of the eight conditions I-VIII, such as at least seven of the eight conditions I-VIII, such as all of the eight conditions I-VIII.

In some embodiments of a TSLP binding polypeptide according to the first aspect, X_4 is E, X_7 is V and X_{20} is W. In some embodiments, X_4 is E, X_7 is V and X_{20} is Y. In some embodiments, X_4 is E, X_7 is V and X_{21} is Q. In some embodiments, X_4 is E, X_7 is V and X_{21} is E. In some embodiments, X_4 is E, X_7 is V and X_{25} is Y. In some embodiments, X_4 is E, X_7 is V and X_{25} is L.

As described in detail in the experimental section to follow, the selection of TSLP binding polypeptide variants has led to the identification of a number of individual TSLP binding motif (*BM*) sequences belonging to the

class defined in the first aspect of the disclosure. These sequences constitute individual embodiments of sequence i) or ii) according to this aspect. The sequences of individual TSLP binding motifs correspond to amino acid positions 8-36 in SEQ ID NO:1-875 presented in the sequence listing. In one embodiment of the TSLP binding polypeptide according to this first aspect, sequence i) corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1-645. 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:1-630 and 645. 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:1-91 and 645. 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:1-27. 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:1-2.

As the skilled person will realize, the function of any polypeptide, such as the TSLP 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 TSLP binding polypeptide, which have retained TSLP binding characteristics.

In this way, encompassed by the present disclosure is a TSLP binding polypeptide comprising an amino acid sequence with 93 % or greater identity, such as 96 % or greater identity, to a polypeptide as defined in i). 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 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 TSLP binding polypeptide as disclosed herein. In other embodiments, such changes may be made only in the non-variable positions, also denoted scaffold amino acid residues. In such cases, changes are not allowed in the variable positions. In other embodiments, such changes may be only in the variable positions.

According to one definition of such “variable positions”, these are positions denoted with an “X” in sequence i) as defined above.

According to another definition, “variable positions” are those positions that are randomized in a selection library of Z variants prior to selection, and may thus for example be positions 2, 3, 4, 6, 7, 10, 11, 17, 18, 20, 21, 25 and 28 in sequence i). This definition of “variable positions” does not include positions 16 and 26, which are scaffold positions in this context, albeit allowed to be either one of two alternatives in each position. Reference is made to Nord *et al.* (1995), Prot Eng 8:601-608, and Löfblom *et al.* (2010), FEBS Letters, 584:2670-2680. Like the TSLP-binding Z variants of the present disclosure, the polypeptides disclosed in Nord *et al.* and Löfblom *et al.* are also based on a scaffold from the Z derivative of domain B of protein A from *Staphylococcus aureus*, although directed to other targets. As shown in Nord *et al.* (see for example Figure 4), the amino acids in positions 23 (corresponding to position 16 in the instant TSLP-binding motif) and 33 (corresponding to position 26 in the instant TSLP-binding motif) are N and S, respectively. As also shown in Löfblom *et al.*, polypeptides with amino acid residues N and S in positions 23 and 33, respectively (corresponding to positions 16 and 26 in the instant TSLP-binding motif; see Figure 2 of Löfblom *et al.*), and polypeptides with amino acid residues T and K in positions 23 and 33, respectively, all have a maintained basic structure and function. Thus, in the context of this definition of “variable positions”, the amino acid residues at positions 16 and 26 form part of the common scaffold, and it is contemplated to have either N or T in scaffold position 16 and either S or K in scaffold position 26.

The term ”% identity”, as used throughout the specification, may for example be calculated as follows. The query sequence is aligned to the target 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 shortest of the aligned sequences may be the query sequence. 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.

In another embodiment, there is provided a TSLP binding polypeptide comprising a binding motif sequence that corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO:1-875; or a sequence having 93 % or greater identity thereto, such as 96 % identity thereto.

In some embodiments, the *BM* as defined 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 Cα 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 TSLP 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 (Wahlberg E *et al.* 2003, PNAS 100(6):3185-3190).

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

iii) K-*[BM]*-DPSQS$X_a$$X_bLLX_c$EAKKL$X_d$$X_e$$X_f$Q (SEQ ID NO:1082);
wherein
[BM] is a TSLP binding motif according to any definition herein;
X_a is selected from A and S;
X_b is selected from E and N;
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 the sequence defined in iii).

In some embodiments, said polypeptide may beneficially exhibit a 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 polypeptide, are also within the scope of the present disclosure. Thus, in some embodiments, sequence iv) has at least 93 %, such as at least 95 %, such as at least 97 % identity to a sequence defined by iii).

In one embodiment, X_a in sequence iii) is A.
In one embodiment, X_a in sequence iii) is S.
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.
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.
In one embodiment, X_e in sequence iii) is S.
In one embodiment, X_dX_e in sequence iii) is selected from EE, ES, SD, 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_dX_e in sequence iii) is SD.
In one embodiment, X_f in sequence iii) is A.
In one embodiment, X_f in sequence iii) is S.
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 S; X_b is E; 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.
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.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A; X_dX_e is ND 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 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.
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 S; X_b is E; 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.
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.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is SD and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A; X_dX_e is SD 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 SD 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 SD 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 SD and X_f is S.

In a further embodiment, which is an embodiment of the first aspect of the disclosure, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-875. In one embodiment, sequence iii) is selected from the amino acid sequences corresponding to positions 7-55 in SEQ ID NO:1-645. In one embodiment, sequence iii) is selected from the amino acid sequences corresponding to positions 7-55 in SEQ ID NO:1-630 and 645. In another

embodiment, sequence iii) is selected from the amino acid sequences corresponding to positions 7-55 in SEQ ID NO:1-91. In one embodiment, sequence iii) is selected from the amino acid sequences corresponding to positions 7-55 in SEQ ID NO:1-27. In yet another embodiment, sequence iii) is selected from the amino acid sequences corresponding to positions 7-55 in SEQ ID NO:1-2.

Also, in a further embodiment, there is provided a TSLP binding polypeptide, which comprises an amino acid sequence selected from:

v) YA-[*BMod*]-AP (SEQ ID NO:1083);
wherein [*BMod*] is a TSLP binding module as defined above; and

vi) an amino acid sequence which has at least 86 % identity to a sequence defined by v).

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences without largely affecting the tertiary structure and the function thereof also fall within the scope of the present disclosure. Thus, in some embodiments, the TSLP binding polypeptides as defined above may for example have a sequence vi) which is at least 88 %, such as 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).

In some embodiments, the TSLP binding motif may form part of a polypeptide comprising an amino acid sequence selected from

ADNNFNK-[*BM*]-DPSQSANLLSEAKKLNESQAPK (SEQ ID NO:1084);
ADNKFNK-[*BM*]-DPSQSANLLAEAKKLNDAQAPK (SEQ ID NO:1085);
ADNKFNK-[*BM*]-DPSVSKEILAEAKKLNDAQAPK (SEQ ID NO:1086);
ADAQQNNFNK-[*BM*]-DPSQSTNVLGEAKKLNESQAPK (SEQ ID NO:1087);
AQHDE-[*BM*]-DPSQSANVLGEAQKLNDSQAPK (SEQ ID NO:1088);
VDNKFNK-[*BM*]-DPSQSANLLAEAKKLNDAQAPK (SEQ ID NO:1089);
AEAKYAK-[*BM*]-DPSESSELLSEAKKLNKSQAPK (SEQ ID NO:1090);
VDAKYAK-[*BM*]-DPSQSSELLAEAKKLNDAQAPK (SEQ ID NO:1091);
VDAKYAK-[*BM*]-DPSQSSELLAEAKKLNDSQAPK (SEQ ID NO:1092);
AEAKYAK-[*BM*]-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1093);
AEAKYAK-[*BM*]-DPSQSSELLSEAKKLSESQAPK (SEQ ID NO:1094);

AEAKFAK-[*BM*]-DPSQSSELLSEAKKLSESQAPK (SEQ ID NO:1095);
AEAKYAK-[*BM*]-DPSQSSELLSEAKKLESSQAPK (SEQ ID NO:1096);
VDAKYAK-[*BM*]-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1097);
VDAKYAK-[*BM*]-DPSQSSELLSEAKKLSESQAPK (SEQ ID NO:1098);
VDAKYAK-[*BM*]-DPSQSSELLSEAKKLESSQAPK (SEQ ID NO:1099);
VDAKYAK-[*BM*]-DPSQSSELLAEAKKLNKAQAPK (SEQ ID NO:1100);
and
AEAKYAK-[*BM*]-DPSQSSELLAEAKKLNKAQAPK (SEQ ID NO:1101);
wherein [*BM*] is an IL-17A binding motif as defined above.

In one embodiment, the TSLP binding polypeptide comprises an amino acid sequence selected from:

vii) VDAKYAK-*[BM]*-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1097); wherein [*BM*] is a TSLP binding motif as defined herein; and

viii) an amino acid sequence which has at least 86 % identity to a sequence defined by vii).

In a further embodiment, sequence vii) is selected from the group consisting of SEQ ID NO:1-636, 639-644 and 646-875, for example selected from the group consisting of SEQ ID NO:1-636 and 639-644.

In another further embodiment, the TSLP binding polypeptide comprises an amino acid sequence selected from:

ix) AEAKFAK-[*BM*]-DPSQSSELLSEAKKLSESQAPK (SEQ ID NO:1095); wherein [*BM*] is a TSLP binding motif as defined herein; and

x) an amino acid sequence which has at least 86 % identity to a sequence defined by ix).

In a further embodiment, sequence ix) is selected from the group consisting of SEQ ID NO:637 and SEQ ID NO:638.

In another further embodiment, the TSLP binding polypeptide comprises an amino acid sequence selected from:

xi) AEAKYAK-[*BM*]-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1093); wherein [*BM*] is a TSLP binding motif as defined herein; and

xii) an amino acid sequence which has at least 86 % identity to a sequence defined by xi).

In a further embodiment, sequence xi) is selected from the group consisting of SEQ ID NO:645 and 876-972.

In one embodiment, the TSLP binding polypeptide of the first aspect binds to TSLP such that the K_D value of the interaction is at most 1 x 10^{-6} M, for example at most 5 x 10^{-7} M, for example at most 1 x 10^{-7} M, for example at most 5 x 10^{-8} M, for example at most 1 x 10^{-8} M.

The terms “TSLP binding” and ”binding affinity for TSLP” as used in this specification refer to a property of a polypeptide which may be tested for example by ELISA, by surface plasmon resonance (SPR) technology, by quartz crystal microbalance (QCM) technology and/or by Kinetic Exclusion Assay (KinExA®).

For example as described in the experimental section below, TSLP binding affinity may be tested in an experiment in which samples of the polypeptide are captured on antibody-coated ELISA plates and biotinylated TSLP 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 Victor^3 (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 TSLP. If a quantitative measure is desired, for example to determine the EC50 value (the half maximal effective concentration) for the interaction, ELISA may also be used. The response of the polypeptide against a dilution series of biotinylated TSLP is measured using ELISA as described above. The skilled person may then interpret the results obtained by such experiments, and EC50 values may be calculated from the results using for example GraphPad Prism 5 and non-linear regression.

TSLP binding affinity may also be tested in an experiment in which TSLP, 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 TSLP, or a fragment thereof, is passed over the chip. 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 TSLP. 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

(Cytiva) or ProteOn XPR 36 (Bio-Rad) instrument. TSLP 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 BIAevaluation 4.1 software, or other suitable software, provided by the instrument manufacturer.

TSLP binding affinity may also be tested using a continuous-flow system for automated analysis based on the Quartz Crystal Microbalance (QCM) technology. To monitor binding interactions, one of the interacting molecules, or fragment thereof, is immobilized on the sensor surface and the sample containing the other one is injected over the sensor surface. Binding data is measured in real-time. The signal output is given in frequency (Hz) and is directly related to changes in mass on the sensor surface. If a quantitative measure is desired, for example to determine a K_D value for the interaction, QCM methods may also be used. Binding values may for example be defined in an Attana A200® (Attana) instrument. For example, TSLP may be 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. Data is collected by Attester Software and K_D values may then be calculated from the results using for example a 1:1 Langmuir binding model and subsequently processed in the Evaluation Software, or other suitable software, provided by the instrument manufacturer.

Another method for determining binding affinity for TSLP is the Kinetic Exclusion Assay (KinExA®; Sapidyne Instruments Inc; Darling and Brault, 2004, Assay and Drug Dev Tech 2(6):647-657) for measurements of the equilibrium binding affinity and kinetics between unmodified molecules in solution. For affinity analysis, the equilibrium dissociation constant, K_D, and the rate of association, k_a, are experimentally determined, while the rate of dissociation, k_d, may be calculated based on the equation $k_d = K_D * k_a$.

A KinExA® K_D analysis requires immobilization of one interaction partner (e.g. the titrated binding partner) to a solid phase, which is then used as a probe to capture the other interaction partner (e.g. the constant binding partner) free in solution once an equilibrium is reached. For each experiment, a series of solutions with a constant concentration of one binding partner and a titration of the other binding partner are equilibrated. The solutions are then briefly exposed to the solid phase and a portion of free constant binding

partner is captured and labeled with a fluorescent secondary molecule. The short contact time with the solid phase is less than the time needed for dissociation of the pre-formed complex in solution, meaning that competition between the solution and the solid phase titrated binding partner is “kinetically excluded”. Since the solid phase is only used as a probe for the free constant binding partner in each sample, the solution equilibrium is not altered during measurements. A K_D value is calculated from signals generated from captured free constant binding partner, which are directly proportional to the concentration of free constant binding partner in the equilibrated sample. The data may be analyzed using the KinExA® Pro software and least squares analysis to fit the optimal solutions for the K_D and the Active Binding site Concentration (ABC) to a curve representative of a stoichiometric relevant model, for instance a 1:1 reversible bi-molecular interaction.

Determination of binding kinetics may be done in a similar format as the equilibrium analysis, except measurements are collected “pre-equilibrium” and the binding signals are a function of time and total concentration of the titrated binding partner. There are two methods that can be used to determine the k_a. The “direct method” holds the concentrations of titrated and constant binding partners fixed, and the solution is probed over time. The amount of the free constant binding partner in the solution will decrease as the sample moves toward equilibrium. The “inject method” holds incubation time and one partner’s concentration fixed, while titrating concentrations of the other partner. As the concentration of the titrated binding partner increases, the amount of free constant binding partner will decrease as more complexes are formed.

The terms “albumin binding” and “binding affinity for albumin” as used in this disclosure refer to a property of a polypeptide which may be tested for example by SPR, by QCM or by KinExA®, in an analogous way to the examples described above for TSLP.

The skilled person will understand that various modifications and/or additions can be made to a TSLP 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 a TSLP 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, the TSLP 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 and/or simplify 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 His_6 tag, a $(HisGlu)_3$ tag (“HEHEHE” tag) or a ”myc” (c-myc) tag or a ”FLAG” tag for interaction with antibodies specific to the tag or immobilized metal affinity chromatography (IMAC) in the case of a His_6-tag.

In one embodiment, there is provided a TSLP binding polypeptide as described herein which comprises additional amino acids at the C-terminal and/or N-terminal end. For example, in one embodiment of the TSLP binding polypeptide as disclosed herein, it consists of any one of the sequences disclosed herein, having from 0 to 15 additional C-terminal and/or N-terminal residues, such as from 0 to 7 additional C-terminal and/or N-terminal residues. In one embodiment, the TSLP binding polypeptide consists of any one of the sequences disclosed herein, having from 0 to 15, such as from 0 to 4, such as 3 additional C-terminal residues.

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

A further polypeptide domain may moreover provide another TSLP binding moiety. Thus, in a further embodiment, there is provided a TSLP binding polypeptide in a multimeric form. Said multimer is understood to comprise at least two TSLP 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 a TSLP 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 TSLP binding polypeptide of the invention may form homo- or heteromultimers, for example homo- or heterodimers. In one embodiment, there is provided a TSLP binding polypeptide, wherein said monomer units are covalently coupled together. In another embodiment, said TSLP binding polypeptide monomer units are expressed as a fusion protein. In one embodiment, there is provided TSLP binding polypeptide in dimeric form. In one particular embodiment, said dimeric form is a homodimeric form. In another embodiment, said dimeric form is a heterodimeric form.

For the sake of clarity, throughout this disclosure, the term “TSLP binding polypeptide” is used to encompass TSLP binding polypeptides in all forms, i.e. monomeric and multimeric forms.

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

Furthermore, it may be beneficial that the TSLP binding polypeptide as defined herein is part of a fusion protein or a conjugate comprising a second or further moieties. Second and further moiety/moieties of the fusion polypeptide or conjugate in such a protein may suitably have a desired biological activity.

Thus, in a second aspect of the present disclosure, there is provided a fusion protein or conjugate comprising

- a first moiety consisting of a TSLP binding polypeptide according to the first aspect; and
- a second moiety consisting of a polypeptide having a desired biological activity.

In one embodiment, said fusion protein or conjugate may additionally comprise further moieties, comprising desired biological activities that can be either the same as or different from the biological activity of the second moiety.

Non-limiting examples of a desired biological activity comprise a therapeutic activity, a binding activity and an enzymatic activity. In one embodiment, the second moiety having a desired biological activity is a therapeutically active polypeptide. In one embodiment, said second moiety is an immune response modifying agent. In another embodiment, said second moiety is an anti-cancer agent.

In one embodiment of the first or second aspect of the disclosure, there is provided a TSLP binding polypeptide, fusion protein or conjugate which comprises an immune response modifying agent. Non-limiting examples of additional immune response modifying agents include immunomodulating agents or other anti-inflammatory agents.

Non-limiting examples of therapeutically active polypeptides are biomolecules, such as molecules selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

Non-limiting examples of binding activities are binding activities which increase the *in vivo* half-life of the fusion protein or conjugate, and binding activities which act to e.g. block, inhibit, activate, increase, antagonize or agonize a biological activity. One example of such a binding activity is a binding activity, which increases the *in vivo* half-life of a fusion protein or conjugate. In one embodiment of said fusion protein or conjugate, the *in vivo* half-life of said fusion protein or conjugate is longer than the *in vivo* half-life of the TSLP binding polypeptide *per se*. In one embodiment, said *in vivo* half-life is increased at least 10 times, such as at least 25 times, such as at least 50 times, such as at least 75 times, such as at least 100 times, compared the *in vivo* half-life of the TSLP binding polypeptide *per se*.

As discussed, the fusion protein or conjugate may comprise at least one further moiety with a binding activity towards a target. In one particular embodiment, said target is albumin, binding to which increases the *in vivo* half-life of said fusion protein or conjugate. In one embodiment, said albumin binding activity is provided by an albumin binding domain (ABD) of streptococcal protein G, or a derivative thereof. Thus, said fusion protein may for example comprise a TSLP binding polypeptide in monomeric or multimeric form (such as a homodimeric or heterodimeric form) as defined herein and an albumin binding domain of streptococcal protein G or a derivative thereof. Derivatives of the albumin binding domain of streptococcal protein G are known to persons of skill in the art, for example from WO2009/016043,

WO2012/004384 and WO2014/048977, all hereby incorporated by reference. It will be appreciated that said albumin binding domain (ABD) may be positioned at the C-terminal end of the TSLP binding polypeptide and/or at the N-terminal end of the TSLP binding polypeptide. Suitably, having the ABD at N-terminal end of the TSLP binding polypeptide reduces or prevents oligomerization.

In another embodiment, there is provided a fusion protein or a conjugate wherein said second moiety having a desired binding activity is a protein based on protein Z, derived from the B domain of protein A from *Staphylococcus aureus*, which has a binding affinity for a target other than TSLP.

In another embodiment, there is provided a fusion protein or a conjugate wherein said second moiety having a desired binding activity is an antibody or antigen binding fragment thereof. As is well known, antibodies are immunoglobulin molecules capable of specific binding to a target (an antigen), such as a carbohydrate, polynucleotide, lipid, polypeptide or other, through at least one antigen recognition site on the immunoglobulin molecule. As used herein, the term “antibody or an antigen binding fragment thereof” encompasses not only full-length or intact polyclonal or monoclonal antibodies, but also antigen-binding fragments thereof, such as Fab, Fab′, $F(ab')_2$, Fab_3, Fv and variants thereof, fusion proteins comprising one or more antibody portions, humanized antibodies, chimeric antibodies, minibodies, diabodies, triabodies, tetrabodies, linear antibodies, single chain antibodies, multispecific antibodies (e.g. bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies and covalently modified antibodies. Further examples of modified antibodies and antigen binding fragments thereof include nanobodies, AlbudAbs, DARTs (dual affinity re-targeting), BiTEs (bispecific T-cell engager), TandAbs (tandem diabodies), DAFs (dual acting Fab), two-in-one antibodies, SMIPs (small modular immunopharmaceuticals), FynomAbs (fynomers fused to antibodies), DVD-Igs (dual variable domain immunoglobulin), CovX-bodies (peptide modified antibodies), duobodies and triomAbs. This listing of variants of antibodies and antigen binding fragments thereof is not to be seen as limiting, and the skilled person is aware of other suitable variants.

In one embodiment, said at least one antibody or antigen binding fragment thereof is selected from the group consisting of full-length antibodies, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, single chain Fab (scFab) fragments, Fc fragments, Fv fragments, single chain Fv (scFv) fragments, $(scFv)_2$, scFv-Fc constructs and domain antibodies. In one embodiment, said at least one antibody or antigen binding fragment thereof is selected from full-length antibodies, Fab fragments and scFv fragments. In one particular embodiment, said at least one antibody or antigen binding fragment thereof is a full-length antibody.

In one embodiment, the antibody or antigen binding fragment thereof is selected from the group consisting of monoclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, and antigen-binding fragments thereof.

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, 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 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 said TSLP binding polypeptide and a further polypeptide domain, such as between a TSLP binding domain as disclosed herein and an antibody or antigen binding fragment thereof. 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. Additional non-limiting examples of linkers include ASGS (SEQ ID NO:1102), GAPGGGGS (SEQ ID NO:1103), GGGGSLVPRGSGGGGS (SEQ ID NO:1104), $(GS)_3$ (SEQ ID NO:1105), $(GS)_4$ (SEQ ID NO:1106), $(GS)_8$ (SEQ ID NO:1107), GGSGGHMGSGG (SEQ ID NO:1108), GGSGGSGGSGG (SEQ ID NO:1109), GGSGG (SEQ ID NO:1110), GGSGGGGG (SEQ ID NO:1111), GGGSEGGGSEGGGSEGGG (SEQ ID NO:1112), AAGAATAA (SEQ ID NO:1113), GGGGG (SEQ ID NO:1114), GGSSG (SEQ ID NO:1115), GSGGGTGGGSG (SEQ ID NO:1116), GSGSGSGSGGSG (SEQ ID NO:1117), GSGGSGGSGGSGGS (SEQ ID NO:1118) and GSGGSGSGGSGGSG (SEQ ID NO:1119), respectively, and GT. The skilled person is aware of other suitable linkers.

In one embodiment, said linker is a flexible linker comprising glycine (G), serine (S) and/or threonine (T) residues. In one embodiment, said linker has a general formula selected from $(G_nS_m)_p$ and $(S_nG_m)_p$, wherein, independently, n = 1-7, m = 0-7, n + m ≤ 8 and p = 1-7. In one embodiment, n = 1-5. In one embodiment, m = 0-5. In one embodiment, p = 1-5. In a more specific embodiment, n = 4, m = 1 and p = 1-4. In one embodiment, said linker is selected from the group consisting of S_4G (SEQ ID NO:1120), $(S_4G)_3$ (SEQ ID NO:1121) and $(S_4G)_4$ (SEQ ID NO:1122). In one embodiment, said linker is selected from the group consisting of G_4S (SEQ ID NO:1123) and $(G_4S)_3$ (SEQ ID NO:1124). In one particular embodiment, said linker is G_4S and in another embodiment said linker is $(G_4S)_3$.

With regard to the description above of fusion proteins or conjugates incorporating a TSLP 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 TSLP binding polypeptide or polypeptides according to the invention on the one hand, and moieties exhibiting the same or 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 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 above aspects furthermore encompass polypeptides in which the TSLP binding polypeptide according to the first aspect, or the TSLP binding polypeptide as comprised in a fusion protein or conjugate according to the second aspect, further comprises a label, such as a label selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds, bioluminescent proteins, enzymes, radionuclides and radioactive particles. Such labels, which a person of skill in the art will be familiar with, may for example be used for detection of the polypeptide.

In some embodiments, the labeled TSLP binding polypeptide is present as a moiety in a fusion protein or conjugate also comprising a second or further moiety having a desired biological activity. The label may in some instances be coupled only to the TSLP binding polypeptide, and in some instances both to the TSLP binding polypeptide and to the second moiety of the fusion protein or conjugate. Furthermore, it is also possible that the label may be coupled to a second moiety and not to the TSLP binding moiety. Hence, in yet another embodiment, there is provided a TSLP binding polypeptide comprising a second moiety, wherein said label is coupled to the second moiety only. Thus, when reference is made to a labeled polypeptide, this should be understood as a reference to all aspects of polypeptides as described herein, including TSLP binding polypeptides, fusion proteins and conjugates comprising a TSLP binding polypeptide.

In further aspects of the present disclosure, there is provided a polynucleotide encoding a TSLP binding polypeptide, fusion protein or conjugate 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 TSLP binding 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 TSLP binding polypeptide or fusion protein 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 or fusion protein as described herein having protected reactive side-chains,

- removal of the protecting groups from the reactive side-chains of the polypeptide or fusion protein, and

- folding of the polypeptide in aqueous solution.

A conjugate as disclosed herein may be produced by the conjugation of at least one TSLP binding polypeptide or fusion protein as described herein to at least one additional moiety. The skilled person is aware of conjugation methods, such as conventional chemical conjugation methods, for example using charged succinimidyl esters or carbodiimides.

It should be understood that the TSLP binding polypeptide according to the present disclosure is typically useful as a therapeutic, diagnostic and/or prognostic agent in its own right. A therapeutic effect may for example be accomplished by antagonizing TSLP action.

Thus, in another aspect, there is provided a composition comprising a TSLP 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 agent, such as at least two additional active agents, such as at least three additional active agents.

The small size and robustness of the TSLP binding polypeptides of the present disclosure confer several advantages over conventional monoclonal antibody-based therapies. Such advantages include advantages in formulation, modes of administration, such as alternative routes of administration, administration at higher molar doses than antibodies and absence of Fc-mediated side effects. In particular when treating acute inflammatory conditions, the short plasma half-life of the polypeptides described herein may be advantageous over monoclonal antibodies which have a long residence time. The agents of the present disclosure are contemplated for oral, respiratory (including inhalation and insufflation), topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual or suppository administration, such as in particular for respiratory, intravenous or subcutaneous administration.

In another aspect of the present disclosure, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition as described herein for use as a medicament, a prognostic agent and/or a diagnostic agent. In one embodiment, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition for use in the treatment, diagnosis or prognosis of a TSLP related disorder or disease, such as an inflammatory disease, an autoimmune disease or a cancer disease.

In one embodiment, said use in diagnosis is carried out *in vivo*. In another embodiment, said use in diagnosis is carried out *in vitro*. In one embodiment, said use in prognosis is carried out *in vivo*. In another embodiment, said use in prognosis is carried out *in vitro*.

In one embodiment, said TSLP binding polypeptide, fusion protein, conjugate or composition is provided for use as a medicament. In a more specific embodiment, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a medicament to modulate TSLP function, such as to modulate TSLP function *in vivo.* As used herein, the term “modulate” refers to changing the activity, such as partially inhibiting or fully inhibiting TSLP function.

In one embodiment, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition for use in the treatment of a TSLP related disorder.

In one embodiment, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition for use in the diagnosis of a TSLP related disorder. In a more specific embodiment, said use in diagnosis is carried out *in vivo*. In another specific embodiment, said use in diagnosis is carried out *in vitro*.

In one embodiment, there is provided a TSLP binding polypeptide, fusion protein, conjugate or composition for use in the prognosis of a TSLP related disorder. In a more specific embodiment, said use in prognosis is carried out *in vivo*. In another specific embodiment, said use in prognosis is carried out *in vitro*.

As used herein, the term “TSLP related disorder or disease” refers to any disorder, disease or condition in which TSLP action plays a role and/or wherein modulation (e.g. inhibition) of TSLP may be beneficial. Such a disorder, disease or condition may be selected from the group consisting of

respiratory diseases, dermatological diseases, allergies, eye diseases, gastrointestinal diseases and cancers.

Non-limiting examples include asthma, atopic dermatitis, atopic keratoconjunctivitis, urticaria, allergic rhinitis, chronic rhinosinusitis with nasal polyps, eosinophilic esophagitis, chronic obstructive pulmonary disease, eosinophilic granulomatosis with polyangiitis (EGPA)/Churg–Strauss syndrome, breast cancer, prurigo nodularis, and bullous pemphigoid.

It is to be understood that said TSLP binding polypeptide, fusion protein, conjugate or composition may be used as the sole diagnostic or prognostic agent or as a companion diagnostic and/or prognostic agent.

In a related aspect, there is provided a method of treatment of a TSLP related disorder, comprising administering to a subject in need thereof an effective amount of a TSLP binding polypeptide, fusion protein, conjugate or composition as described herein. In a more specific embodiment of said method, the TSLP binding polypeptide, fusion protein, conjugate or composition as described herein modulates TSLP function *in vivo*. The skilled person will appreciate that any description in relation to the use of TSLP binding polypeptide, fusion protein, conjugate or composition as described herein for treatment of a disease or disorder is equally relevant for the related therapeutic method. For the sake of brevity, such description will not be repeated here.

In another aspect of the present disclosure, there is provided an *in vitro* method of detecting TSLP, comprising providing a sample suspected to contain TSLP, contacting said sample with a TSLP binding polypeptide, fusion protein, conjugate or composition as described herein, and detecting the binding of the TSLP binding polypeptide, fusion protein, conjugate or composition to indicate the presence of TSLP 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.

In another aspect of the present disclosure, there is provided a diagnostic or prognostic method for determining the presence of TSLP in a subject, the method comprising the steps:

a) contacting the subject, or a sample isolated from the subject, with a TSLP binding polypeptide, fusion protein, conjugate or composition as described herein; and

b) obtaining a value corresponding to the amount of the TSLP binding polypeptide, fusion protein, conjugate or composition that has bound in said subject or to said 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 subject or sample and before obtaining a value.

In one embodiment, said method further comprises a step of comparing said value to a reference. Said reference may be by a numerical value, a threshold or a visual indicator, for example based on a color reaction. The skilled person will appreciate that different ways of comparison to a reference are known in the art and may be suitable for use.

In one embodiment of such a method, said subject is a mammalian subject, such as a human subject. In one embodiment, said method is performed *in vivo*. In another embodiment, said method is performed *in vitro*.

In certain embodiments of the various medical uses and methods of treatment, diagnosis and prognosis disclosed herein, said TSLP related disorder is selected from the group consisting of inflammatory diseases, autoimmune diseases and cancer diseases. Thus, said TSLP related disorder may be selected from respiratory diseases, dermatological diseases, allergies, eye diseases, gastrointestinal tract diseases and cancers.

Examples of specific indications within these categories, which are also embodiments of TSLP related disorders within the context of the present disclosure for which a polypeptide with affinity for TSLP is considered to have therapeutic, diagnostic or prognostic relevance, are asthma, atopic dermatitis, atopic keratoconjunctivitis, urticaria, allergic rhinitis, chronic rhinosinusitis with nasal polyps, eosinophilic esophagitis, chronic obstructive pulmonary disease (COPD), eosinophilic granulomatosis with polyangiitis (EGPA)/Churg–Strauss syndrome, breast cancer, prurigo nodularis, and bullous pemphigoid.

In another embodiment of any one of the foregoing therapeutic aspects of the disclosure, the TSLP related disorder is selected from the group consisting of cryoglobulinemia, pulmonary fibrosis, idiopathic pulmonary fibrosis, Netherton syndrome, atherosclerosis, systemic sclerosis, lichen planus, pancreatic cancer, Behçet's disease, eczema herpeticum, eosinophilic gastroenteritis, multiple sclerosis, thrombosis induced by Kawasaki disease, IgG4-related disease and endometriosis.

More information concerning TSLP and its role as target for therapeutic intervention, or as biomarker for diagnosis and/or prognosis, is found in the reviews by Tsilingiri *et al.*, Varricchi *et al.*, Gauvreau *et al.* and Markovic and Savvides, all referred to above in the Background section.

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.

Brief description of the figures

Figure 1 shows examples of circular dichroism (CD) spectra collected as described in Example 3 for N- or C-terminally His-tagged Z variants in two different scaffolds. Melting curve of (A) ZTP631 (SEQ ID NO:631) and (C) ZTP637 (SEQ ID NO:637); CD spectra collected before (black line) and after (grey line) heat-induced denaturation of (B) ZTP631 and (D) ZTP637.

Figure 2 shows an example of the sensorgrams obtained for the His_6-tagged Z variant ZTP631 (SEQ ID NO:631) when analyzed for the interaction to immobilized human TSLP measured by Biacore, as described in Example 3. ZTP631 was injected at concentrations of 11 nM (solid line), 33 nM (dashed line), and 100 nM (dotted line).

Figure 3 shows concentration dependent inhibition of TSLP-induced TARC production in human PBMC by the indicated Z variants, as described in Example 4.

Figure 4 shows examples of circular dichroism (CD) spectra collected as described in Example 7 for matured C-terminally His-tagged Z variants in two different scaffolds. Melting curve of (A) ZTP876 (SEQ ID NO:876) and (C) ZTP967 (SEQ ID NO:967); CD spectra collected before (black line) and after (grey line) heat-induced denaturation of (B) ZTP876 and (D) ZTP967.

Figure 5 shows three examples of sensorgrams obtained for matured Z-ABD variants when analyzed for the interaction to immobilized TSLP measured by Biacore, as described in Example 7. ZTPA028 (SEQ ID

NO:1000) (solid line), ZTPA029 (SEQ ID NO:1001) (dashed line), and ZTPA030 (SEQ ID NO:1002) (dotted line), respectively, were injected in an SCK experiment at successive concentrations of 3.3, 10, 30, and 90 nM.

Figure 6 shows examples of sensorgrams obtained in a Biacore kinetic analysis against TSLP from different species, demonstrating binding to cynomolgus TSLP, but not to rat or mouse TSLP. TSLP at concentrations 4 nM (solid line), 16 nM (dashed line), and 64 nM (dotted line) were injected over HSA captured ZTPA001 (SEQ ID NO:973) in fusion with ABD.

Figure 7 shows the result of a dot blot performed with TSLP binding Z variants ZTPA104 (SEQ ID NO:1076; panel A) and ZTPA001 (SEQ ID NO:973; panel B), as well as with an IL-17A binding control polypeptide (panel C). Z variants were incubated with a membrane on which highly abundant plasma proteins; HSA (1), IgG (2), alpha-2-macroglobulin (3), alpha 1-antitrypsin (4), complement component 3c (C3c) (5), haptoglobulin (6), alpha 1-antichymotrypsin (7), C4 complement (8), IgE (9), hemopexin (10), transthyretin (11), Fc fragment from IgG (12), holo-transferrin (13), fibrinogen (14), IgA (15), IgM (16); structurally related IL-2 family of cytokines; IL-2 (17), IL-4 (19), IL-7 (21), IL-9 (22), IL-15 (23), and IL-21 (25); unrelated cytokines; IL-3 (18), IL-5 (20), and IL-17A (24); and TSLP proteins; sfTSLP (26), full-length TSLP uncleaved (27), full-length TSLP cleaved (28) were applied, all of human origin. Development of the membrane using an anti-Z polyclonal antibody resulted in dots for positive binding events. Panel D shows the result of ZTPA104 pre-incubated with 10× excess of HSA before use in a dot blot with a subset of the proteins in panel A–C: HSA (1), IgG (2), complement component 3c (C3c) (5), alpha 1-antichymotrypsin (7), C4 complement (8), IgE (9), Fc fragment from IgG (12), holo-transferrin (13), fibrinogen (14), IgA (15), IgM (16), sfTSLP (26), full-length TSLP uncleaved (27), full-length TSLP cleaved (28).

Figure 8 shows a diagram of exemplary results from the PathHunter® eXpress IL7R/CRLF2 cell assay described in Example 8, from analysis of ZTPA002 (SEQ ID NO:974; light grey circles), ZTPA001 (SEQ ID NO:973; dark grey triangles) and ZTPA093 (SEQ ID NO:1065; black diamonds). The results are presented as inhibitory profile curves with relative light units (RLU) as measured by an EnSpire multimode reader on the y-axis and inhibitor concentration on the x-axis.

Figure 9 shows the serum concentration versus time profiles in individual cynomolgus monkeys administered i.v. with ZTPA001 (SEQ ID NO:973) and analyzed as described in Examples 9 (A) and 10 (B).

Figure 10 shows production of cytokines and chemokines in TSLP-induced cynomolgus model described in Example 10. Levels of IL-2 (A), IL-5 (B), TARC (C) and MDC (D) in serum are shown at 4 and 8 h after intravenous injection of 62.5 µg/kg TSLP in the absence (left side of the graph) or presence (right side of the graph) of ZTPA001 (SEQ ID NO:973) injected 2 h prior to injection of TSLP. Cytokine/chemokine serum levels are represented in pg/mL; LLOQ=lower limit of quantification.

Figure 11 shows the mean serum concentration versus time profiles of ZTPA001 (SEQ ID NO:973; closed circles) and ZTPA104 (SEQ ID NO:1076; closed squares) in rats. A mean value was included in the graph only if all values (n=3) at a given timepoint were above the LLOQ.

Figure 12 shows concentration of ZTPA001 (SEQ ID NO:973) in (A) BAL fluid and (B) lung tissue homogenate in the male (square) and female (circle) cynomolgus monkey assessed after administration by inhalation. No difference was observed between left (L) or right (R) lung or between tissue sampling locations, i.e. proximal (prox) or distal.

Figure 13 shows ZTPA001 (SEQ ID NO:973) plasma concentration versus time profiles in individual cynomolgus monkeys, assessed after administration by inhalation.

Figure 14 shows the TSLP binding capacity of ZTPA001 (SEQ ID NO:973) in plasma samples from cynomolgus monkeys, assessed after administration by inhalation. Data of two analyses is displayed and depicted as mean ± standard deviation of duplicates.

Examples

Summary

The following Examples disclose the development of novel Z variant molecules targeting the human thymic stromal lymphopoietin (TSLP), based on phage display technology. The polypeptides selected as described herein were sequenced, and their amino acid sequences are listed in the sequence listing with the sequence identifiers SEQ ID NO:1-875. The Examples further describe the characterization of these selected TSLP binding polypeptides,

variants and derivatives thereof, as well as fusions proteins comprising them, and demonstrate their *in vitro* and *in vivo* functionality.

Example 1

Selection and screening of TSLP binding Z variants

Summary

In this Example, recombinantly produced human TSLP was used as target in phage display selections using two different phage libraries of Z variants. Selected clones were DNA sequenced, produced in *E. coli* and assayed against TSLP in ELISA (enzyme-linked immunosorbent assay).

Materials and methods

Biotinylation of target protein: Recombinantly produced human TSLP (R&D Systems, cat. no.1398-TS/CF; SEQ ID NO:1079) was biotinylated using No-Weigh EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) at a 10× molar excess, according to the manufacturer's recommendations. The reaction was performed at 4°C for 2.5 h followed by incubation at room temperature (RT) for 1 h. The biotinylation was performed in a dialysis cassette that directly, following the biotinylation, was used for buffer exchange to phosphate buffered saline (PBS: Dulbecco's Gibco DPBS; 9.6 mM phosphate, 138 mM NaCl, 2.67 mM KCl, pH 7.4).

Phage display selection of TSLP binding Z variants: Two different libraries of random variants of protein Z displayed on bacteriophage, constructed in phagemid pAY02592 or pAY04242, respectively, essentially as described in Grönwall *et al.*, 2007 J Biotechnol 128:162-183, were used to select TSLP binding polypeptides. In these libraries, an albumin binding domain (ABD, domain GA3 of protein G from *Streptococcus* strain G148; SEQ ID NO:1078) is used as fusion partner to the Z variants. The libraries are denoted Zlib006Naive.II and Zlib008Naive.II, respectively, and have a size of 1.5×10^{10} and 2.9×10^{10} library members (Z variants).

Production of phage stock from phagemid library Zlib006Naive.II was essentially performed as described earlier (see e.g. PCT publication WO2017/072280) using *E. coli* RRIΔM15 cells (Rüther *et al.*,1982 Nucleic Acids Res 10:5765-5772) from a glycerol stock of the library.

Production of phage stock from phagemid library "Naive.II was performed as follows. *E. coli* XL-1 Blue cells (Agilent technologies, cat. no. 200268) from a glycerol stock containing the phagemid library Zlib008Naive.II

were inoculated in 20 L of TSB+YE medium [30 g/L tryptic soy broth; 5.0 g/L yeast extract] supplemented with 11 g/L glucose monohydrate, 100 µg/mL carbenicillin, and 10 mg/mL tetracycline hydrochloride. The culture was grown at 37°C in a fermenter (Belach Bioteknik, BR20). When the cells reached an optical density at 600 nm (OD_{600}) of 0.62, approximately 4 L of the cultivation were infected using a 10 × molar excess of M13K07 helper phage (New England Biolabs, cat. no. N0315S). The cells were incubated for 30 min, whereupon the fermenter was filled up to 20 L with cultivation medium (3.05 g/L $(NH_4)_2SO_4$; 6.1 g/L yeast extract; 3.65 g/L K_2HPO_4; 5.45 g/L KH_2PO_4; 2.29 g/L $Na_3C_6H_5O_7 \cdot 2\ H_2O$; 0.1 mL/L Breox FMT30 antifoaming agent); 16 mL of a trace element solution [129 mM $FeCl_3$; 36.7 mM $ZnSO_4$; 10.6 mM $CuSO_4$; 78.1 mM $MnSO_4$; 94.1 mM $CaCl_2$, dissolved in 1.2 M HCl]; 10.4 mL of a vitamin solution [500 mg/L DL-pantothenic acid, calcium salt; 500 mg/L choline chloride; 500 mg/L folic acid; 1000 mg/L myo-inositol; 500 mg/L niacinamide; 50 mg/L riboflavin; 500 mg/L thiamine hydrochloride]; 36.8 ml of 30 g/L thiamine hydrochloride; 53 mL of 1.217 M $MgSO_4$; 16 mL of 100 mg/mL carbenicillin; 14.4 mL of 25% NH_4OH. The culture was incubated for 60 min. 20 mL 50 mg/mL kanamycin and 2 mL 1 M isopropyl-β-D-1-thiogalactopyranoside (IPTG) were added. The temperature was lowered to 30°C. A glucose-limited fed-batch cultivation was started where a 600 g/L glucose solution was fed to the reactor (15 g/h in the start, with a linear increase to 75 g/h after 20 h, and then constant feed until harvest). pH was controlled at 7 through the automatic addition of 25% NH_4OH, air was supplemented (10 L/min), and the stirrer was set to keep the dissolved oxygen level above 30%. The culture was harvested 23 h after induction with IPTG. The cells in the cultivation were removed by centrifugation.

For both libraries, the phage particles were precipitated from the supernatant twice using polyethylene glycol/sodium chloride (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.

Selection against biotinylated TSLP was performed in solution in tracks 1, 3, 4, and 6 or using a solid phase of streptavidin beads (SA beads, Dynabeads M-280 Streptavidin, Thermo Fisher) in tracks 2, 5, and 7. In tracks 1, 2, 4, and 5 selection was carried out at 37°C and in tracks 3, 6, and 7 at RT. As selection proceeded, the tracks were further divided according to target concentration and number and/or time of washes.

In order to reduce the amount of background binders, pre-selection was performed in cycle 1–3 using SA beads and in cycle four using neutravidin beads (NA beads, Sera-Mag SpeedBeads Neutravidin-Coated Magnetic Particles, Cytiva). During pre-selection, the phage stock was incubated with coated beads end-over-end for 30–60 min at RT. Beads used in the pre-selections or selection were pre-blocked with PBS supplemented with 3% bovine serum albumin (BSA, Sigma) and 0.1% Tween20 (PBSTB) and all tubes used were of non-stick type (AM12450 Nonstick RNase-free Microfuge Tubes, Ambion).

Selection was performed in solution in PBSTB supplemented with 1.5 µM human serum albumin (HSA, Recombumin, Novozyme) (PBSTBH) at RT or 37°C. The time for selection was between 60–120 min followed by catch of target-phage particle complexes on beads alternating between SA beads or NA beads. For solid-phase selection, the biotinylated TSLP was already immobilized on SA beads prior to the selection. Finally, the different beads with target-phage particle complexes were washed with PBS supplemented with 0.1% Tween-20 (PBST0.1%).

In addition to the above, some tracks in cycle three were also performed where selection was made in solution followed by catch of target-phage particle complexes in wells of 96-well high binding polystyrene plates (Corning 3690) coated with neutravidin protein (NA Pierce, cat. no. 31000) diluted to 5 µg/mL in carbonate buffer (Sigma). These plates were subsequently rigorously washed with PBST0.1%.

In the final selection cycle, log phase bacteria were infected with eluate and diluted before being spread onto TBAB plates (30 g/L tryptose blood agar base, Oxoid) supplemented with 0.2 g/L ampicillin in order to form single colonies to be used in ELISA screening.

An overview of the selection strategy, describing an increased stringency in successive cycles with a lowered target concentration and an increased number of washes, is shown in Table 2. Unless noted otherwise, washes were performed for 1 min using PBST. Elution was carried out as described in WO2009/077175.

Table 2: Overview of the selection against TSLP using two primary libraries

Cycle	Selection track	Phage stock from library or selection track(s)	Target conc. (nM)	Selection in solution (S) or type of solid phase: NA beads (B), SA in polystyrene plate (P)	Number of washes	Temperature during selection and wash
1	1	Zlib008Naive.II	100	S	2	37°C
1	2	Zlib008Naive.II	100	B	2	37°C
1	3	Zlib008Naive.II	100	S	2	RT
1	4	Zlib006Naive.II	100	S	2	37°C
1	5	Zlib006Naive.II	100	B	2	37°C
1	6	Zlib006Naive.II	100	S	2	RT
1	7	Zlib006Naive.II	100	B	2	RT
2	8	1, 2	50	S	4	37°C
2	9	1, 2	50	B	4	37°C
2	10	1, 2	20	S	6	37°C
2	11	3	50	S	4	RT
2	12	3	50	B	4	RT
2	13	3	20	S	6	RT
2	14	4, 5	50	S	4	37°C
2	15	4, 5	50	B	4	37°C
2	16	4, 5	20	S	6	37°C
2	17	6, 7	50	S	4	RT
2	18	6, 7	50	B	4	RT
2	19	6, 7	20	S	6	RT
3	20	8, 9, 10	20	S	6	37°C
3	21	8, 9, 10	5	S	8	37°C
3	22	11, 12, 13	20	S	6	RT
3	23	11, 12, 13	5	S	8	RT
3	24	9, 12	10	S, P	10	37°C
3	25	9, 12	10	S, P	20	37°C
3	26	9, 12	10	S, P	40	37°C
3	27	14, 15, 16	20	S	6	37°C
3	28	14, 15, 16	5	S	8	37°C
3	29	17, 18, 19	20	S	6	RT
3	30	17, 18, 19	5	S	8	RT
3	31	15, 18	10	S, P	10	37°C
3	32	15, 18	10	S, P	20	37°C
3	33	15, 18	10	S, P	40	37°C
4	34	20	5	S	8	37°C
4	35	20	1	S	10	37°C
4	36	21	1	S	10	37°C
4	37	21	0.1	S	12	37°C
4	38	22	5	S	8	RT
4	39	23	1	S	10	RT
4	40	27	5	S	8	37°C
4	41	27	1	S	10	37°C
4	42	28	1	S	10	37°C
4	43	28	0.1	S	12	37°C
4	44	29	5	S	8	RT
4	45	30	1	S	10	RT

Amplification and preparation of phage particles: Amplification of phage particles between the different selection cycles was performed as follows. *E. coli* strain XL1-Blue (Agilent technologies, cat. no. 200268) was used for phage amplification and M13K07 helper phage was used in 100 × excess in the first cycle and 50 × in the following cycles. XL-1 Blue was cultivated in TSB medium (Tryptic Soy Broth, 30 g/L) supplemented with 1% glucose and 1 µg/ml tetracycline at 37°C to early log phase and thereafter infected with phage particles. 10–100 × excess of bacteria was used compared to the phage particle amount. The infection was allowed for 30 min at 37°C after which the medium volume was doubled by addition of TSB medium supplemented with 1% glucose, 1 µg/mL tetracycline and 200 µg/mL of carbenicillin. After incubation for approximately 1 h at 37°C, helper phage were added and incubated for 1.5 h at 37°C. Superinfected bacteria were pelleted at 3500 × *g* and resuspended in 50 mL (100 mL after first cycle) TSB+YE medium supplemented with 25 µg/mL kanamycin and 0.1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) and incubated at 30°C overnight. The overnight cultures were pelleted and phage particles in the supernatant were precipitated twice with PEG/NaCl. Finally, the phage particles were re-suspended in selection buffer before entering the next selection cycle. In the last selection cycle, log phase bacteria were infected with eluate and diluted before being spread onto TBAB plates (30 g/L tryptose blood agar base, Oxoid) supplemented with 0.2 g/L ampicillin in order to form single colonies for use in ELISA screening.

Production of Z variants for ELISA: Z variants were produced by inoculating single colonies from the selections into 1.2 mL TSB-YE medium supplemented with 100 µg/mL ampicillin and 1 mM IPTG in deep-well plates (Nunc). The plates were incubated with rotation for 22–24 h at 37°C. Cells were pelleted by centrifugation, re-suspended in 150 µL PBST0.05% (PBS supplemented with 0.05%Tween20) sealed with aluminum foil and incubated at 82°C for 20 min in a water bath. Plates were quickly spun down to remove droplets from the foil and then the heat-treated suspension were subjected to a filtration step using a 96-well filter plate (Merck Millipore). The final filtrated supernatant (Heat Treated (HT) lysate), with the soluble part of the extract, contained the Z variants as fusions to ABD, expressed as AQHDEALE-[ZTP###]-VDYV-[ABD]-YVPG (SEQ ID NO:1125; Grönwall *et al. supra*). ZTP### refers to sequences of individual, 58 amino acid residue, TSLP binding Z variants.

Sequencing: All individually picked clones were subjected to a sequencing procedure. PCR fragments were amplified from single colonies, sequenced and analyzed essentially as described in WO2015/189430.

ELISA screening of Z variants against TSLP: The binding of Z variants to TSLP was analyzed in ELISA assays. Half-area 96-well ELISA plates (Corning) were coated at 4°C overnight with 50 µL of 2 µg/mL of an anti-ABD goat antibody (produced in-house) diluted in PBS. The antibody solution was poured off and the wells were blocked with 100 µL of Blocker® casein in PBS (Thermo Scientific) for 1 h at RT. The blocking solution was discarded and 50 µL HT lysate, diluted 1:8 in PBST0.05%, were added to the wells and incubated for 1 h at RT. As a negative control, HT lysate prepared using the ABD moiety alone was added. The supernatants were poured off and the wells were washed 4 × with PBST0.05%. Then, 50 µL of biotinylated TSLP at a concentration of 10 nM diluted in Blocker® casein in PBS were added to each well, respectively. The plates were incubated for 2 h at RT followed by washes as described above. Streptavidin conjugated HRP (Thermo Scientific), diluted 1:30,000 in Blocker® casein in PBS, was added to the wells and the plates were incubated for 45 min. After washing as described above, 50 µL TMB substrate (1-Step Ultra TMB-ELISA Pierce ThermoFisher Scientific) was added to the wells and the plates were treated according to the manufacturer's recommendations. The absorbance at 450 nm was measured using a multi-well plate reader (EnSpire, Perkin Elmer). In addition, binders were subjected to an ELISA assay using the steps and reagents mentioned above but with all steps performed at 37°C. Also, a subset of binders was subjected to an ELISA assay using the protocol above but with the following concentrations of biotinylated TSLP: 3.3 nM, 1.7 nM or 0.37 nM, respectively.

Results

Phage display selection of TSLP binding Z variants: Individual clones were obtained after three to four cycles of phage display selections against biotinylated TSLP.

Sequencing: Sequencing was performed for clones obtained after three or four cycles of selection. Each variant was given a unique identification number ###, and individual variants are referred to as ZTP###. The amino acid sequences of the 58 amino acid residues long Z variants are listed in the sequence listing as SEQ ID NO:631-644, 809-828 and 846-875.

The deduced TSLP 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 against TSLP: The clones obtained after three and four cycles of selection were produced as soluble crude samples in 96-well plates and screened for TSLP binding activity in ELISA. Binders were shown to give a response between 0.5 and 3.2 AU at a target concentration of 10 nM TSLP, corresponding to at least 3.5 × the blank control. The ELISA performed at 37°C gave the approximate same result as for the ELISA performed at RT. The binders assayed against biotinylated TSLP at concentrations of 3.3 nM, 1.7 nM or 0.37 nM were shown to give a response between 1.9 and 3.2 AU with the blank control at 0.08 AU, 0.3 and 3.0 AU with the blank control at 0.095 AU, and 0.06 and 1.5 AU with the blank control at 0.06 AU, respectively.

Example 2
Production of TSLP binding Z variants

Summary

This Example describes the general procedure for subcloning and production of His-tagged Z variants and Z variants in fusion with an albumin binding domain, which are used throughout the characterization experiments that follow.

Materials and methods

Subcloning of Z variants with a His_6-tag: DNA encoding Z variants ZTP632 (SEQ ID NO:632), ZTP634 (SEQ ID NO:634), ZTP633 (SEQ ID NO:633), ZTP635 (SEQ ID NO:635), ZTP636 (SEQ ID NO:636), and ZTP631 (SEQ ID NO:631) was amplified from the Zlib006Naive.II library vector pAY02592. DNA encoding Z variants ZTP637 (SEQ ID NO:637) and ZTP638 (SEQ ID NO:638) was amplified from the Zlib008Naive.II library vector pAY04242. A subcloning strategy for construction of monomeric Z variant molecules with an N-terminal His_6-tag was applied to Zlib006Naive.II library members and a subcloning strategy with a C-terminal His_6-tag was applied to Zlib008Naive.II library members, both using standard molecular biology techniques and essentially as described in PCT publication WO2009/077175. The Z gene fragments were subcloned into T7 promoter driven expression

vectors, resulting in the encoded sequences MGSSHHHHHHLQ-[ZTP###]-VD (SEQ ID NO:1126) and [ZTP###]-LEHHHHHH (SEQ ID NO:1127), respectively.

Subcloning of Z variant in fusion with ABD: A gene encoding the Z variant ZTP631 (SEQ ID NO:631) cloned in a custom plasmid was ordered from ATUM (Newark, CA). The Z variant was cloned as a fusion protein with an ABD variant PP013 (SEQ ID NO:1077). The construct encoded by the expression vector was in the format [ZTP631]-ASGS-[PP013] (ZTPA092; SEQ ID NO:1064).

Expression of TSLP binding Z variants: Production of Z variants was accomplished essentially as follows: *E. coli* T7E2 cells (GeneBridges) were transformed with plasmids containing sequence verified gene fragments of each respective TSLP binding Z variant and cultivated at 37°C in in 50 mL scale using the EnPresso protocol (Enpresso GmbH). In order to induce protein expression, IPTG was added to a final concentration of 0.5 mM after 8 h of cultivation and the temperature set to 30°C. After induction, the cultivations were incubated for 16 h before the cells were harvested by centrifugation.

Purification of TSLP binding Z variants with a His_6-tag: Approximately 1–2 g of each cell pellet was re-suspended 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). Z variants were subjected to a second purification step by reverse phase chromatography (RPC), for which each Z variant was loaded onto a 1 mL Resource 15RPC column (GE Healthcare), pre-equilibrated with RPC solvent A (0.1% trifluoroacetic acid (TFA), 10% acetonitrile (ACN), 90% water). After column wash with RPC solvent A, bound proteins were eluted with a linear gradient of 0–60% RPC solvent B (0.1% TFA, 80% ACN, 20% water) for 18 mL. The buffer was then exchanged to DPBS (Corning) using PD-10 desalting columns.

Protein concentrations were determined by absorbance measurements at 280 nm. Samples with a concentration of less than approximately 1 mg/ml

were concentrated using Amicon Ultra-4, Ultracel-3K filters (Merck Millipore). 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.

Purification of TSLP binding Z variants in fusion with ABD:

Approximately 5 g of 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, clarification by centrifugation and filtration (0.45 µm filter), the supernatant was applied on a gravity flow column with 5 mL agarose immobilized with an anti-ABD ligand (produced in-house). After washing with TST-buffer and 5 mM NH_4Ac pH 5.5 buffer, the ABD fused Z variant was eluted with 0.1 M HAc. The eluate was then subjected to a second purification step by RPC. 10% ACN was added to each eluate before loaded on a 3 mL Resource RPC column (GE Healthcare) equilibrated with solvent A (10% ACN, 0.1% TFA, 90% milli-Q-water) and eluted using a gradient of 0–60 % solvent B (80% ACN, 0.1% TFA, 20% milli-Q-water) for 54 mL. Eluted fraction was analyzed by SDS-PAGE and HPLC-MS and pooled. The buffer of the eluate was exchanged to DPBS using PD-10 desalting columns. Determination of protein concentration, as well as analysis of purity and identity, was performed as described above for Z variants with a His_6-tag.

Results

Production of TSLP binding Z variants: The TSLP binding Z variants with His_6-tag or in fusion with ABD were successfully cloned and expressed as soluble gene products in *E. coli*. Each DNA construct was verified by DNA sequencing. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the TSLP binding Z variant. The correct identity and molecular weight of each Z variant were confirmed by HPLC-MS analysis.

Example 3

Characterization of primary TSLP binding Z variants

Summary

In this Example, N-terminally His_6-tagged Z variants ZTP632 (SEQ ID NO:632), ZTP634 (SEQ ID NO:634), ZTP633 (SEQ ID NO:633), ZTP635 (SEQ ID NO:635), ZTP636 (SEQ ID NO:636) and ZTP631 (SEQ ID NO:631); and the C-terminally His_6-tagged Z variants ZTP637 (SEQ ID NO:637) and

ZTP638 (SEQ ID NO:638) were characterized in terms of secondary structure, stability, and binding profile. The melting temperature and secondary structure content were analyzed by circular dichroism (CD) spectroscopy. Biacore was used to characterize the interactions of the Z variants with TSLP.

Materials and methods

Circular dichroism (CD) spectroscopy analysis: The His_6-Z and Z-His_6 variants produced as described in Example 2 were diluted to 0.5 mg/mL in PBS. 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 (Tm). In the VTM, absorbance was measured at 221 nm while the temperature was raised from 20°C 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.

Biacore affinity analysis: Affinities (K_D) for TSLP were determined for the TSLP binding His_6-Z and Z-His_6 variants using a Biacore 8K instrument (Cytiva). In the experiment, human TSLP was immobilized on the carboxylated dextran layer of one CM5 chip surface (Cytiva) to immobilization levels of approximately 210–280 RU. The immobilization was performed using amine coupling chemistry according to the manufacturer's protocol and using HBS-EP+ (Cytiva) as running buffer. One flow cell surface was activated and deactivated for use as blank during analyte injections. In the kinetic experiment, HBS-EP+ was used as running buffer and the flow rate was 30 µL/min. The analyte, i.e. the His_6-Z or Z-His_6 variant, was diluted in HBS-EP+ buffer to a concentration of 100, 33 and 11 nM, respectively, and injected over the flow cells. After injection of the analyte concentrations for 2 min, a dissociation time of 10 min followed. The surfaces were regenerated by one injection of regeneration buffer (10 mM glycine pH 1.5, Cytiva) and a waiting period of 10 min was set before next injection round. A blank cycle with HBS-EP+ was run before or close to each analyte cycle for background subtraction. Target-binding analyses were performed using the Biacore 8K Evaluation Software (Cytiva) and a Langmuir 1:1 model. Double referencing was applied to all data prior to kinetic fit, i.e. responses obtained from a blank

surface and from a blank cycle where buffer was injected instead of analyte were subtracted.

Results

CD analysis: The CD spectra determined for eight TSLP binding Z variants with a His_6 tag showed that all variants have an α-helical structure at 20°C as judged from the typical minima at 208 and 222 nm. Reversible folding was seen for all Z variants when spectra measured before and after heating to 90°C were superimposed. The melting temperatures (Tm) are summarized in Table 3. Exemplary CD spectra of His_6-ZTP631 and ZTP637-His_6 are shown in Figure 1.

Table 3: Melting temperatures (Tm)

Z variant	SEQ ID NO	Tm (°C)
ZTP631	631	51
ZTP637	637	69
ZTP638	638	65
ZTP632	632	61
ZTP634	634	58
ZTP633	633	54
ZTP635	635	48
ZTP636	636	65

Biacore affinity analysis: The interactions of His_6-Z and Z-His_6 variants with TSLP were analyzed in a Biacore 8K instrument by injecting various concentrations of the purified Z variants over surfaces with immobilized TSLP. A summary of the dissociation equilibrium constants (K_D) based on data from three concentrations of each TSLP binder (11, 33 and 100 nM, respectively), injected over TSLP surfaces, is given in Table 4. An example of sensorgrams obtained for one TSLP binding polypeptide (ZTP631, SEQ ID NO:631) is displayed in Figure 2.

Table 4: Affinity constants

Z variant	SEQ ID NO	K_D (M)
ZTP631	631	2.9×10^{-9}
ZTP637	637	3.0×10^{-9}
ZTP638	638	3.0×10^{-9}
ZTP632	632	5.6×10^{-9}
ZTP634	634	1.5×10^{-9}
ZTP633	633	2.4×10^{-9}

ZTP635	635	1.7×10^{-9}
ZTP636	636	6.0×10^{-9}

Example 4

Characterization of TSLP binding polypeptides in an *in vitro* blocking assay

Summary

This Example describes the evaluation of selected Z variants in terms of their ability to block the effect of TSLP-induced thymus and activation-regulated chemokine (TARC) production in human peripheral blood mononuclear cells (PBMC).

Material and methods

100,000 PBMC per well were seeded in assay medium [RPMI 1640 (Gibco), 5% human serum (BIOIVT) and 1% Pen-Strep (Lonza)] in 96-well culture plates (Greiner Bio One). Serially titrated His-tagged Z variants (diluted in assay medium in seven steps from 10 to 0.0002 µM final concentration in cell assay plate) and TSLP (1 ng/mL final concentration in cell assay plate) were preincubated for 1 h at RT before being added to the cells. The cells with the added reagents were incubated at 37°C for 24 h in a CO_2 incubator. The supernatant was collected by transferring 120 µL from each well to a V-bottom dilution plate (Greiner Bio One), centrifuged for 5 min at 400 × g. 2 × 60 µL were thereafter transferred to two duplicate U-bottom plates (Greiner Bio One) and the plates were sealed and stored at -80°C until further analysis. The TARC levels were measured using Human CCL17/TARC-DuoSet® ELISA (R&D Systems, cat. no. DY364). The ELISA was performed essentially as described by the manufacturer, but with the exceptions a) half area ELISA plates were used, b) half of the stated volumes were used and c) the plates were washed 4 times in 175 µL wash buffer. The absorbance at 450 nm was measured using an EnSpire plate reader.

Results

The Z variants ZTP631 (SEQ ID NO:631), ZTP632 (SEQ ID NO:632), ZTP633 (SEQ ID NO:633), ZTP634 (SEQ ID NO:634), ZTP635 (SEQ ID NO:635), ZTP636 (SEQ ID NO:636), ZTP673 (SEQ ID NO:637) and ZTP638 (SEQ ID NO:638) were shown to block TSLP-induced TARC production in human PBMC in a concentration dependent manner (Figure 3).

Example 5

Design and construction of a maturation library of TSLP binding Z variants

Summary

In this Example, a new library was designed based on the TSLP binding variants described in Example 2, 3 and 4. The maturation library contained approximately 5.9×10^9 individual clones.

Materials and methods

Design of an affinity maturation TSLP library: A new library was designed based on the sequences of the TSLP binding Z variants selected, produced and characterized as described in Examples 1–4. 13 surface exposed positions in the Z molecule scaffold were biased towards certain amino acid residues, according to a strategy mainly based on the binding motifs of the Z variants defined in SEQ ID NO:631-644, 809-828 and 846-875. The design of the new library is shown in Table 5, wherein percentages of the amino acids used in each of the 13 randomized positions are indicated.

Table 5: Design of the TSLP maturation library

Amino acid		Position in BM												
		2	3	4	6	7	10	11	17	18	20	21	25	28
		Position in full length Z variant												
		9	10	11	13	14	17	18	24	25	27	28	32	35
A	Ala	100	0	0	0	16.7	0	6.3	5.9	5.6	0	5.6	5.6	0
C	Cys	0	0	0	0	0	0	0	0	0	0	0	0	0
D	Asp	0	0	14.3	0	0	0	6.3	5.9	5.6	0	5.6	5.6	0
E	Glu	0	0	14.3	0	0	0	6.3	5.9	5.6	0	5.6	5.6	0
F	Phe	0	0	0	0	16.7	0	6.3	5.9	5.6	0	5.6	5.6	0
G	Gly	0	0	0	0	0	0	0	5.9	5.6	0	5.6	5.6	0
H	His	0	0	14.3	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0
I	Ile	0	0	0	0	16.7	0	6.3	5.9	5.6	0	5.6	5.6	0
K	Lys	0	0	0	0	0	0	6.3	5.9	5.6	0	5.6	5.6	0
L	Leu	0	0	0	100	16.7	0	6.3	5.9	5.6	0	5.6	5.6	0
M	Met	0	0	0	0	16.7	0	0	0	5.6	0	5.6	5.6	0
N	Asn	0	0	14.3	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0
P	Pro	0	0	0	0	0	0	0	0	0	0	0	0	0
Q	Gln	0	0	14.3	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0
R	Arg	0	0	0	0	0	0	6.3	5.9	5.6	0	5.6	5.6	100
S	Ser	0	0	14.3	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0
T	Thr	0	0	14.3	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0
V	Val	0	100	0	0	16.7	0	6.3	5.9	5.6	0	5.6	5.6	0
W	Trp	0	0	0	0	0	100	6.3	5.9	5.6	14.3	5.6	5.6	0
Y	Tyr	0	0	0	0	0	0	6.3	5.9	5.6	14.3	5.6	5.6	0

Two oligonucleotides, one forward and one reverse complementary, with complementary 3'-ends were generated using TRIM technology. These oligos were ordered from Ella Biotech GmbH (Martinsried, Germany).

The construction of the library was performed essentially as described earlier (e.g. PCT publication WO2017/072280) in a vector denoted pAY02592, but with the following exceptions. Transformation was done using approximately 230 ng per electroporation into electrocompetent XL-1 Blue cells. Cells were pooled after the electroporation, incubated in recovery medium (Lucigen) for 60 min at 37°C and thereafter cultivated in 3 L medium TSB-YE medium, supplemented with 10 µg/mL tetracycline and 100 µg/mL carbenicillin. The library quality and the distribution of amino acids were verified by sequencing essentially as described in WO2009/077175.

Preparation of phage stock: Cells from a glycerol stock containing the phagemid library were inoculated into 2 L TSB supplemented with 100 µg/mL carbenicillin, 10 µg/mL tetracycline and 1% glucose and cultivated at 80 rpm and 37°C. When the cultivation reached an optical density at 600 nm (OD_{600})

of 0.7, the cultivation was infected using a 50 × molar excess of M13K07 helper phage and the cells were incubated for 1.5 h at 37°C. Cells were pelleted and resuspended in 2 L TSB+YE supplemented with 100 µg/mL carbenicillin, 25 µg/mL kanamycin and 0.1 mM IPTG. The cultivation was incubated at 30°C and 70 rpm and harvested after 21 h. The cells in the cultivation were removed by centrifugation (10,700 × *g*, 30 min at 4°C). The phage particles were precipitated from the supernatant twice using PEG/NaCl and filtered as described in Example 1, and finally dissolved in PBSB and glycerol. Phage stocks were stored at −80°C until use in selection.

Results

Library construction: The new library was designed based on the set of TSLP binding variants described in Examples 1–4. The theoretical size of the designed library was 4.7×10^8 Z variants. The actual size of the library, determined by titration after transformation to *E. coli.* XL-1 Blue cells, was 5.9×10^9 transformants. The library quality was tested by sequencing of 96 transformants and by comparing their actual sequences with the theoretical design. Sequence analysis of individual library members verified a distribution of codons in accordance with the theoretical design. The library was designated Zlib006TSLP.I.

Example 6

Selection and screening of affinity matured TSLP binding Z variants

Summary

In this Example, TSLP was used as target in phage display selections using a TSLP maturation phage library of Z variants. Selected clones were DNA sequenced, produced in *E. coli* periplasmic fractions and assayed against different target proteins in ELISA and Biacore.

Materials and methods

Biotinylation of proteins: Human TSLP was biotinylated using No-Weigh EZ-Link Sulfo-NHS-LC-Biotin as described in Example 1.

Phage display selection of TSLP binding Z variants: Phage display selection was performed using a phage stock of the newly produced maturation library. Selections against biotinylated TSLP was performed in solution in all tracks and essentially as described in Example 1, but with the specific conditions described in Table 6.

Washing was performed using streptavidin coated beads (Sera-Mag SpeedBeads Streptavidin-Coated Magnetic Particles, Cytiva) as solid phase in cycle 1, 3, and 4, while neutravidin beads were used in cycle 2. In some tracks the washing step was performed by catch of target-phage particle complexes in wells of 96-well high binding polystyrene plates (Corning) coated with SA protein (Millipore) diluted to 5 mg/mL in carbonate buffer. In washes performed overnight, non-biotinylated TSLP was added in the washing buffer. Selection was made at 37°C or at RT and using preblocked tubes (Protein LowBind, Eppendorf). In order to reduce the degree of unspecific binding, pre-selection was performed in cycle 1–3 using the corresponding solid phase used in that specific cycle and track. In addition, in some tracks the phage stock was preheated at 70°C for 15 min followed by a spin down at 13,000 rpm in a centrifuge before use in selection.

As selection proceeded, the tracks were further divided according to target concentration and number and/or time of washes.

Table 6: Overview of the selection against TSLP using the maturation library

Cycle	Selection track	Phage stock from library or selection track	Pre-heated phage stock	Target conc. (nM)	Number of short washes (1 min)	Number of long washes (30 min)	Number of over night washes (17.5 h)	Temperature during selection and wash
1	1	Zlib006TSLP.I		50	3			37°C
1	2	Zlib006TSLP.I	Yes	50	3			37°C
1	3	Zlib006TSLP.I		50	3			RT
2	4	1		25	6			37°C
2	5	1		10	10			37°C
2	6	2	Yes	25	6			37°C
2	7	2	Yes	10	10			37°C
2	8	3		25	6			RT
3	9	4		10	10	1		37°C
3	10	5		1	16	4		37°C
3	11	6	Yes	10	10	1		37°C
3	12	7	Yes	1	16	4		37°C
3	13	8		10	10	1		RT
4	14	9		2	16	1		37°C
4	15	10		0.25	24	1		37°C
4	16	9+10		0.25	32	4	1	37°C
4	17	11	Yes	2	16	1		37°C
4	18	12	Yes	0.25	24	1		37°C
4	19	11+12	Yes	0.25	32	4	1	37°C
4	20	9–13		0.25	32	4	1	37°C
4	21	9–13		0.25	32	4	1	RT
4	22	13		1	24	4		RT
3	23	4–8		10	45	1		37°C
1	24	Zlib006TSLP.I		10	45	1		37°C

Amplification and preparation of phage particles: Phage stock preparation and amplification of phage between selection cycles were performed essentially as described in Example 1. In the last selection cycle, log phase bacteria were infected with eluate and spread on TBAB plates supplemented with 0.2 g/L ampicillin in order to form single colonies for use in ELISA screening.

Production of Z variants for ELISA: The Z variants were produced and the HT lysate of each individual variant was prepared as described in Example 1. The final filtered supernatant with the soluble part of the extract contained the Z variants as fusions to ABD, expressed as AQHDEALE-[ZTP###]-VDYV-[ABD]-YVPG (SEQ ID NO:1125; Grönwall *et al. supra*). ZTP### refers to individual, 58 amino acid residue Z variants.

ELISA screening of Z variants: The binding of Z variants to TSLP was analyzed in a set of ELISA assays. Each Z variant was analyzed against biotinylated TSLP at concentrations 1, 0.2, 0.04 and 0 nM, respectively. The ELISA was performed essentially as described in Example 1, with the exception that high binding 384-well plates (Greiner) were used. In addition, a selected subset of the binders was subjected to an ELISA where a target concentration of 0.2 nM was used. All samples were run in triplicates. In addition, yet another selected subset of binders was subjected to an ELISA using the target concentration 0.2 nM and with all samples run in triplicates. As negative control, a HT lysate containing the fusion protein ABD with no Z fusion partner, i.e. AQHDEALEVDYV-[ABD]-YVPG (SEQ ID NO:1128), was used on all plates. The absorbance at 450 nm was measured using a multi-well plate reader, EnSpire (Perkin Elmer).

Sequencing: In parallel with the ELISA screening, all clones were sequenced. PCR fragments were amplified from single colonies, sequenced and analyzed as described in Example 1.

Results

Phage display selection of TSLP binding Z variants: Individual clones were obtained after one to four cycles of phage display selections against biotinylated TSLP.

ELISA screening of Z variants: Clones obtained after one to four cycles of selection were produced in 96-well plates and screened for binding activity against TSLP. In a first ELISA all clones were run against TSLP at concentrations of 1, 0.2, 0.04 and 0 nM, respectively. In two following ELISA

assays, subsets of binders were assayed against 0.2 nM TSLP in triplicates. Binders were regarded as positive if the response was 3 × the background response at 0.2 nM TSLP (above 0.26 AU) in the first set together with a sound target dependent response in the other concentrations, or if average response was 3 × the background response (above 0.195 AU or 0.293 AU, respectively) in either of the two latter ELISA.

Sequencing: Sequencing was performed for clones obtained after one to four cycles of selection. Each variant was given a unique identification number ###, and individual variants are referred to as ZTP###. The amino acid sequences of the 58 amino acid residues long Z variants are listed in the sequence listing as SEQ ID NO:1-630, 646-808 and 829-845. The deduced TSLP 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.

Example 7

Production and characterization of matured TSLP binding Z variants

Summary

This Example describes the cloning, production and characterization of matured Z variants. CD spectroscopy was used to determine the melting temperature and assess the secondary structure content. SPR was used to determine the target binding affinity and kinetic values as well as to analyze binding to TSLP from different species. The specificity was analyzed in a dot blot assay to assess any potential off-target binding to selected high abundant plasma proteins or to other related or unrelated proteins. TSLP is a member of the interleukin 2 (IL-2) family of cytokines that also includes IL-4, IL-7, IL-9, IL-15, and IL-21. Each member of the IL-2 cytokine family consists of a four alpha helix bundle and shares the common cytokine receptor γ chain. However, the amino acid sequence similarity is low, but to confirm specificity towards TSLP of the Z variants selected here, all cytokines in the IL-2 cytokine family were included in the dot blot assay. In addition, the unrelated IL-17A cytokine was included in the specificity assay.

Materials and methods

Subcloning of Z variants in fusion with ABD: A subset of clones, chosen based on scoring in the ELISA assays, were ordered from Twist

Biosciences (San Francisco, CA) as cloned genes in a custom vector. In the genes ordered from Twist, the N-terminal amino acids in positions 1 and 2 (originally V and D, respectively) of the Z variants were mutated to the amino acid residues A and E, respectively (SEQ ID NO:876-966). One set of these Z variants was then further mutated in scaffold positions as follows: Y5F, N52S and D53E (SEQ ID NO 967-972). The synthesized Z variants were subcloned into an expression vector containing the ABD variant PP013 (SEQ ID NO:1077). The constructs encoded by the expression vectors were in the format [ZTP###]-ASGS-[PP013] and denoted ZTPA### (SEQ ID NO:973-1070). Furthermore, the two Z variants ZTP970 (SEQ ID NO:970) and ZTP876 (SEQ ID NO:876) were subcloned as fusions to ABD with a set of different linkers. The constructs were made according to the methods described in Example 2 and were in the format [ZTP970]-A$(EAAAK)_2$-AGS-[PP013] (ZTPA099; SEQ ID NO:1071), [ZTP970]-A$(EAAAK)_4$-AGS-[PP013] (ZTPA100; SEQ ID NO:1072), [ZTP970]-$(KEAAA)_2$-KAK-[PP013] (ZTPA101; SEQ ID NO:1073), [ZTP970]-$(KEAAA)_2$-[PP013] (ZTPA102; SEQ ID NO:1074), [ZTP970]-$(GGGGS)_3$-GS-[PP013] (ZTPA103; SEQ ID NO:1075), and GS-[PP013]-GGGGS-[ZTP876] (ZTPA104; SEQ ID NO:1076). One Z variant (SEQ ID NO:645) was created in fusion with PP013 in the format [ZTP645]-ASGS-[PP013] (ZTPA105; SEQ ID NO:1129)

Subcloning of Z variants in fusion with His_6: A subset of Z variants was subcloned with a His_6-tag according to the methods described in Example 2. The cloned variants were in the format [ZTP###]-LEHHHHHH (SEQ ID NO:1127).

Expression of TSLP binding Z variants: Expression of TSLP binding Z variants was performed either as described in Example 2 or in a smaller scale in 24-well format. Production in 24-well format followed the same procedure as described in Example 2, with the exception of using a 3 mL culture volume and keeping the temperature at 37°C after the addition of IPTG.

Purification of TSLP binding Z variants in fusion with ABD in 96-well plate format: Approximately 0.1 mg of cell pellet was resuspended in TST-buffer with 3 mM EDTA, cells were disrupted by 90°C heat treatment and subsequent DENARASE® (c-LEcta) (in 5 mM $MgSO_4$) treatment followed by centrifugation and finally filtration through a 3.0/0.2 µm filter (Pall). The clarified lysates were stored frozen and when thawed, passed through a 0.45 µm filter plate (AcroPrep) before being applied on a 1 mL filter plate (1.2 µm, Acroprep) prepacked with 100 µL anti-ABD ligand (produced in-house). After

washing with TST buffer and 5 mM NH_4Ac pH 5.5 buffer, the ABD fused Z variant was eluted with 0.1 M HAc. All wash and elution steps were performed using centrifugal force. Buffer exchange to DPBS was performed using a PD MultiTrap G-25 desalting plate (Cytiva).

Protein concentrations were determined by absorbance measurements at 280 nm. The identity of each purified Z variant was confirmed using LC/MS analysis.

Purification of TSLP binding Z variants in fusion with ABD: Appr. 4 g of cell pellet was re-suspended in TST-buffer supplemented with DENARASE® (c-Lecta). After cell disruption, clarification by centrifugation and filtration (0.45 µm filter), the supernatant was applied on a gravity flow column with 2 × 1 mL agarose immobilized with an anti-ABD ligand (produced in-house). After washing with TST-buffer and 5 mM NH_4Ac pH 5.5 buffer, the ABD fused Z variant was eluted with 0.1 M HAc. The eluate was then subjected to a second purification step by RPC. 10% ACN was added to each eluate before loaded on a 1 mL Resource RPC column (Cytiva) equilibrated with solvent A (10% ACN, 0.1% TFA, 90% milli-Q-water) and eluted using a gradient of 0–60% solvent B (80% ACN, 0.1% TFA, 20% milli-Q-water) for 18 mL. Eluted fraction was analyzed by SDS-PAGE and HPLC-MS and pooled. The buffer of the eluate was exchanged to DPBS using PD-10 desalting columns (Cytiva).

Protein concentrations were determined by absorbance measurements at 280 nm. Samples with a concentration of less than approximately 1 mg/mL were concentrated using Amicon Ultra-4, Ultracel-3K (Merck Millipore). 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.

Purification of TSLP binding Z variant in fusion with His_6: Z variants cloned as fusions to a C-terminal His_6 were purified using an IMAC strategy as described in Example 2.

CD analysis: A subset of Z variants cloned as Z-His_6 was subjected to a CD analysis essentially as described in Example 3.

Biacore kinetic screen: The affinity and kinetic values for binding to TSLP were determined for the Z-ABD variants, produced in this Example using the 96-well strategy, as well as the Z-ABD variant ZTPA092 (SEQ ID NO:1064) produced in Example 2, using a Biacore 8K instrument. TSLP was immobilized on the surfaces of a CM5 chip as described in Example 3 giving an immobilization level of approximately 510-550 RU. A single-cycle kinetic

(SCK) method (a series of analyte injections in one cycle with no regeneration between) was used for injection of the respective Z-ABD variant. A blank cycle with HBS-EP+ was run before each analyte cycle for background subtraction. Z variants were injected at 3.3, 10, 30, and 90 nM, respectively, over sensor chip surfaces immobilized with TSLP. After injection for 240 s of each of the four analyte concentrations, a dissociation time of 15 min followed. The flow rate was 30 µL/min and HBS-EP+ was used as running buffer. The surfaces were regenerated by three injections of regeneration buffer (10 mM glycine pH 1.5) followed by a waiting period before next injection round. The obtained sensorgrams were reference subtracted and analyzed with Biacore Insight Evaluation software using the Langmuir 1:1 binding model to determine kinetic constants; associate rate constant (k_{on}), dissociation rate constant (k_{off}) and the dissociation equilibrium constant (K_D).

Biacore kinetic analysis against TSLP from different species: The affinity and kinetic values were determined for the Z-ABD variants ZTPA002 (SEQ ID NO:974), ZTPA001 (SEQ ID NO:973), and ZTPA093 (SEQ ID NO:1065) against a set of TSLP from different species: cynomolgus (cynoTSLP, Sino Biological cat. no. 90911-C08H), rat (rTSLP, MyBiosource, cat. no. MBS2010776) and mouse (mTSLP, R&D Systems, cat. no. 555-TS-010/CF) using a Biacore 8K instrument. HSA was diluted to 10 µg/mL in immobilization buffer and immobilized on a CM5 sensor chip by amine chemistry, using an Amine coupling kit type 2 (Cytiva), 10 mM sodium acetate pH 4.5 as immobilization buffer, and HBS-EP+ as running buffer, all according to the manufacturer's instructions. Both flow cells of each channel were immobilized (in sequence) to approximately 6500 RU.

Multi cycle kinetics (MCK) with ligand capture was used to assess the binding characteristics. Z variants were captured on immobilized HSA in flow cell 2 by injecting 20 nM at 5 µL/min for 150 s (capture response approximately 50–100 RU), followed by analyte injection in a concentration series, 4 nM, 16 nM, and 64 nM, over both flow cell 1 and 2. Binding interactions were assessed in HBS-EP+ running buffer. Association time was 180 s (30 µL/min) and dissociation time was 460 s (30 µL/min). Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to analysis. Regeneration buffer was 10 mM HCl (two pulses of 30 s / 30 µL/min).

Dot blot analysis: The binding of the Z-ABD variants ZTPA104 (SEQ ID NO:1076) and ZTPA001 (SEQ ID NO:973) to full length human TSLP in two

different forms: hTSLP uncleaved (Acro Biosystems, cat. no. TSP-H52Ha) and hTSLP cleaved (Acro Biosystems, cat. no. TSP-H52Hb), and to short form of TSLP (sfTSLP; SEQ ID NO:1080; synthesized at Almac, Craigavon, UK) was assessed. In addition, the Z variants were assayed against a set of different high abundant plasma proteins: HSA (Recombumin Elite, Albumedix, cat. no. 205-005); human IgG (Sigma, cat. no. G4386); alpha-2-macroglobulin (Human A2M, Sino Biological, cat. no.10952-H08B); alpha 1-antitrypsin (SERPINA1 Human, ProSpec, cat. no. PRO-529); complement component 3c (C3c, Lee Biosolutions, cat. no.194-32); haptoglobulin, (ProSpec, cat. no. PRO-567); alpha 1-antichymotrypsin (SERPINA3, Fitzgerald/Kem-En-Tech, cat. no. 30-AC47); C4 complement (Complement Technology, cat. no. A105); IgE (Fitzgerald/Kem-En-Tech, cat. no. 31-AI01); hemopexin (Human HPX, Sino Biological, cat. no. 10870-H08H); transthyretin (TTR, Sino Biological, cat. no. 12091-H08H); IgG Fc fragment, (Jackson ImmunoResearch/Nordic BioSite, cat. no. 009-060-008); holo-transferrin human (Sigma, cat. no. T4132); fibrinogen (Abcamab, cat. no. 81752); IgA (Bethyl Laboratories/Nordic Biosite, cat. no. P80-102); IgM, (Sigma, cat. no. I8260); structurally related IL-2 family of cytokines: (IL-2, Peprotech, cat. no. 200-02); IL-4 (R&D Systems, cat. no. 6507-IL/CF); IL-7 (kindly received through a research collaboration); IL-9 (R&D Systems, cat. no. 209-ILB-010/CF); IL-15 (Peprotech, cat. no. 200-15); and IL-21 (R&D Systems, cat. no. 8879-IL-010/CF), as well as unrelated IL-3 (R&D Systems, cat. no. 203-IL-010/CF), IL-5 (R&D Systems, cat. no. 205-IL-025/CF), and IL-17A (Peprotech, cat. no. 200-17). A nitrocellulose membrane was divided into three areas whereby proteins were applied to create identical sections on the membrane. 1 µL of each protein (0.1 mg/mL) were immobilized at a predefined position on each section. The membrane was blocked with Blocker® casein for 2 h at RT. The blocking solution was removed and the membrane was cut into three identical strips. Each strip was incubated for 1 h at RT with 2 µg/mL of either of the two different TSLP binding Z variants ZTPA104 (SEQ ID NO:1076) and ZTPA001 (SEQ ID NO:973), or an IL-17A binding Z variant included as a control polypeptide, all diluted in Blocker® casein; or Blocker® casein only (negative control) and then washed 4 × 2 min in PBST. Bound Z variant was detected by first adding 5 µg/mL of goat anti-Z variant Ig antibody (in house produced), 1 h at RT and then washed in PBST for 4 × 2 min. Second, anti-goat antibody-HRP conjugate (DAKO, cat. no. P0449), diluted 1:10000 was added and incubated for 1.5 h at RT and washed in PBST for 5 × 5 min. All dilutions

were made in Blocker® casein and all incubations and washing steps were carried out with gentle rocking. The signal was developed by adding approximately 5 mL SuperSignal™ West Pico PLUS (Thermo Scientific) followed by incubation for 5 min. The resulting chemiluminescence was detected using a Luminescent Image Analyzer (Fuji Film, Fuji Photo Film) and the picture was taken after 20 s exposure.

In addition, the same protocol was used for another assay performed as described above, but in the presence of HSA and against the subset of proteins derived from human plasma or serum: IgG, complement component 3c (C3c), alpha 1-antichymotrypsin, C4 complement, IgE, Fc fragment from IgG, holo-transferrin, fibrinogen, IgA and IgM; as well as against HSA, sfTSLP, full-length TSLP uncleaved and full-length TSLP cleaved. Z variant ZTPA104 (SEQ ID NO:1076) was diluted in Blocker® casein supplemented with 10× molar excess of HSA and incubated at RT for 30 min before assayed against the proteins on the nitrocellulose membrane.

Results

Production of TSLP binding Z variants: The TSLP binding Z variants in fusion with ABD were expressed as soluble gene products in *E. coli*. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the TSLP binding Z variants. The correct identity and molecular weight of each Z variant were confirmed by HPLC-MS analysis.

CD analysis: The CD spectra determined for TSLP binding Z variants with a C-terminal His_6 tag showed that all variants have an α-helical structure at 20°C as judged from the typical minima at 208 and 222 nm. Reversible folding was seen for all Z variants when spectra measured before and after heating to 90°C were superimposed. The melting temperatures (Tm) are summarized in Table 7. Exemplary CD spectra are shown for Z variants ZTP876 and ZTP967 in Figure 4.

Table 7: Melting temperatures (Tm)

Z variant	SEQ ID NO	Tm (°C)
ZTP878	878	49
ZTP879	879	57
ZTP880	880	52
ZTP881	881	71
ZTP882	882	52
ZTP883	883	52
ZTP884	884	52
ZTP885	885	60
ZTP886	886	68
ZTP887	887	52
ZTP888	888	55
ZTP889	889	57
ZTP890	890	66
ZTP891	891	57
ZTP892	892	55
ZTP893	893	57
ZTP894	894	52
ZTP895	895	52
ZTP896	896	55
ZTP877	877	70
ZTP876	876	74
ZTP897	897	52
ZTP898	898	52
ZTP899	899	52
ZTP900	900	46
ZTP901	901	55
ZTP967	967	72

Biacore kinetic screen: Respective Z variant in fusion with ABD were injected at different concentrations over surfaces with immobilized TSLP and analyzed for their binding. A summary of the calculated kinetic parameters is presented in Table 8. Exemplary sensorgrams are shown for Z-ABD variants ZTPA028, ZTPA029 and ZTPA030 in Figure 5.

Table 8: Kinetic constants for Z variants binding to TSLP

Z variant	SEQ ID NO	k_a (1/Ms)	k_d (1/s)	K_D (M)
ZTPA092	1064	4.8×10^{5}	1.6×10^{-3}	3.2×10^{-9}
ZTPA028	1000	3.8×10^{5}	1.5×10^{-3}	4.1×10^{-9}
ZTPA029	1001	3.3×10^{5}	1.5×10^{-3}	4.6×10^{-9}
ZTPA030	1002	5.8×10^{5}	1.5×10^{-3}	2.6×10^{-9}
ZTPA054	1026	5.9×10^{5}	1.8×10^{-3}	3.1×10^{-9}

ZTPA003	975	3.7×10^{5}	1.6×10^{-3}	4.4×10^{-9}
ZTPA055	1027	5.7×10^{5}	1.7×10^{-3}	2.9×10^{-9}
ZTPA056	1028	3.7×10^{5}	1.2×10^{-3}	3.4×10^{-9}
ZTPA057	1029	4.6×10^{5}	1.2×10^{-3}	2.6×10^{-9}
ZTPA058	1030	4.6×10^{5}	9.1×10^{-4}	2.0×10^{-9}
ZTPA059	1031	3.0×10^{5}	8.0×10^{-4}	2.7×10^{-9}
ZTPA060	1032	1.1×10^{5}	3.4×10^{-4}	2.9×10^{-9}
ZTPA061	1033	3.2×10^{5}	9.3×10^{-4}	2.9×10^{-9}
ZTPA062	1034	5.4×10^{5}	1.2×10^{-3}	2.3×10^{-9}
ZTPA004	976	3.1×10^{5}	1.2×10^{-3}	3.9×10^{-9}
ZTPA031	1003	3.2×10^{5}	9.8×10^{-4}	3.0×10^{-9}
ZTPA063	1035	5.1×10^{5}	1.2×10^{-3}	2.3×10^{-9}
ZTPA032	1004	6.2×10^{5}	1.5×10^{-3}	2.5×10^{-9}
ZTPA033	1005	3.2×10^{5}	7.1×10^{-4}	2.2×10^{-9}
ZTPA005	977	3.5×10^{5}	1.4×10^{-3}	4.0×10^{-9}
ZTPA006	978	1.5×10^{5}	3.6×10^{-4}	2.4×10^{-9}
ZTPA034	1006	3.4×10^{5}	1.3×10^{-3}	3.7×10^{-9}
ZTPA007	979	4.1×10^{5}	2.0×10^{-3}	4.8×10^{-9}
ZTPA035	1007	3.2×10^{5}	1.5×10^{-3}	4.7×10^{-9}
ZTPA008	980	3.4×10^{5}	1.6×10^{-3}	4.8×10^{-9}
ZTPA036	1008	4.6×10^{5}	1.2×10^{-3}	2.6×10^{-9}
ZTPA009	981	3.1×10^{5}	1.5×10^{-3}	4.7×10^{-9}
ZTPA010	982	6.2×10^{5}	1.1×10^{-3}	1.8×10^{-9}
ZTPA011	983	1.1×10^{5}	3.6×10^{-4}	3.1×10^{-9}
ZTPA012	984	3.3×10^{5}	1.4×10^{-3}	4.2×10^{-9}
ZTPA037	1009	3.1×10^{5}	6.9×10^{-4}	2.3×10^{-9}
ZTPA038	1010	4.1×10^{5}	9.1×10^{-4}	2.2×10^{-9}
ZTPA013	985	4.7×10^{5}	1.1×10^{-3}	2.3×10^{-9}
ZTPA014	986	5.4×10^{5}	1.6×10^{-3}	2.9×10^{-9}
ZTPA015	987	1.3×10^{5}	3.4×10^{-4}	2.6×10^{-9}
ZTPA016	988	7.0×10^{5}	1.5×10^{-3}	2.1×10^{-9}
ZTPA039	1011	2.7×10^{5}	7.2×10^{-4}	2.7×10^{-9}
ZTPA017	989	4.7×10^{5}	2.2×10^{-3}	4.7×10^{-9}
ZTPA018	990	3.4×10^{5}	1.1×10^{-3}	3.2×10^{-9}
ZTPA040	1012	5.5×10^{5}	1.4×10^{-3}	2.5×10^{-9}
ZTPA041	1013	3.9×10^{5}	1.1×10^{-3}	2.8×10^{-9}
ZTPA019	991	4.3×10^{5}	2.3×10^{-3}	5.4×10^{-9}
ZTPA020	992	3.0×10^{5}	1.4×10^{-3}	4.6×10^{-9}
ZTPA021	993	3.7×10^{5}	8.1×10^{-4}	2.2×10^{-9}
ZTPA042	1014	5.6×10^{5}	2.5×10^{-3}	4.5×10^{-9}
ZTPA043	1015	4.4×10^{5}	1.1×10^{-3}	2.5×10^{-9}
ZTPA002	974	1.5×10^{5}	4.0×10^{-4}	2.7×10^{-9}
ZTPA044	1016	5.8×10^{5}	1.3×10^{-3}	2.3×10^{-9}
ZTPA045	1017	3.2×10^{5}	8.9×10^{-4}	2.7×10^{-9}
ZTPA046	1018	3.0×10^{5}	1.7×10^{-3}	5.7×10^{-9}
ZTPA064	1036	4.2×10^{5}	9.7×10^{-4}	2.3×10^{-9}
ZTPA065	1037	3.0×10^{5}	9.4×10^{-4}	3.1×10^{-9}

ZTPA001	973	1.1×10^{5}	3.6×10^{-4}	3.4×10^{-9}
ZTPA047	1019	3.2×10^{5}	1.1×10^{-3}	3.3×10^{-9}
ZTPA066	1038	3.8×10^{5}	1.0×10^{-3}	2.8×10^{-9}
ZTPA048	1020	4.6×10^{5}	1.5×10^{-3}	3.2×10^{-9}
ZTPA049	1021	1.0×10^{5}	4.0×10^{-4}	4.0×10^{-9}
ZTPA050	1022	2.7×10^{5}	9.7×10^{-4}	3.6×10^{-9}
ZTPA022	994	2.6×10^{5}	1.2×10^{-3}	4.5×10^{-9}
ZTPA051	1023	4.5×10^{5}	9.9×10^{-4}	2.2×10^{-9}
ZTPA052	1024	3.5×10^{5}	1.6×10^{-3}	4.7×10^{-9}
ZTPA027	999	1.0×10^{5}	3.3×10^{-4}	3.3×10^{-9}
ZTPA067	1039	7.1×10^{5}	3.3×10^{-3}	4.6×10^{-9}
ZTPA053	1025	4.2×10^{5}	1.3×10^{-3}	3.2×10^{-9}
ZTPA068	1040	3.7×10^{5}	1.3×10^{-3}	3.5×10^{-9}
ZTPA069	1041	4.7×10^{5}	1.5×10^{-3}	3.2×10^{-9}
ZTPA070	1042	6.8×10^{5}	1.5×10^{-3}	2.3×10^{-9}
ZTPA071	1043	5.9×10^{5}	1.6×10^{-3}	2.8×10^{-9}
ZTPA072	1044	5.4×10^{5}	1.2×10^{-3}	2.3×10^{-9}
ZTPA073	1045	3.0×10^{5}	2.6×10^{-3}	8.7×10^{-9}
ZTPA074	1046	5.5×10^{5}	1.3×10^{-3}	2.3×10^{-9}
ZTPA075	1047	5.8×10^{5}	1.4×10^{-3}	2.4×10^{-9}
ZTPA023	995	5.2×10^{5}	2.0×10^{-3}	3.8×10^{-9}
ZTPA076	1048	2.4×10^{5}	7.4×10^{-4}	3.1×10^{-9}
ZTPA077	1049	4.6×10^{5}	9.8×10^{-4}	2.1×10^{-9}
ZTPA078	1050	3.4×10^{5}	1.1×10^{-3}	3.3×10^{-9}
ZTPA024	996	5.1×10^{5}	1.3×10^{-3}	2.6×10^{-9}
ZTPA025	997	5.7×10^{5}	1.9×10^{-3}	3.3×10^{-9}
ZTPA079	1051	3.1×10^{5}	5.7×10^{-4}	1.8×10^{-9}
ZTPA080	1052	3.6×10^{5}	1.3×10^{-3}	3.6×10^{-9}
ZTPA081	1053	5.0×10^{5}	1.3×10^{-3}	2.6×10^{-9}
ZTPA082	1054	1.9×10^{5}	1.2×10^{-3}	5.9×10^{-9}
ZTPA083	1055	4.2×10^{5}	1.4×10^{-3}	3.3×10^{-9}
ZTPA084	1056	5.0×10^{5}	1.2×10^{-3}	2.5×10^{-9}
ZTPA085	1057	3.6×10^{5}	1.3×10^{-3}	3.5×10^{-9}
ZTPA086	1058	2.5×10^{5}	1.1×10^{-3}	4.3×10^{-9}
ZTPA087	1059	2.8×10^{5}	1.2×10^{-3}	4.2×10^{-9}
ZTPA026	998	3.9×10^{5}	1.3×10^{-3}	3.4×10^{-9}
ZTPA088	1060	4.1×10^{5}	2.2×10^{-3}	5.4×10^{-9}
ZTPA089	1061	3.7×10^{5}	8.6×10^{-4}	2.3×10^{-9}
ZTPA090	1062	3.7×10^{5}	1.2×10^{-3}	3.3×10^{-9}
ZTPA091	1063	3.9×10^{5}	1.2×10^{-3}	3.2×10^{-9}

Biacore kinetic analysis against TSLP from different species: The affinity and kinetic values of binding to TSLP from cynomolgus, rat, and mouse, respectively, were determined for the Z-ABD variants ZTPA002 (SEQ ID NO:974), ZTPA001 (SEQ ID NO:973), and ZTPA093 (SEQ ID NO:1065)

using a capture strategy in a Biacore 8K instrument. HSA was immobilized followed by injections of respective Z-ABD and subsequently TSLP from different species. A summary of the calculated kinetic parameters based on data from three concentrations of cynomolgus TSLP protein (4, 16, and 64 nM), injected over Z-ABD surfaces, is given in Table 9. No binding could be detected to TSLP from rat or mouse. An example of sensorgrams obtained for one TSLP binding polypeptide (ZTPA001, SEQ ID NO:973) is displayed in Figure 6.

Table 9: Kinetic constants for Z variants binding to cynomolgus TSLP

Z variant	SEQ ID NO	k_a (1/Ms)	k_d (1/s)	K_D (M)
ZTPA002	974	5.0×10^5	1.4×10^{-3}	2.8×10^{-8}
ZTPA001	973	4.1×10^5	1.4×10^{-3}	3.4×10^{-8}
ZTPA093	1065	1.2×10^5	3.8×10^{-3}	3.3×10^{-8}

Dot blot analysis: The specificity of ZTPA104 and ZTPA001 was assessed using a dot blot assay. These Z-ABD variants were allowed to bind to a nitrocellulose membrane containing dots of 28 different proteins including two forms of full-length hTSLP (uncleaved or cleaved) and sfTSLP, 16 abundant plasma proteins, as well as six structurally related cytokines and three unrelated cytokines. The result of the Z-ABD variants ZTPA104 and ZTPA001, compared with an IL-17A binding Z variant included as negative control, is shown in Figure 7 A–C. To investigate if the weak dots (dots 5, 7, 14 and 16) seen in Figure 7 A-C against some of the proteins purified from plasma or serum could be due to binding of remaining trace amounts of HSA, binding in the presence of 10× molar excess of HSA was also performed against those proteins and the result of this analysis is shown in Figure 7D. Of the tested proteins in this dot blot, binding could only be detected against full-length hTSLP and HSA.

Example 8

Characterization of TSLP binding polypeptides in an *in vitro* potency assay

Summary

A PathHunter® eXpress IL7R/CRLF2 Dimerization assay (Eurofins/DiscoverX) was used to evaluate the potency of polypeptides targeting TSLP. The PathHunter® Dimerization assay detects TSLP induced dimerization of the IL-7 receptor alpha-chain (IL-7Rα) and the TSLP receptor

alpha chain 1 (TSLP-R; encoded by CRLF). The assay uses the Enzyme Complementation Technology (EFC), which is based on two recombinant β-galactosidase enzyme fragments, the enzyme acceptor (EA) and the enzyme donor (ED) that are fused to one of the two TSLP receptor subunits each. Binding of TSLP to one receptor subunit induces receptor dimerization and subsequent enzyme fragment complementation, leading to an active enzyme that can generate a chemiluminescent signal upon substrate hydrolyzation.

Materials and methods

On the day of the assay, 10,000 cells per well were seeded in Cell Plating 25 Reagent (Eurofins/DiscoverX) in a white 96-well TC plates (Eurofins/DiscoverX) and incubated for 4 h at 37°C, in a CO_2 incubator. During this time, Z variants in fusion with ABD variant PP013 (SEQ ID NO:1077) were serially titrated in Cell Plating 25 Reagent containing Recombumin Elite (Albumedix). TSLP (R&D Systems, cat. no. 1398-TS/CF, SEQ ID NO:1079) was diluted to a final concentration of 1 ng/mL. Serially titrated polypeptides and TSLP target protein were pre-incubated together for 30 min before added to the cells. 10 µL of mixed polypeptides and TSLP protein were added to the cells, which were incubated for 16 h at 37°C. After the incubation, 110 µL of Flash Detection Reagent (1:4 mix of Flash Cell Assay Buffer and Flash Substrate Reagent, both from Eurofins/DiscoverX) were added to each well and plates were incubated for 1 h at RT in the dark. The luminescent signal was measured in an EnSpire Multimode reader (PerkinElmer).

Results

All tested TSLP binding Z variants in fusion with ABD showed potency of inhibiting TSLP-induced dimerization. The calculated IC50 values, ranging between 0.03 and 23 nM, are presented in Table 10. A representative graph is shown in Figure 8 by way of illustration.

Table 10: Calculated IC50 values from the PathHunter® eXpress IL7R/CRLF2 Dimerization assay

Z-ABD variant	SEQ ID NO	IC50 (nM)
ZTPA092	1064	4.2[1]
ZTPA003	975	9.1
ZTPA004	976	7
ZTPA005	977	22.8

ZTPA006	978	0.5[1]
ZTPA007	979	13.9
ZTPA008	980	8.4
ZTPA009	981	10.3
ZTPA010	982	2.7
ZTPA011	983	0.6[1]
ZTPA012	984	17.2
ZTPA013	985	9.3
ZTPA014	986	14.3
ZTPA015	987	0.4[1]
ZTPA016	988	9.6
ZTPA017	989	8.9
ZTPA018	990	5.1
ZTPA019	991	19
ZTPA020	992	20.3
ZTPA021	993	5.3
ZTPA002	974	0.4[2]
ZTPA001	973	0.5[2]
ZTPA022	994	15.9
ZTPA027	999	0.4[1]
ZTPA023	995	12.2
ZTPA024	996	16.5
ZTPA025	997	14
ZTPA026	998	13.5
ZTPA093	1065	0.5[1]
ZTPA094	1066	0.5
ZTPA095	1067	0.6
ZTPA096	1068	0.5
ZTPA097	1069	0.6
ZTPA098	1070	0.5
ZTPA099	1071	1.5
ZTPA100	1072	4.2
ZTPA101	1073	2.9
ZTPA102	1074	0.9
ZTPA103	1075	0.9
ZTPA104	1076	0.03

[1] *Mean value of two IC50 determinations*

[2] *Mean value of three IC50 determinations*

Example 9

Pharmacokinetic study of ZTPA001 in non-human primates

Summary

This Example describes a single dose pharmacokinetic (PK) study of ZTPA001 (SEQ ID NO:973) administered intravenously (i.v.) to cynomolgus monkeys. Blood samples were collected up to three weeks post dose and analyzed using an antibody-based sandwich PK-ELISA.

Materials and methods

PK study in NHP: ZTPA001 was administered at 1.33 mg/kg by a bolus i.v. injection to non-naïve, non-fasted cynomolgus monkeys (n=3; 5.0-5.3 kg) on Day 1. A dose volume of 0.5 mL/kg was used for each injection. Blood samples (0.5 mL) were collected at the following time points: predose, at 10 and 30 min and 1, 6, 10, 26, 50, 72, and 96 h postdose and at days 6, 7, 8, 11, 13, 15, 18, and 22. Blood samples were allowed to clot at RT for at least 30 min prior to centrifugation. After centrifugation, sera were extracted, aliquoted and stored at −70°C until further analysis.

Quantification of ZTPA001 by ELISA: 96-well half area plates were coated with mouse anti-Z monoclonal antibody (2 µg/mL) in PBS (50 µL/well) and incubated overnight at 4°C. Plates were washed in PBST and blocked with Blocker® casein (ThermoScientific) for 1.5 h at 22°C. ZTPA001 standard was titrated in a 1.5-fold dilution series (50-1.3 pM) in 1% cynomolgus serum pool in Blocker® casein. Serum samples were diluted 1:100 in Blocker® casein followed by 1:50 dilution in 1% cynomolgus serum pool and 1:4 serial dilution in 1% cynomolgus serum pool. Calibration standards and diluted serum samples were added to coated ELISA plates (50 µL/well) and incubated for 1.5 h at 22°C. Following washing with PBST, a rabbit anti-ABD polyclonal antibody (2 µg/mL) was added. After incubation for 1.5 h at 22°C, the plates were washed with PBST and 100 ng/mL HRP-conjugated donkey anti-rabbit IgG (Jackson Immuno Research cat. no. 711-035-152) was added to each well. After one additional hour of incubation and subsequent washing in PBST, the plates were developed with TMB (50 µL/well) for 15 min at RT and the reactions were stopped by addition of 0.2M H_2SO_4 (50 µL/well). The absorbance at 450 nm was measured in a microplate plate reader (PerkinElmer Enspire LF).

Evaluation of the PK profile: ZTPA001 serum concentration versus time profiles were evaluated by non-compartmental analysis using linear up-log down extrapolation in Phoenix WinNonlin version 8.3.

Results

The PK profile of ZTPA001 in cynomolgus monkey after a single i.v. dose is shown in Figure 9A. In cynomolgus monkeys 1 and 2, the terminal half-life was determined to 3–4 days with an area under the concentration versus time curve to the last observed time point of 11 h*mg/mL.

Example 10

Proof of mechanism study of ZTPA001 in non-human primates

Summary

This Example describes a single dose proof of mechanism (POM) study of ZTPA001 administered i.v. to cynomolgus monkeys. Blood samples were collected up to three weeks post ZTPA001 dose and analyzed using an antibody-based sandwich PK-ELISA. Cytokine and chemokine levels were determined on the Meso Scale Discovery platform (MSD).

Materials and methods

POM study in NHP: TSLP was administered by i.v. bolus at 6.25, 31.25 and 62.5 µg/kg to non-fasted female cynomolgus monkeys (n=3; 3.5-4.1 kg; one naïve and two non-naïve) on Days 1, 8, and 16, respectively. A dose volume of 0.5 mL/kg was used for each injection. Serum samples were obtained at predose, and at 4, 8, 24, and 48 h post TSLP administration. On Day 30, ZTPA001 was administered as a single 1.33 mg/kg dose by i.v. (bolus) on Day 1. Two hours after the i.v. ZTPA001 administration, 62.5 µg TSLP was administered via i.v. injection. A dose volume of 0.5 mL/kg was used for each injection. Blood samples (0.5 mL) for ZTPA001 serum concentrations were collected at the following time points: predose, 10 and 130 min (10 min after TSLP dosing) and 3, 6, 10, 26, 50, 72, and 96 h post ZTPA001 dose and at days 8, 11, 15, 18, and 22. Blood samples for cytokine and chemokine levels were obtained at predose, and at 4, 8, 24, and 48 h post TSLP administration. The blood samples were allowed to clot at RT for at least 30 min prior to centrifugation. After centrifugation, sera were extracted, aliquoted and stored at −70°C until further analysis.

Quantification of ZTPA001 by ELISA: The analysis was performed as described in Example 9.

Evaluation of the PK profile: ZTPA001 serum concentration versus time profiles were evaluated by non-compartmental analysis using linear up-log down extrapolation in Phoenix WinNonlin version 8.3.

Quantification of cytokines and chemokines by MSD: Cytokine and chemokine analysis were performed on sera obtained at predose, 4, 8, 24 and 48 h post TSLP administration, on Days 1, 8, 16 and 30. Cytokines (IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A, IFN-γ, GM-CSF) and chemokines (MIP-1β, eotaxin-3, TARC, IP-10, MIP-1α, IL-8 HA, MCP-1, MDC, MCP-4) were measured using V-PLEX Plus NHP Cytokines 24-Plex kit (Meso Scale Discovery, Rockville, MD, cat. no. K15058G) and analyzed on a MESO QuickPlex SQ 120 reader (Meso Scale Discovery, Rockville, MD). Serum samples were diluted 1:4 and analyzed according to the manufacturer's instructions for cytokine panel 1 (NHP) kit, proinflammatory panel 1 (NHP) kit and chemokine panel 1 (NHP) kit, respectively. The cytokine and chemokine serum concentration were evaluated by MSD Discovery Workbench version 4.0.

Results

The ZTPA001 serum concentration versus time relationship in cynomolgus monkey after a single i.v. dose is shown in Figure 9B. Mean terminal half-life was determined to 4 days with a mean area under the concentration versus time curve to the time of the last quantifiable concentration of 5.7 h*mg/mL. Of the 24 cytokines and chemokines measured in NHP PoM study, 16 were above both LOD (limit of detection) and LLOQ (lower limit of quantification), thereby meeting the acceptance criteria (IL-2, IL-5, IL-6, IL-7, IL-8, IL-12/IL-23p40, IL-15, IL-16, VEGF-A, MIP-1α, MIP-1β, TARC, IP-10, MCP-1, MDC and MCP-4). Eight of the cytokines and chemokines did not meet the acceptance criteria as they were below LOD or LLOQ (IL-1β, IL-10, IL-17A, TNF-β, IFN-γ, GM-CSF, eotaxin-3 and IL-8 HA). TSLP administration at a dose of 6.25 µg/kg did not lead to upregulation of cytokines/chemokines level. TSLP administration at a dose of 31.25 µg/kg resulted in increased production in a few of the cytokines/chemokines, most prominent for IL-2, TARC, MDC, MCP-1 and MCP-4. The highest dose of 62.5 ug/kg TSLP triggered upregulation of several proinflammatory and asthma associated cytokines/chemokines, most prominent for: IL-2, IL-5, IL-6,

IL-8, IL-16, TARC, MDC, MCP-1, MCP-4 and IP-10 with the highest concentration reached at 4-8 h post administration. A single i.v. injection of ZTPA001 resulted in a reduction of several of those proinflammatory cytokines and chemokines. The serum concentration of asthma associated cytokines and chemokines versus time profile after TSLP administration in the absence or presence of ZTPA001 are presented in Figure 10, which shows that a single i.v. dose of ZTPA001 leads to reduction of asthma associated cytokines.

Example 11

Pharmacokinetic study of ZTPA001 and ZTPA104 in rat

Summary

This Example describes a single dose pharmacokinetic (PK) study of ZTPA001 (SEQ ID NO:973) and ZTPA104 (SEQ ID NO:1076) administered intravenously (i.v.) to Sprague-Dawley rats. Blood samples were collected up to three weeks postdose and serum concentrations were analyzed using an antibody-based sandwich PK-ELISA.

Materials and methods

PK study in rat: ZTPA001 and ZTPA104, respectively, were administered at 1.2 mg/kg by a i.v. injection to male Sprague-Dawley rats (Charles River, Germany; n=6/test item) on Day 1. A dose volume of 5 mL/kg was used for each injection. Due to the 8 mL/kg blood sampling restriction, blood sampling time points were divided into two cohorts as follows: cohort A (n=3 per test item) were bled predose, at 5 min and 1, 8, 72, 168, 264, 408 and 504 h postdose; and cohort B (n=3 per test item) were bled predose, at 30 min and 3, 24, 120, 216, 336 and 456 h postdose. Blood samples were allowed to clot at RT for at least 30 min prior to centrifugation. After centrifugation, sera were extracted, aliquoted and stored at −80 °C until further analysis.

Quantification by ELISA: The ELISA assay was performed essentially as described in Example 9, but with the following exceptions: Exception 1: ZTPA001 standard was titrated in a 1.5-fold dilution series (50-0.87 pM) in 1 % rat serum pool (Sprague Dawley; Bio IVT) in Blocker® casein. Serum samples were diluted 1:100 in Blocker® casein followed 1:5 serial dilution in 1 % rat serum pool. Exception 2: ZTPA104 standard was titrated in a 1.5-fold dilution series (25-0.43 pM) in 1 % rat serum pool in Blocker® casein. Serum

samples were diluted 1:100 in Blocker® casein followed by 1:100 dilution in 1 % rat serum pool (except the samples taken at predose) and 1:5 serial dilution in 1 % rat serum pool. The lower limit of quantification (LLOQ) was 1.03 ng/mL for ZTPA001 and 0.52 ng/mL for ZTPA104.

Evaluation of the PK profile: Mean ZTPA001 and ZTPA104 serum concentrations versus time profiles were evaluated by non-compartmental analysis using linear up-log down extrapolation in Phoenix WinNonlin version 8.3. Concentration values under the LLOQ were excluded from the analysis.

Results

The PK profiles of ZTPA001 and ZTPA104 in rat after a single i.v. dose are shown in Figure 11. The two polypeptides show a similar PK profile with a maximum serum concentration (C_{max}) of 41 and 35 µg/mL, a dose normalized area under the concentration versus time curve to the time of last quantifiable serum concentration or extrapolated to infinity (AUC/dose) of 1.2 and 1.1 h*kg/mL, and a terminal half-life ($t_{1/2}$) of 29 and 36 h for ZTPA001 and ZTPA104, respectively.

Example 12

Inhalation study of ZTPA001 in non-human primates

Summary

This Example describes a study of repeated administration of ZTPA001 (SEQ ID NO:973) via inhalation to cynomolgus monkeys. Blood, bronchoalveolar lavage (BAL) and lung tissue samples were collected approximately 24 h after the final dose of ZTPA001 and analyzed by LC-MS/MS for compound concentration. Retained TSLP binding functionality of systemically distributed ZTPA001 was confirmed by ELISA.

Materials and methods

Inhalation study in NHP: ZTPA001 was administered nominally at 1.2, 6.7 or 12 mg/kg via a daily 1-hour inhalation for 7 doses to cynomolgus monkeys (3.2-4.7 kg; one male and one female per dose level). Test aerosols were generated using a jet nebulizer to facilitate inhalation by face mask. Blood samples (0.5 ml) were collected in K_2EDTA blood tubes on Day 1 (first administration) and Day 7 (last administration) at the following time points: at predose, immediately after dosing, and at 1, 3, 7 and 23 h post end of administration. Plasma was obtained by centrifugation at 2000 × g for 10 min at 4°C within 2 h of blood collection. After centrifugation plasma was

extracted, aliquoted and stored at -70°C until further analysis. At termination, bronchoalveolar lavage (BAL) and tissue samples of the proximal and distal lung were obtained and stored frozen at -70°C until bioanalysis.

Quantification of ZTPA001: Plasma, lung tissue homogenate and BAL fluid concentrations were determined at by liquid chromatography tandem mass spectrometry (LC-MS/MS). The SMART Digest Trypsin kit (Thermo Fisher Scientific, cat. no 60113-01) was used for digestion of the samples. A unique signature tryptic peptide was used for quantification and a corresponding stable isotope labeled peptide was used as internal standard.

Evaluation of the PK profile: Profiles of mean ZTPA001 serum concentrations versus time were evaluated by non-compartmental analysis using linear up-log down extrapolation in Phoenix WinNonlin version 8.3.

TSLP binding ELISA: Four samples with high plasma levels were selected to be tested in a human TSLP binding ELISA. These samples had been collected at Day 7, 23 h post end of administration and originated from one male and one female monkey administrated with a nominal dose of 6.7 mg/kg, and one male and one female monkey administrated with a nominal dose of 12 mg/kg. The TSLP binding capacity of the plasma samples was compared to ZTPA001 spiked into cynomolgus plasma in the same range as the samples (Spike low) or spiked at a higher concentration of ZTPA001 (Spike high). A negative control of non-spiked blank plasma was also included.

Two half area assay plates were coated at 4°C overnight with 1 µg/mL recombinant human TSLP (RnD Systems, cat. no. 1398-TC/CF) in PBS. On the following day, the plates were washed with PBST 0.05% and blocked for 1 h at 22°C in Blocker® casein in PBS. Meanwhile, the samples and controls were diluted in matrix (Blocker® casein in PBS supplemented with 1% cynomolgus plasma): The samples and the Spike low control were each diluted to 100 nM and the Spike high control was diluted to 5000 nM. Duplicate dilutions were made for all samples and controls, and 3-fold dilution series were made in matrix. The samples were added to the wells of the blocked plates, incubated for 1 h at 22°C and washed in PBST 0.05%. A monoclonal mice anti-ABD Ig was used for primary detection and an anti-mouse Ig-HRP for secondary detection. The plates were developed with TMB substrate and the reactions subsequently stopped by addition of 0.2 M H_2SO_4. The absorbance at 450 nm was measured using an EnSpire plate reader.

Results

Repeated administration of ZTPA001 by inhalation resulted in high BAL fluid and lung tissue levels with distribution to serum/plasma observed already after the first dose. At 24 h after initiation of the last of seven daily inhaled doses, a higher dose resulted in increased ZTPA001 concentrations in BAL (Figure 12A). In lung tissue homogenate the ZTPA001 concentrations were more variable, but generally the concentrations were lowest in the 1.2 mg/kg/day dose group (Figure 12B). No differences between left or right lung or between tissue sampling locations (proximal or distal) were observed. The ZTPA001 plasma concentration versus time relationship in cynomolgus monkeys after the first (Day 1) and last (Day 7) daily administration of ZTPA001 is shown in Figure 13. On Day 1, the ZTPA001 concentration in plasma gradually increased over time with maximum concentration reached at 23 h post end of inhalation, i.e. predose Day 2. Repeated administration further increased ZTPA001 plasma concentrations on Day 7.

The TSLP binding capacity of ZTPA001 in four selected plasma samples (showing high concentration of ZTPA001 as determined by LC-MS/MS) from four different animals were assessed in a human TSLP binding ELISA. The titration curves of the test samples, the Spike low control and the Spike high control overlapped well (Figure 14), i.e. all four test samples displayed similar binding to human TSLP as the spiked controls. For the negative control, no binding to TSLP was detected. This indicates that ZTPA001 retains the human TSLP binding function after being nebulized, inhaled and systemically distributed. Furthermore, since an anti-ABD antibody is used to detect ZTPA001 in the TSLP ELISA, the results also indicate that the ABD moiety of the ZTPA001 polypeptide is intact.

ITEMIZED LISTING OF EMBODIMENTS

1. TSLP binding polypeptide, comprising a TSLP binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EAVX_4ALX_7EIW\,X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LRD$ (SEQ ID NO:1081)

wherein, independently of each other,

X_4 is selected from D, E, and H;
X_7 is selected from I, L, M and V;
X_{11} is selected from A, D, E, K, N, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, T, W and Y;
X_{21} is selected from D, E, G, H, K, M, N, Q and R;
X_{25} is selected from A, H, I, K, L, Q, R, V and Y; and
X_{26} is selected from K and S;

and

ii) an amino acid sequence which has at least 93 % identity to the sequence defined in i).

2. TSLP binding polypeptide according to item 1, wherein, in sequence i),

X_4 is selected from D, E and H;
X_7 is selected from I, L, M and V;
X_{11} is selected from A, D, E, K, N, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, T, W and Y;

X_{21} is selected from D, E, G, H, K, M, N, Q and R;

X_{25} is selected from A, H, I, K, L, Q, R, V and Y; and

X_{26} is selected from K and S.

3. TSLP binding polypeptide according to any preceding item, wherein, in sequence i),

X_4 is selected from E and H;

X_7 is selected from I, L and V;

X_{11} is selected from A, D, E, Q, R, S and T;

X_{16} is selected from N and T;

X_{17} is selected from A, D, E, G, H, K, N, Q, R, S, T, V, W and Y;

X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;

X_{20} is selected from H, N, Q, W and Y;

X_{21} is selected from D, E, H, K, M, N, Q and R;

X_{25} is selected from H, I, K, L, Q, R, V and Y; and

X_{26} is selected from K and S.

4. TSLP binding polypeptide according to any preceding item, wherein, in sequence i),

X_4 is selected from E and H;

X_7 is selected from I, L and V;

X_{11} is selected from A, D, E, Q, S and T;

X_{16} is T;

X_{17} is selected from D, E, G, H, N, Q, R, S, W and Y;

X_{18} is selected from A, D, E, F, G, H, I, L, N, Q, R, S, T, W and Y;

X_{20} is selected from H, W and Y;

X_{21} is selected from D, E, H, N and Q;

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

X_{26} is K.

5. TSLP binding polypeptide according to any preceding item, wherein, in sequence i),

X_4 is E;

X_7 is selected from I and V;

X_{11} is selected from A, D, E, Q, S and T;

X_{16} is T;

X_{17} is selected from D, E, G, H, N, Q, R and Y;

X_{18} is selected from A, D, E, F, G, I, L, N, Q, R, S, T and Y;

X_{20} is selected from H, W and Y;

X_{21} is selected from D, E, H, N and Q;

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

X_{26} is K.

6. TSLP binding polypeptide according to any preceding item, wherein, in sequence i),

X_4 is E;

X_7 is V;

X_{11} is selected from A and T;

X_{16} is T;

X_{17} is R;

X_{18} is selected from D and E;

X_{20} is W;

X_{21} is Q;

X_{25} is Y; and

X_{26} is K.

7. TSLP binding polypeptide according to any preceding item, wherein sequence i) fulfills at least four of the eight conditions I-VIII:

I. X_4 is E or H;

II. X_7 is selected from I, L and V;

III. X_{11} is selected from A, D, E, Q, S and T;

IV. X_{16} is T;

V. X_{20} is selected from H, W and Y;

VI. X_{21} is selected from D, E, H, N and Q;

VII. X_{25} is selected from I, L, R, V and Y; and

VIII. X_{26} is K.

8. TSLP binding polypeptide according to item 7, wherein sequence i) fulfils at least five of the eight conditions I-VIII.

9. TSLP binding polypeptide according to item 8, wherein sequence i) fulfils at least six of the eight conditions I-VIII.

10. TSLP binding polypeptide according to item 9, wherein sequence i) fulfils at least seven of the eight conditions I-VIII.

11. TSLP binding polypeptide according to item 10, wherein sequence i) fulfils all of the eight conditions I-VIII.

12. TSLP binding polypeptide according to any one of items 1-5, wherein X_4 is E, X_7 is V and X_{20} is W.

13. TSLP binding polypeptide according to any one of items 1-5, wherein X_4 is E, X_7 is V and X_{21} is Q.

14. TSLP binding polypeptide according to any one of items 1-5, wherein X_4 is E, X_7 is V and X_{25} is Y.

15. TSLP binding polypeptide according to item 1, wherein the binding motif sequence corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO: 1-875.

16. TSLP binding polypeptide according to item 15, 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:1-645.

17. TSLP binding polypeptide according to item 16, 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:1-91.

18. TSLP binding polypeptide according to item 17, 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:1-27.

19. TSLP binding polypeptide according to item 18, 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:1-2.

20. TSLP binding polypeptide according to any preceding item, wherein said binding motif forms part of a three-helix bundle protein domain.

21. TSLP binding polypeptide according to item 20, wherein said binding motif essentially forms part of two helices with an interconnecting loop, within said three-helix bundle protein domain.

22. TSLP binding polypeptide according to item 20 or 21, wherein said three-helix bundle protein domain is selected from bacterial receptor domains.

23. TSLP binding polypeptide according to any one of items 20-22, wherein said three-helix bundle protein domain is selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof.

24. TSLP 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]*-DPSQS$X_a$$X_bLLX_c$EAKKL$X_d$$X_e$$X_f$Q (SEQ ID NO:1082);

wherein

[BM] is a TSLP binding motif as defined in any one of items 1-19;

X_a is selected from A and S;

X_b is selected from E and N;

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 the sequence defined in iii).

25. TSLP binding polypeptide according to item 24, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-875.

26. TSLP binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

v) YA-[*BMod*]-AP (SEQ ID NO:1083);

wherein [*BMod*] is as defined in item 24 or 25; and

vi) an amino acid sequence which has at least 86 % identity to a sequence defined by v).

27. TSLP binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

vii) VDAKYAK-*[BM]*-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1097);

wherein [*BM*] is as defined in any one of items 1-19; and

viii) an amino acid sequence which has at least 86 % identity to a sequence defined by vii).

28. TSLP binding polypeptide according to item 27, wherein sequence vii) is selected from the group consisting of SEQ ID NO:1-636, 639-644 and 646-875.

29. TSLP binding polypeptide according to any one of items 1-25, which comprises an amino acid sequence selected from:

ix) AEAKFAK-[*BM*]-DPSQSSELLSEAKKLSESQAPK (SEQ ID NO:1095);

wherein [*BM*] is as defined in any one of items 1-19; and

x) an amino acid sequence which has at least 86 % identity to a sequence defined by ix).

30. TSLP binding polypeptide according to item 29, wherein sequence ix) is selected from the group consisting of SEQ ID NO:637 and SEQ ID NO:638.

31. TSLP binding polypeptide according to any one of items 1-26, which comprises an amino acid sequence selected from:

xi) AEAKYAK-[*BM*]-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1093);

wherein [*BM*] is as defined in any one of items 1-19; and

xii) an amino acid sequence which has at least 86 % identity to a sequence defined by xi).

32. TSLP binding polypeptide according to item 31, wherein sequence xi) is selected from the group consisting of SEQ ID NO:645 and 876-972.

33. TSLP binding polypeptide according to any preceding item, which is capable of binding to TSLP such that the K_D value of the interaction with TSLP is at most 1×10^{-6} M, for example as at most 5×10^{-7} M, for example at most 1×10^{-7} M, for example at most 5×10^{-8} M, for example at most 1×10^{-8} M.

34. TSLP binding polypeptide according to any preceding item, which comprises additional amino acids at the C-terminal and/or N-terminal end.

35. TSLP binding polypeptide according to item 34, wherein said additional amino acid(s) improve(s) production, purification, stabilization *in vitro* or *in vivo*, coupling or detection of the polypeptide.

36. TSLP binding polypeptide according to any preceding item in multimeric form, comprising at least two TSLP binding polypeptide monomer units, whose amino acids may be the same or different.

37. TSLP binding polypeptide according to item 36, wherein said TSLP binding polypeptide monomer units are covalently coupled together.

38. TSLP binding polypeptide according to item 36, wherein said TSLP binding polypeptide monomer units are expressed as a fusion protein.

39. TSLP binding polypeptide according to any one of items 36-38, in dimeric form.

40. Fusion protein or conjugate comprising

- a first moiety consisting of a TSLP binding polypeptide according to any preceding item; and
- a second moiety consisting of a polypeptide having a desired biological activity.

41. Fusion protein or conjugate according to item 40, wherein said desired biological activity is a therapeutic activity.

42. Fusion protein or conjugate according to item 40, wherein said desired biological activity is a binding activity.

43. Fusion protein or conjugate according to item 40, wherein said desired biological activity is an enzymatic activity.

44. Fusion protein or conjugate according to item 42, wherein said desired binding activity is albumin binding activity which increases in vivo half-life of the fusion protein or conjugate.

45. Fusion protein or conjugate according to item 44, wherein said second moiety comprises the albumin binding domain of streptococcal protein G or a derivative thereof.

46. Fusion protein or conjugate according to item 45, which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:973-1076 and 1129.

47. Fusion protein or conjugate according to item 46, which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:973-1076.

48. Fusion protein or conjugate according to item 47, which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:973-999 and 1064-1076.

49. Fusion protein or conjugate according to item 48, which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:973, 974, 1065 and 1076.

50. Fusion protein or conjugate according to item 49, which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:973 and 1076.

51. Fusion protein or conjugate according to item 50, which comprises the amino acid sequence SEQ ID NO:973.

52. Fusion protein or conjugate according to item 50, which comprises the amino acid sequence SEQ ID NO:1076.

53. Fusion protein or conjugate according to item 42, wherein said binding activity acts to block biological activity.

54. Fusion protein or conjugate according to item 41, wherein the second moiety is a therapeutically active polypeptide.

55. Fusion protein or conjugate according to any one of items 40-43 and 53-54, wherein the second moiety is selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

56. Fusion protein or conjugate according to any one of items 40-43, wherein the second moiety is selected from the group consisting of an antibody and an antigen binding fragment thereof.

57. Fusion protein or conjugate according to item 56, wherein said antibody or antigen binding fragment thereof is selected from the group consisting of of full-length antibodies, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, single chain Fab (scFab) fragments, Fc fragments, Fv fragments,

single chain Fv (scFv) fragments, $(scFv)_2$, scFv-Fc constructs and domain antibodies.

58. Fusion protein or conjugate according to item 57, wherein said at least one antibody or antigen binding fragment thereof is selected from the group consisting of full-length antibodies, Fab fragments and scFv fragments.

59. Fusion protein or conjugate according to item 58, wherein said at least one antibody or antigen binding fragment thereof is a full-length antibody.

60. Fusion protein according to any one of items 40-59, wherein the second moiety further comprises a linker.

61. TSLP binding polypeptide, fusion protein or conjugate according to any preceding item, further comprising a label, for example selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds, bioluminescent proteins, enzymes, radionuclides and radioactive particles.

62. Polynucleotide encoding a TSLP binding polypeptide, fusion protein, or conjugate according to any preceding item.

63. Expression vector encoding a polynucleotide according to item 62.

64. Host cell comprising an expression vector according to item 63.

65. Method of producing a polypeptide, fusion protein or conjugate according to any one of items 1-60 comprising

- culturing a host cell according to item 64 under conditions permissive of expression of said polypeptide from said expression vector; and
- isolating said polypeptide.

66. Composition comprising a TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-61 and at least one pharmaceutically acceptable excipient or carrier.

67. TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-60 or a composition according item 66 for oral, respiratory, topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual or suppository administration, such as for respiratory, intravenous or subcutaneous administration.

68. TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-60 or a composition according to item 66 for use as a medicament, a diagnostic agent or a prognostic agent.

69. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to item 68 as a medicament.

70. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to item 68 as a diagnostic agent and/or a prognostic agent.

71. TSLP binding polypeptide, fusion protein, conjugate or composition for use as a medicament according to item 69, wherein said polypeptide, fusion protein, conjugate or composition modulates TSLP function in vivo.

72. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 68-71 in the treatment, prognosis or diagnosis of a TSLP related disorder.

73. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to item 72, wherein said TSLP related disorder is selected from inflammatory, autoimmune and cancer diseases.

74. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to item 72 or 73, wherein said TSLP related disorder is selected from the group consisting of respiratory diseases, dermatological diseases, allergies, eye diseases, gastrointestinal diseases and cancers.

75. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 72-74, wherein said TSLP related disorder or disease is selected from the group consisting of asthma, atopic dermatitis, atopic keratoconjunctivitis, urticaria, allergic rhinitis, chronic rhinosinusitis with nasal polyps, eosinophilic esophagitis, chronic obstructive pulmonary disease (COPD), eosinophilic granulomatosis with polyangiitis (EGPA)/Churg–Strauss syndrome, breast cancer, prurigo nodularis, and bullous pemphigoid.

76. Method of treatment of a TSLP related disorder, comprising administering to a subject in need thereof an effective amount of a TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-60 or a composition according to item 66.

77. Method according to item 76, wherein said TSLP related disorder is selected from inflammatory, autoimmune and cancer diseases.

78. Method according to item 77, wherein said TSLP related disorder is selected from the group consisting of respiratory diseases, dermatological diseases, allergies, eye diseases, gastrointestinal diseases and cancers.

79. Method according to item 78, wherein said TSLP related disorder or disease is selected from the group consisting of asthma, atopic dermatitis, atopic keratoconjunctivitis, urticaria, allergic rhinitis, chronic rhinosinusitis with nasal polyps, eosinophilic esophagitis, chronic obstructive pulmonary disease

(COPD), eosinophilic granulomatosis with polyangiitis (EGPA)/Churg–Strauss syndrome, breast cancer, prurigo nodularis, and bullous pemphigoid.

80. Method of detecting TSLP *in vitro*, comprising providing a sample suspected to contain TSLP, contacting said sample with a TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-61 or a composition according to item 66, and detecting the binding of the TSLP binding polypeptide, fusion protein, conjugate or composition to indicate the presence of TSLP in the sample.

81. Method for determining the presence of TSLP in a subject, comprising the steps of:

a) contacting the subject, or a sample isolated from the subject, with a TSLP binding polypeptide, fusion protein or conjugate according to any one of items 1-61 or a composition according to item 66, and

b) obtaining a value corresponding to the amount of the TSLP binding polypeptide, fusion protein, conjugate or composition that has bound in said subject or to said sample.

82. Method according to item 81, further comprising a step of comparing said value to a reference.

83. Method according to item 81 or 82, wherein said subject is a mammalian subject, such as a human subject.

84. Method according to any one of items 81-83, wherein the method is performed *in vivo*.

CLAIMS

1. TSLP binding polypeptide, comprising a TSLP binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EAVX_4ALX_7EIW\,X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LRD$ (SEQ ID NO:1081)

wherein, independently of each other,

X_4 is selected from D, E, and H;
X_7 is selected from I, L, M and V;
X_{11} is selected from A, D, E, K, N, Q, R, S and T;
X_{16} is selected from N and T;
X_{17} is selected from A, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W and Y;
X_{18} is selected from A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
X_{20} is selected from H, N, Q, T, W and Y;
X_{21} is selected from D, E, G, H, K, M, N, Q and R;
X_{25} is selected from A, H, I, K, L, Q, R, V and Y; and
X_{26} is selected from K and S;

and

ii) an amino acid sequence which has at least 93 % identity to the sequence defined in i).

2. TSLP binding polypeptide according to claim 1, wherein, in sequence i),
X_4 is E;
X_7 is V;
X_{11} is selected from A and T;
X_{16} is T;
X_{17} is R;
X_{18} is selected from D and E;
X_{20} is W;
X_{21} is Q;
X_{25} is Y; and
X_{26} is K.

3. TSLP binding polypeptide according to claim 1, wherein the binding motif sequence corresponds to the sequence from position 8 to position 36 in a sequence selected from the group consisting of SEQ ID NO: 1-875, for example selected from the group consisting of SEQ ID NO:1-645.

4. TSLP binding polypeptide according to any preceding claim, wherein said binding motif forms part of a three-helix bundle protein domain.

5. TSLP binding polypeptide according to any preceding claim, which comprises a binding module (*BMod*), the amino acid sequence of which is selected from:
iii) K-*[BM]*-DPSQSX$_a$X$_b$LLX$_c$EAKKLX$_d$X$_e$X$_f$Q (SEQ ID NO:1082);
wherein
[BM] is a TSLP binding motif as defined in any one of claims 1-3;
X$_a$ is selected from A and S;
X$_b$ is selected from E and N;
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 the sequence defined in iii).

6. TSLP binding polypeptide according to claim 5, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-875.

7. TSLP binding polypeptide according to any preceding claim, which comprises an amino acid sequence selected from:
xi) AEAKYAK-[*BM*]-DPSQSSELLSEAKKLNDSQAPK (SEQ ID NO:1093);
wherein [*BM*] is as defined in any one of claims 1-3; and
xii) an amino acid sequence which has at least 86 % identity to a sequence defined by xi).

8. TSLP binding polypeptide according to claim 7, wherein sequence xi) is SEQ ID NO:645 and 876-972.

9. TSLP binding polypeptide according to any preceding claim, which is capable of binding to TSLP such that the K_D value of the interaction with TSLP is at most 1 x 10^{-6} M.

10. Fusion protein or conjugate comprising
- a first moiety consisting of a TSLP binding polypeptide according to any preceding claim; and
- a second moiety consisting of a polypeptide having a desired biological activity.

11. Polynucleotide encoding a TSLP binding polypeptide or fusion protein according to any preceding claim.

12. Composition comprising a TSLP binding polypeptide, fusion protein or conjugate according to any one of claims 1-10 and at least one pharmaceutically acceptable excipient or carrier.

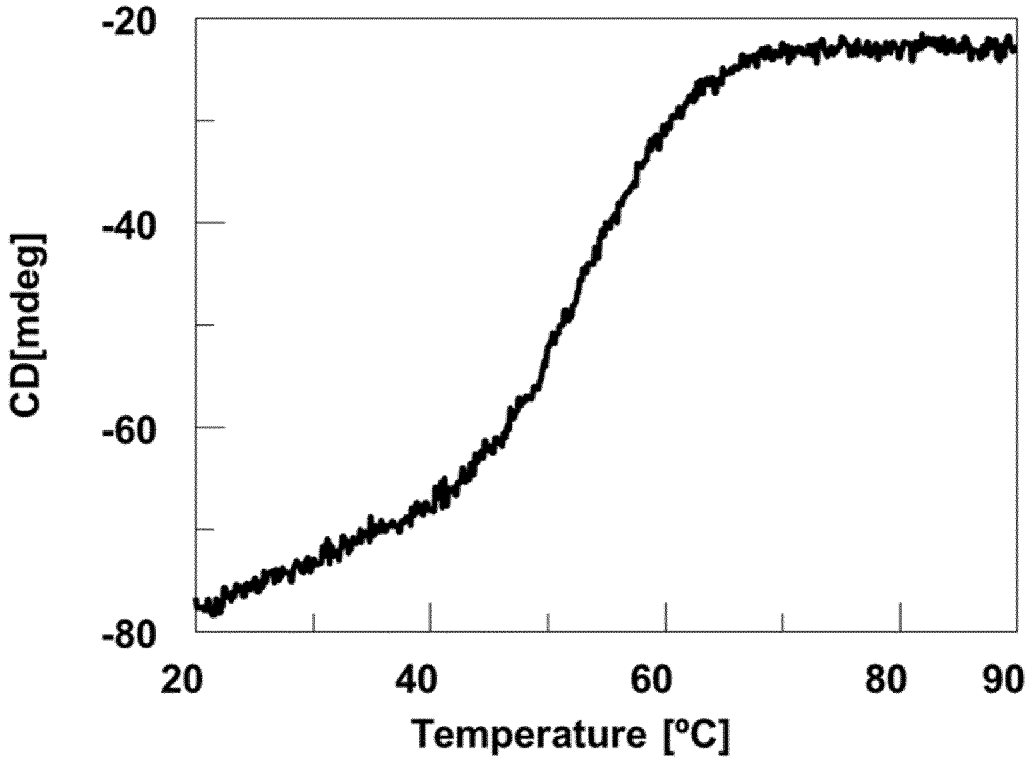
13. TSLP binding polypeptide, fusion protein or conjugate according to any one of claims 1-10 or composition according to claim 12 for use as a medicament, a diagnostic agent or a prognostic agent.

14. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to claim 13 in the treatment, prognosis or diagnosis of a TSLP-related disorder or disease, such as an inflammatory disease, an autoimmune disease or a cancer disease.

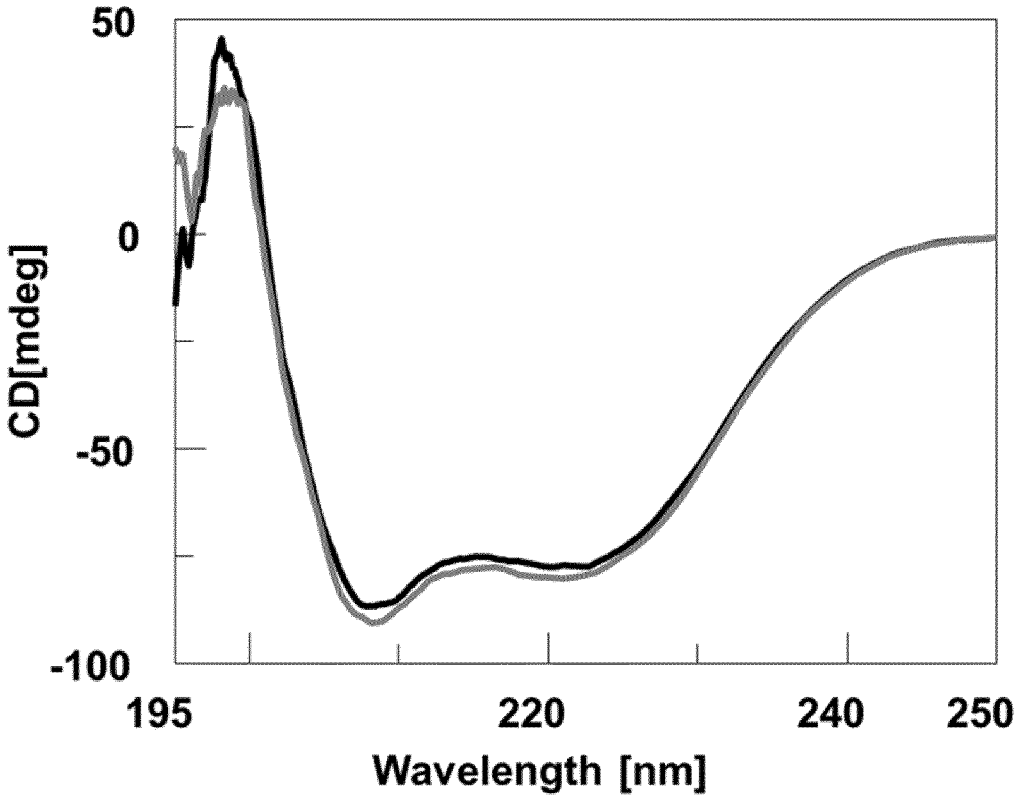
15. TSLP binding polypeptide, fusion protein, conjugate or composition for use according to claim 14, wherein said disorder or disease is selected from the group consisting of asthma, atopic dermatitis, atopic keratoconjunctivitis, urticaria, allergic rhinitis, chronic rhinosinusitis with nasal polyps, eosinophilic esophagitis, chronic obstructive pulmonary disease, eosinophilic granulomatosis with polyangiitis (EGPA)/Churg–Strauss syndrome, breast cancer, prurigo nodularis, and bullous pemphigoid.

Figure 1A-B

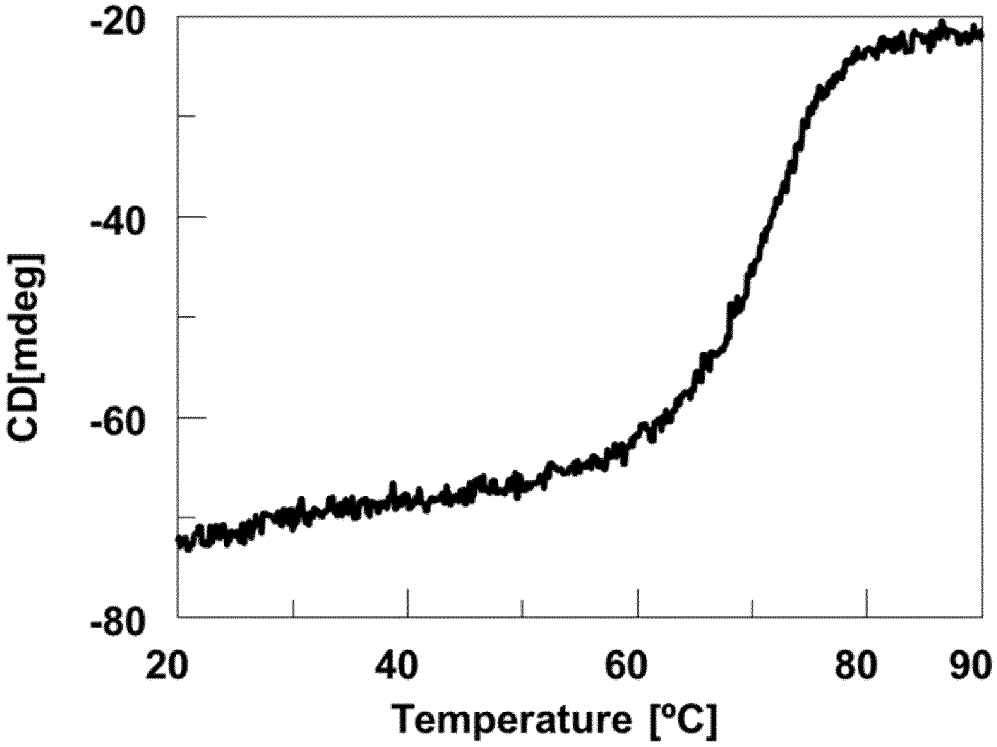
A



B



C



D

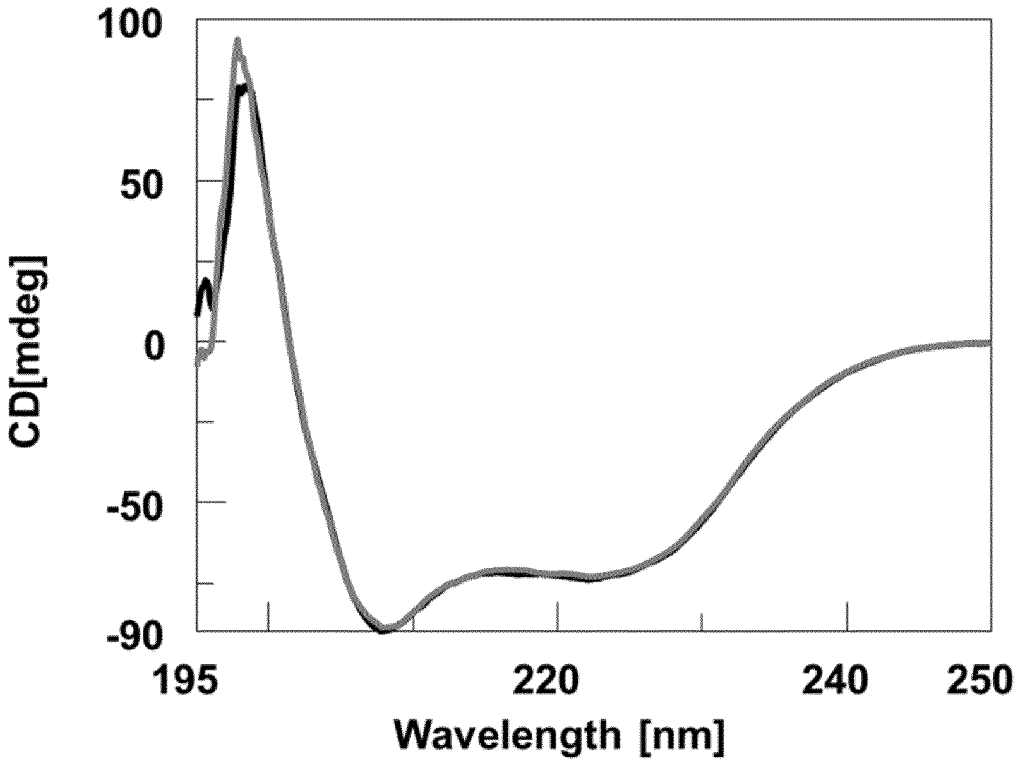


Figure 1C-D

Figure 2

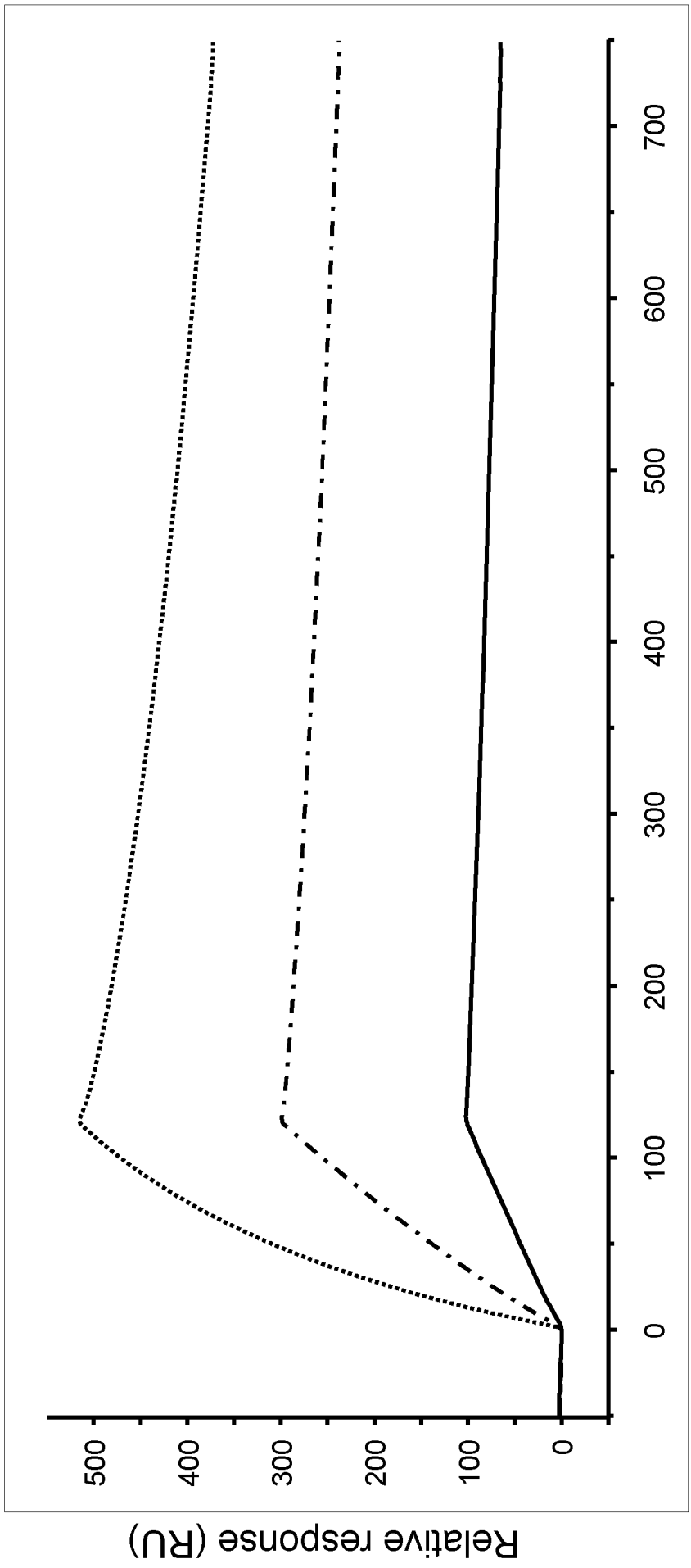

Relative response (RU)
500
400
300
200
100
0
0
100
200
300
400
500
600
700
Time (s)

Figure 3

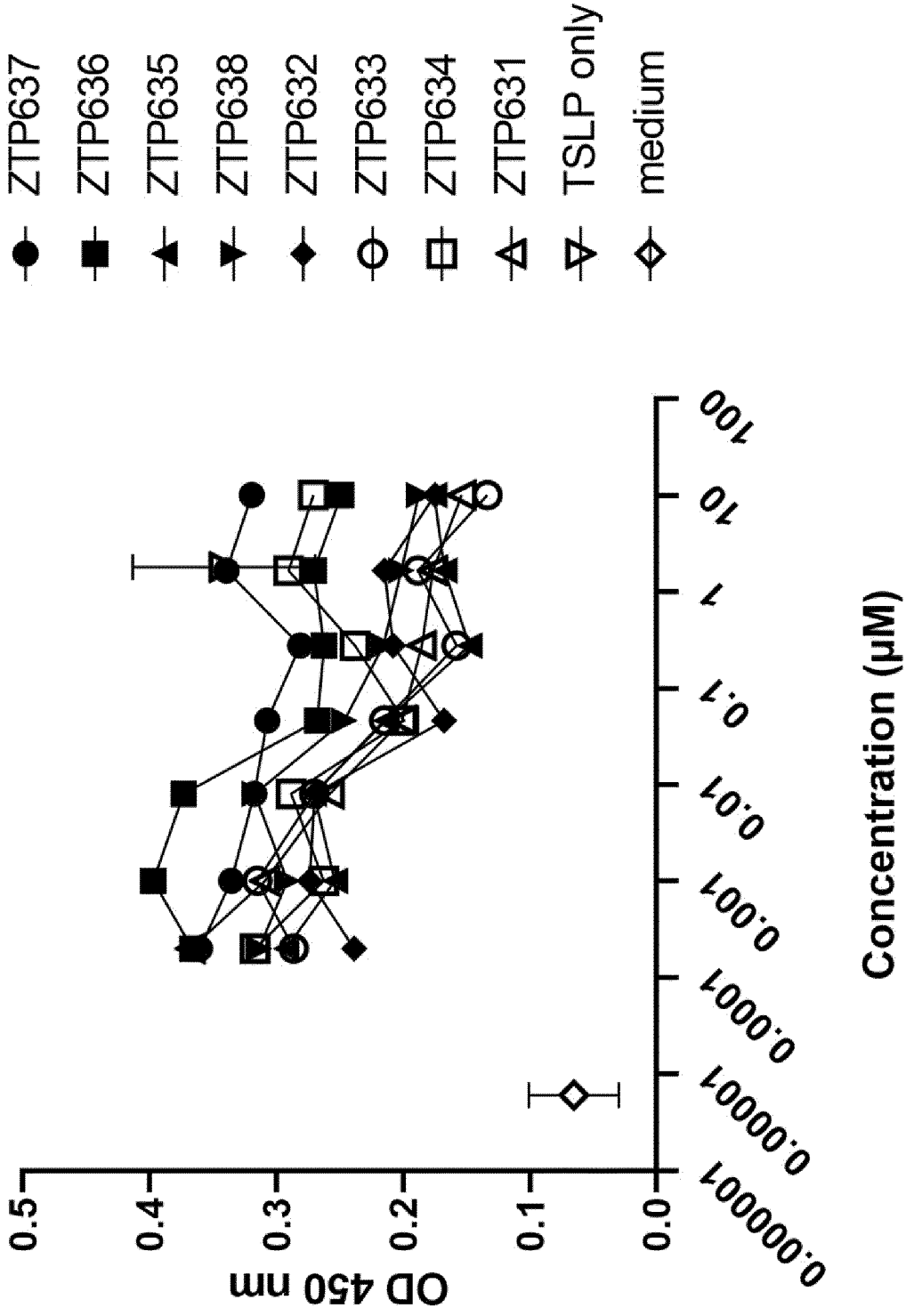
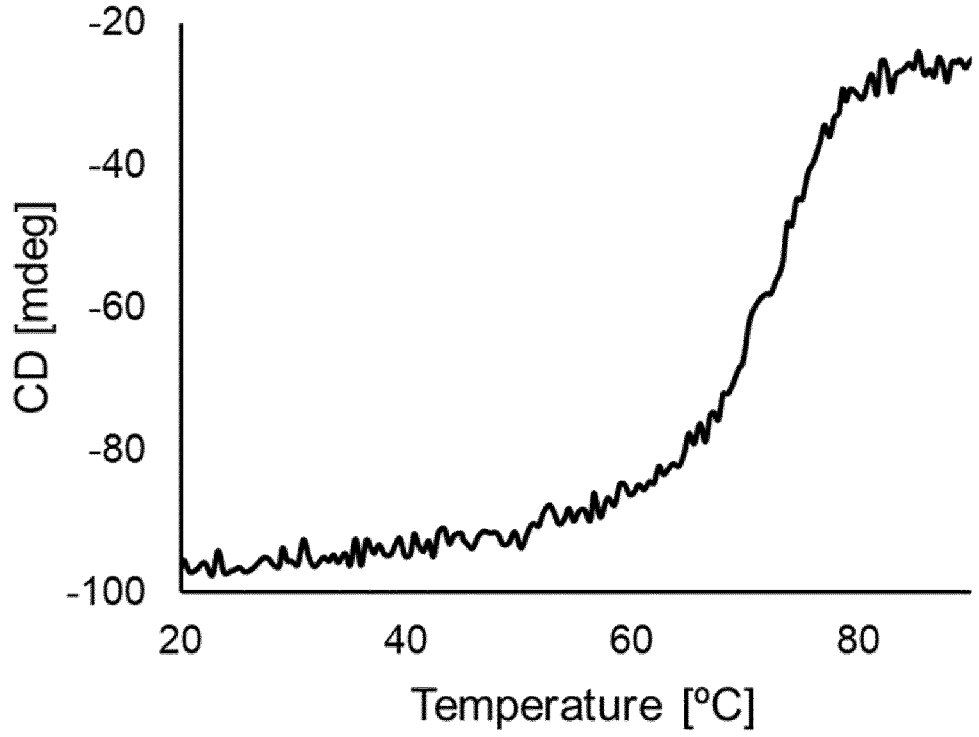

ZTP637
ZTP636
ZTP635
ZTP638
ZTP632
ZTP633
ZTP634
ZTP631
TSLP only
medium
OD 450 nm
0.5
0.4
0.3
0.2
0.1
0.0
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
Concentration (µM)

Figure 4A-B

A



B

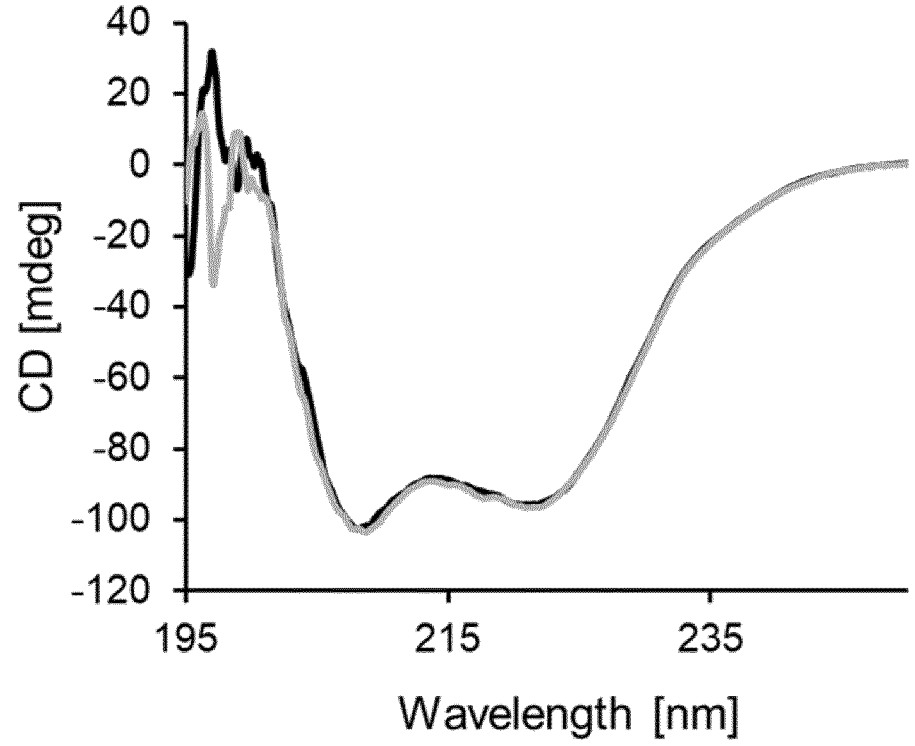
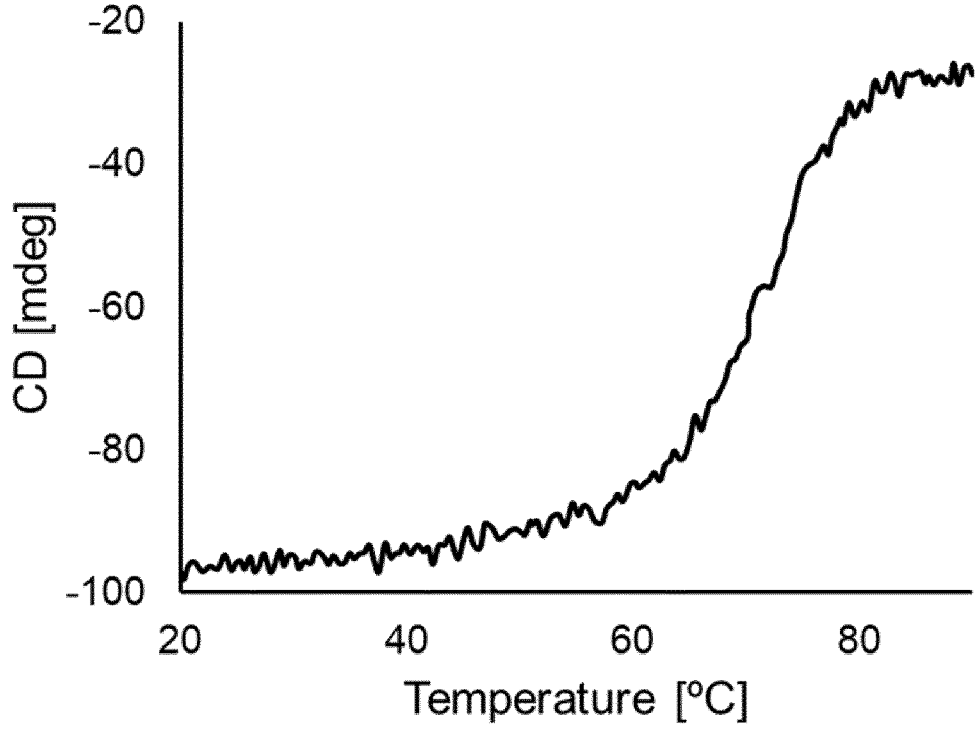


Figure 4C-D

C



D

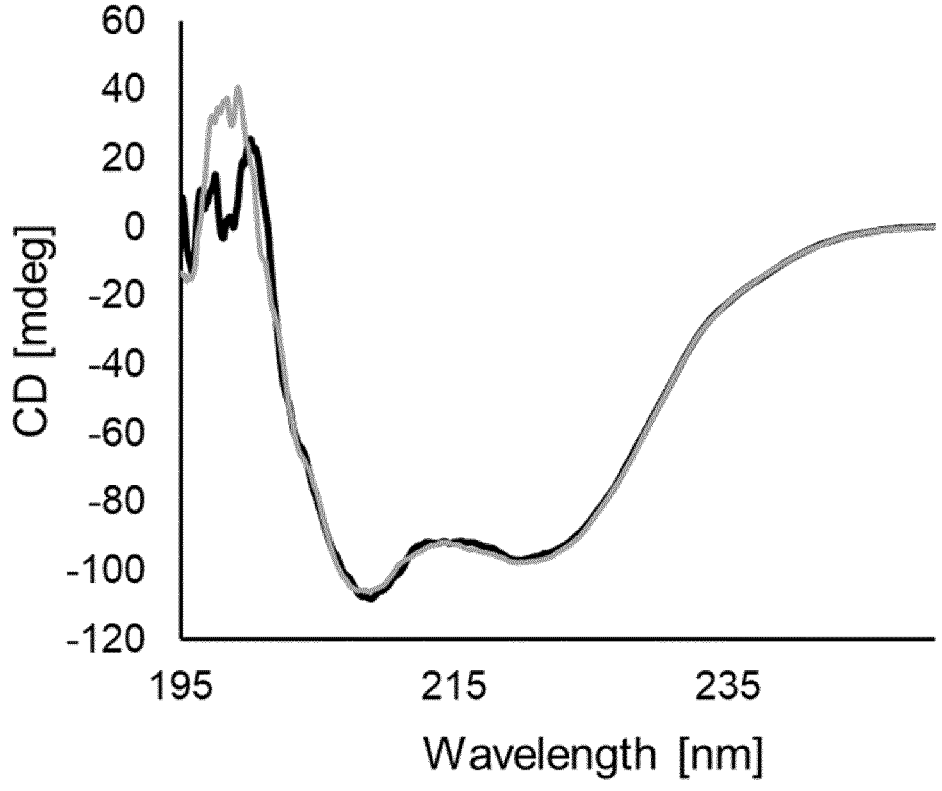


Figure 5

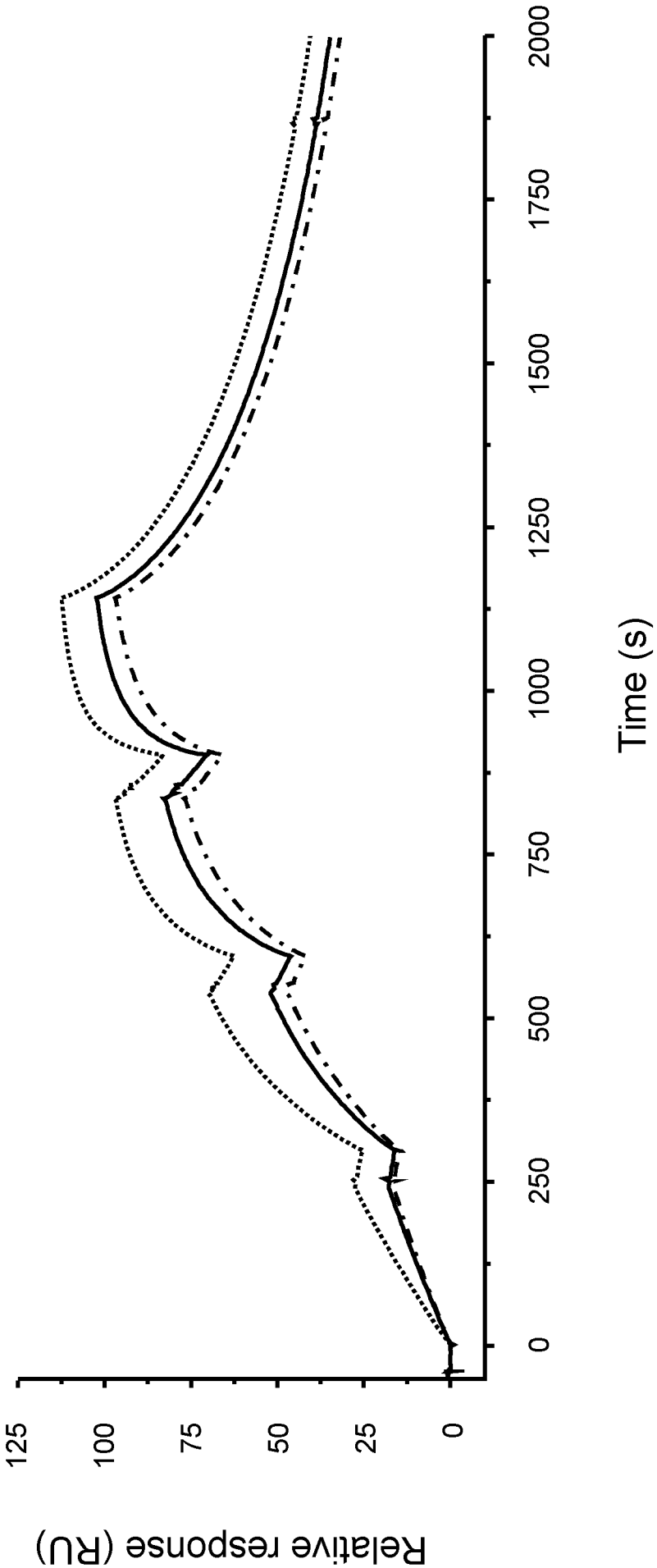

Relative response (RU)
125
100
75
50
25
0
0
250
500
750
1000
1250
1500
1750
2000
Time (s)

Figure 6

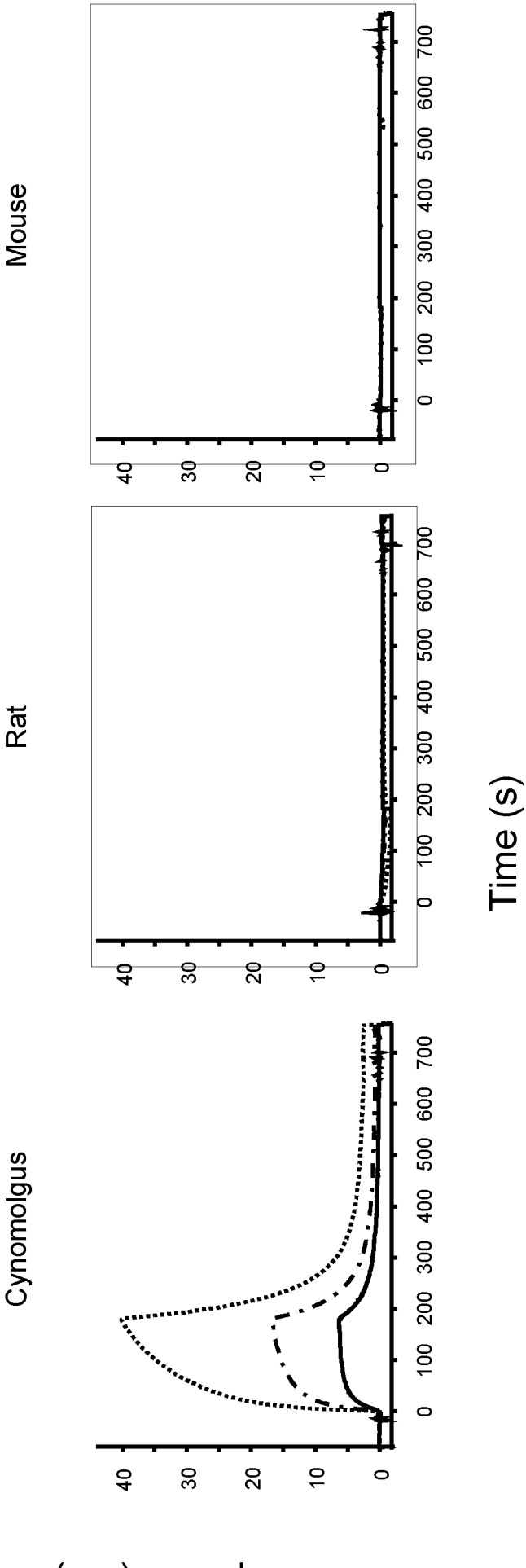

Cynomolgus
Rat
Mouse
Relative response (RU)
Time (s)
0
10
20
30
40
0
100
200
300
400
500
600
700

Figure 7A-D

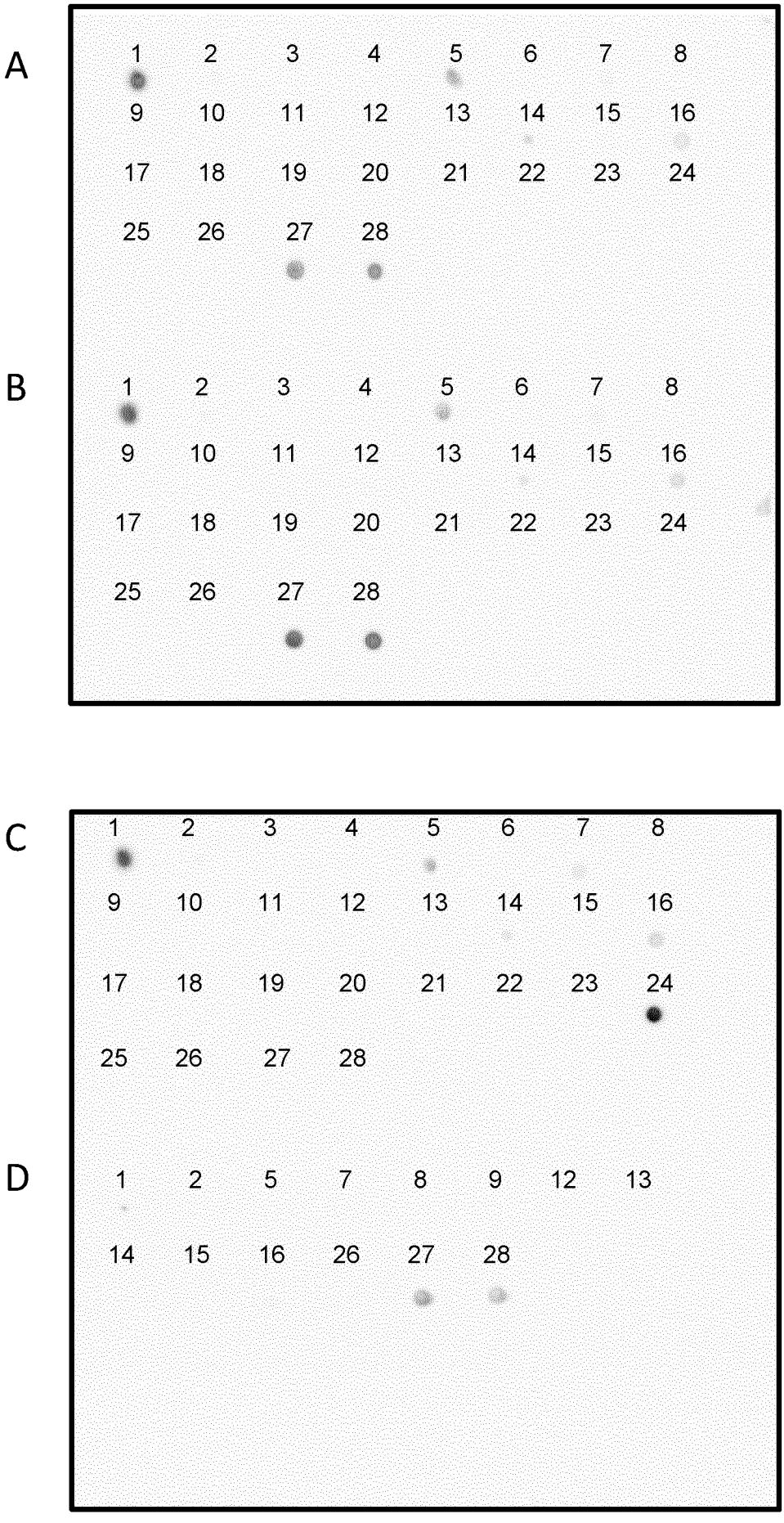
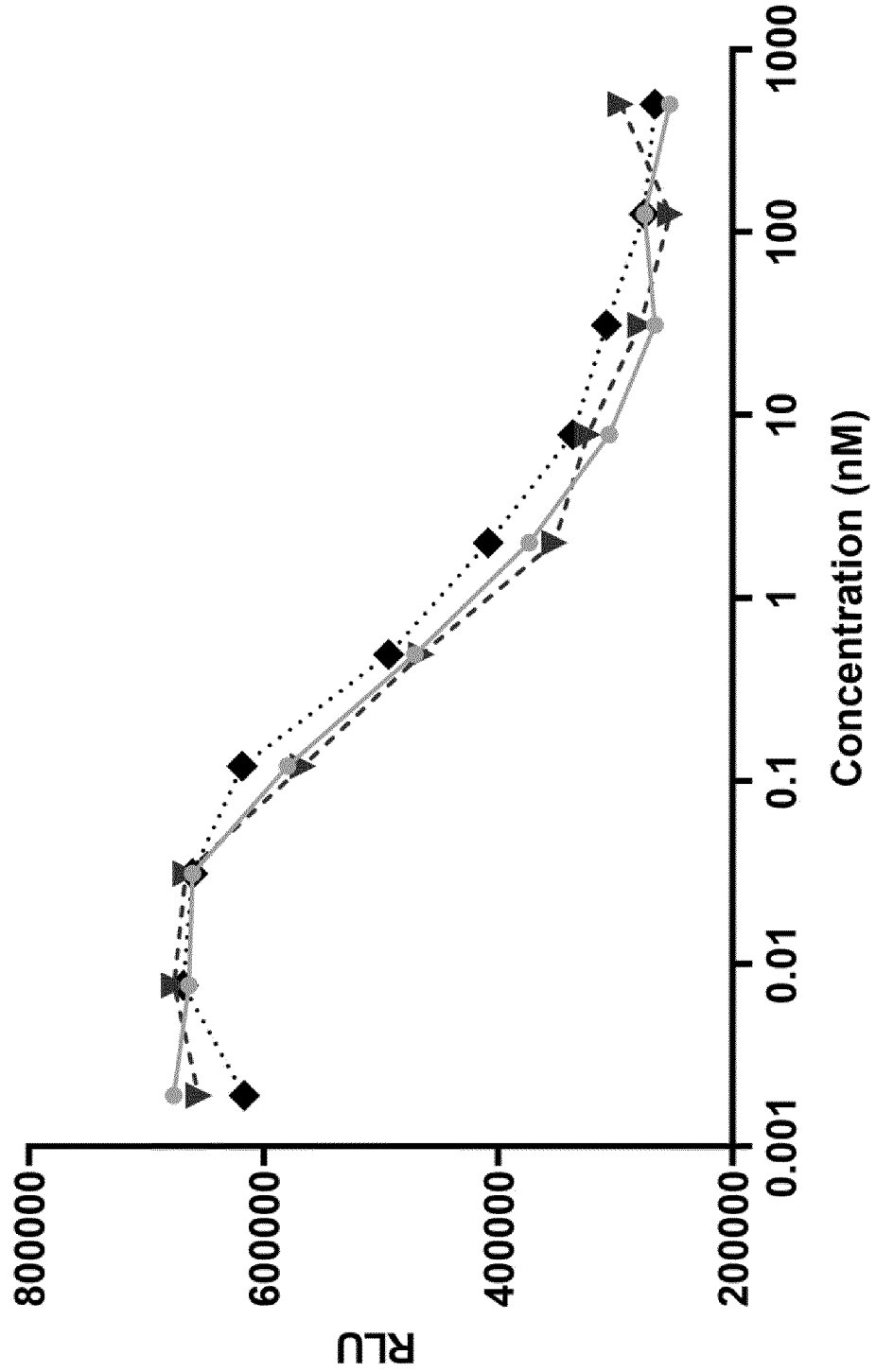
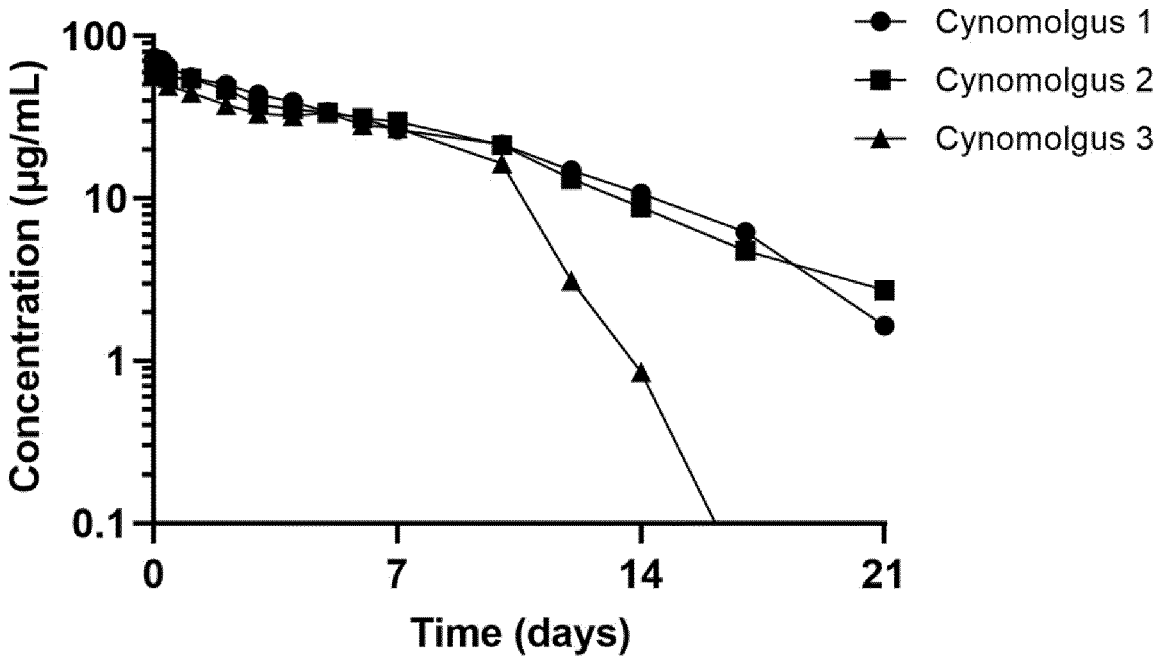

A
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24
25 26 27 28
B
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24
25 26 27 28
C
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24
25 26 27 28
D
1 2 5 7 8 9 12 13
14 15 16 26 27 28

Figure 8


RLU
800000
600000
400000
200000
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

A



B

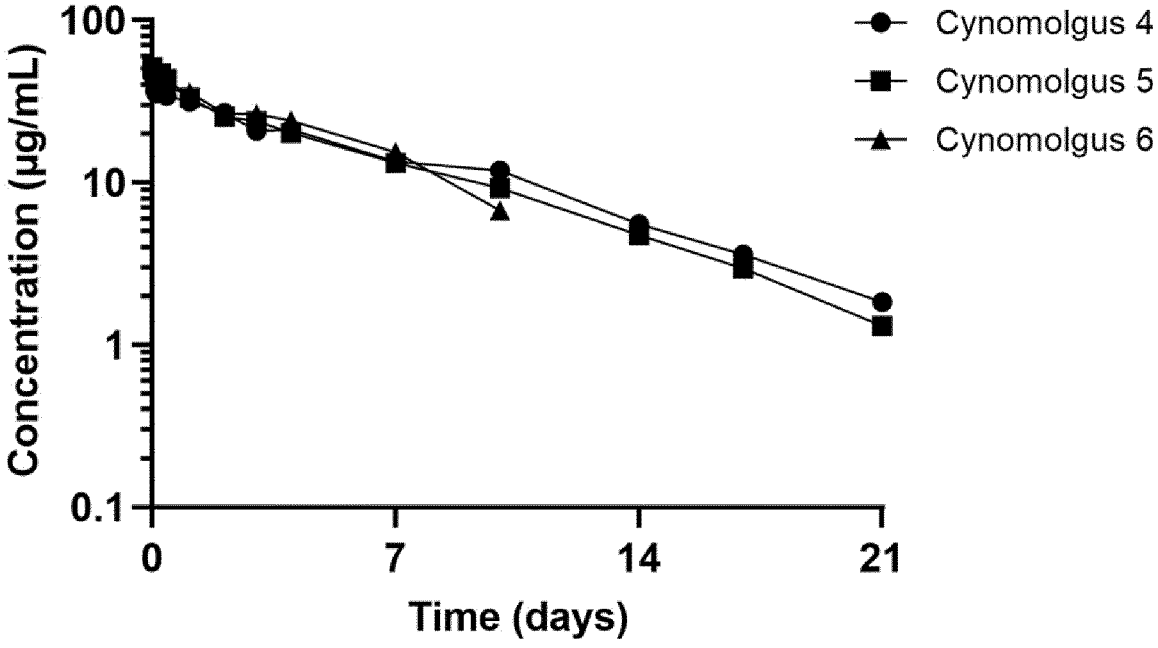


Figure 9A-B

Figure 10A-B

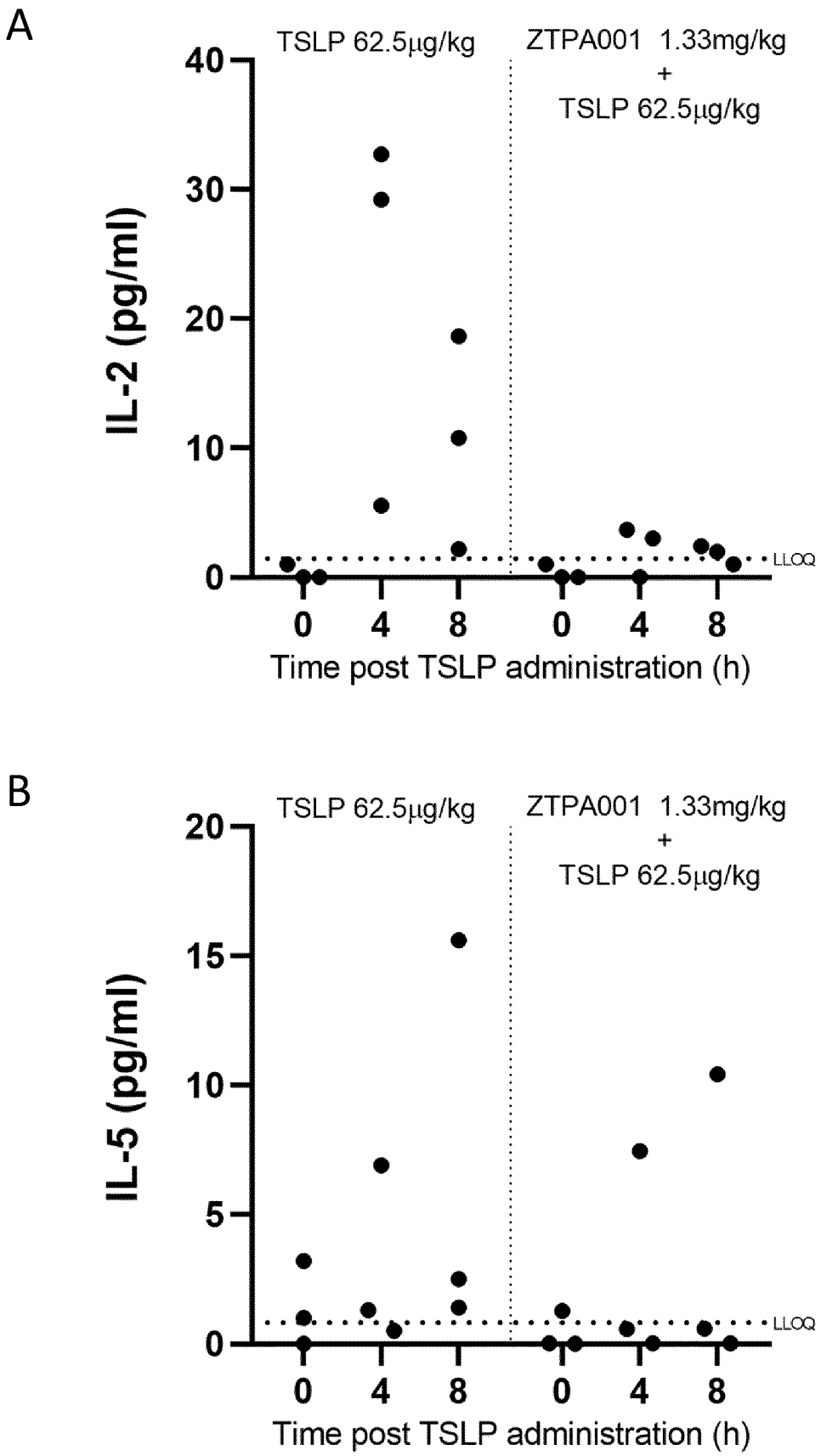

A
TSLP 62.5µg/kg
ZTPA001 1.33mg/kg
+
TSLP 62.5µg/kg
IL-2 (pg/ml)
40
30
20
10
0
LLOQ
0
4
8
0
4
8
Time post TSLP administration (h)
B
TSLP 62.5µg/kg
ZTPA001 1.33mg/kg
+
TSLP 62.5µg/kg
IL-5 (pg/ml)
20
15
10
5
0
LLOQ
0
4
8
0
4
8
Time post TSLP administration (h)

Figure 10C-D

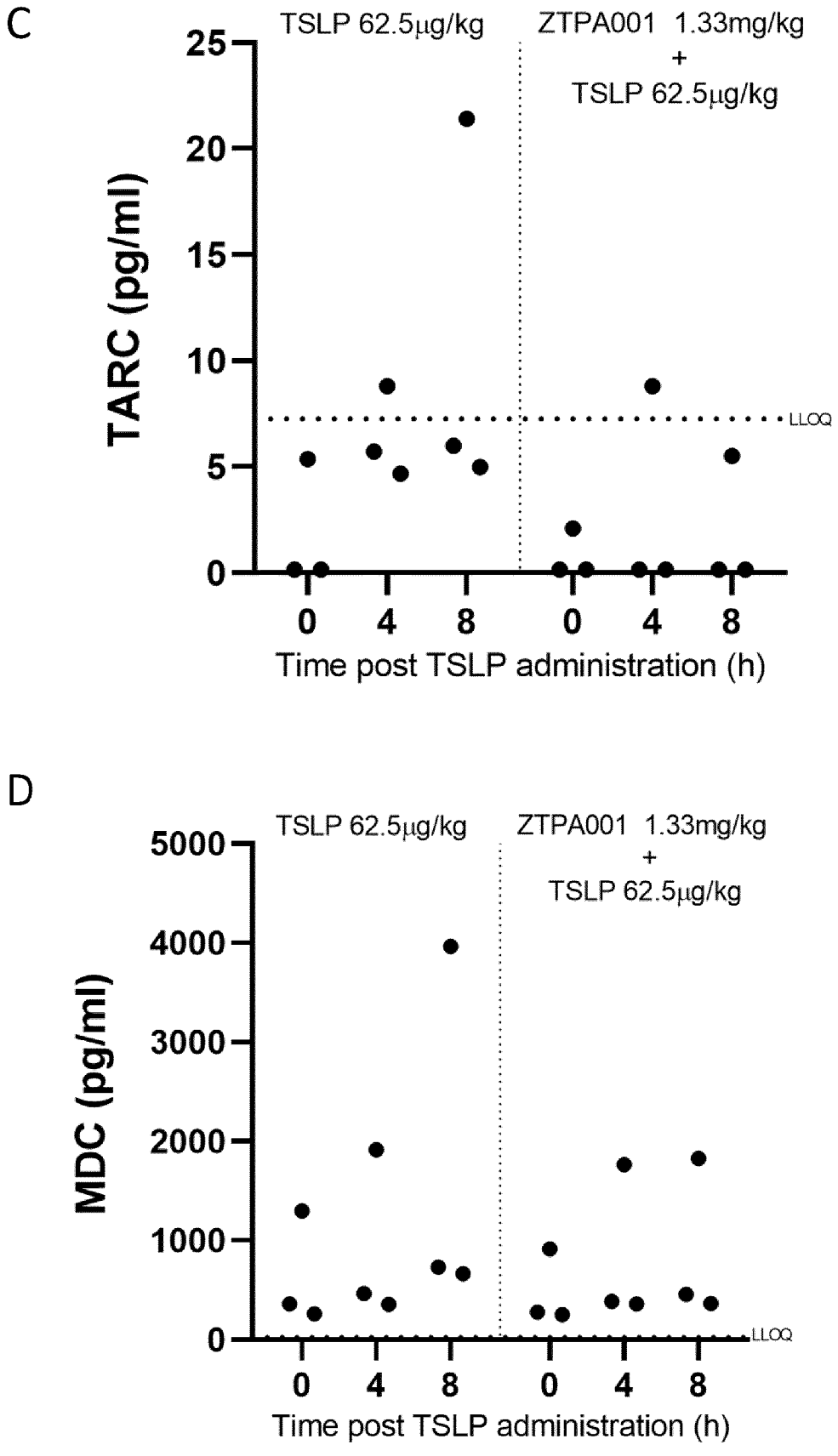

C
TSLP 62.5µg/kg
ZTPA001 1.33mg/kg
+
TSLP 62.5µg/kg
TARC (pg/ml)
25
20
15
10
5
0
LLOQ
0
4
8
0
4
8
Time post TSLP administration (h)
D
TSLP 62.5µg/kg
ZTPA001 1.33mg/kg
+
TSLP 62.5µg/kg
MDC (pg/ml)
5000
4000
3000
2000
1000
0
LLOQ
0
4
8
0
4
8
Time post TSLP administration (h)

Figure 11

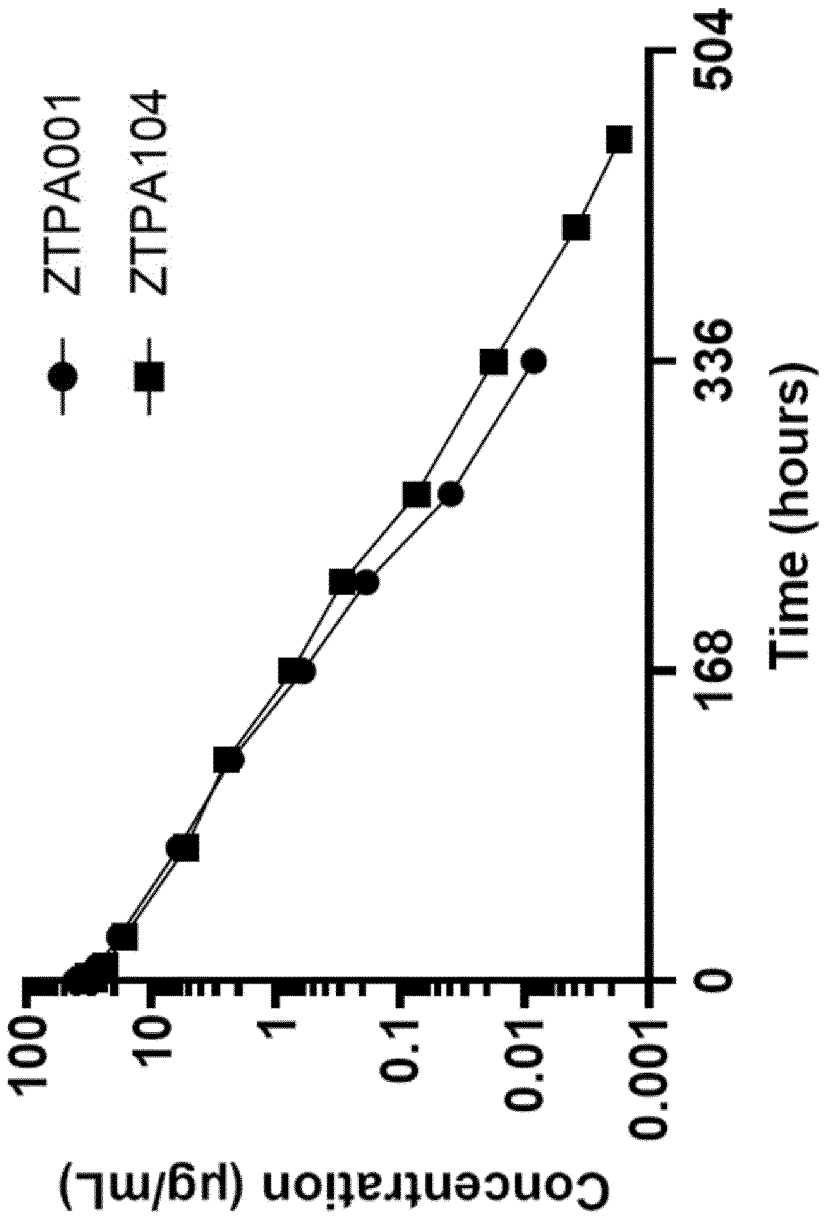

ZTPA001
ZTPA104
Concentration (µg/mL)
100
10
1
0.1
0.01
0.001
0
168
336
504
Time (hours)

Figure 12

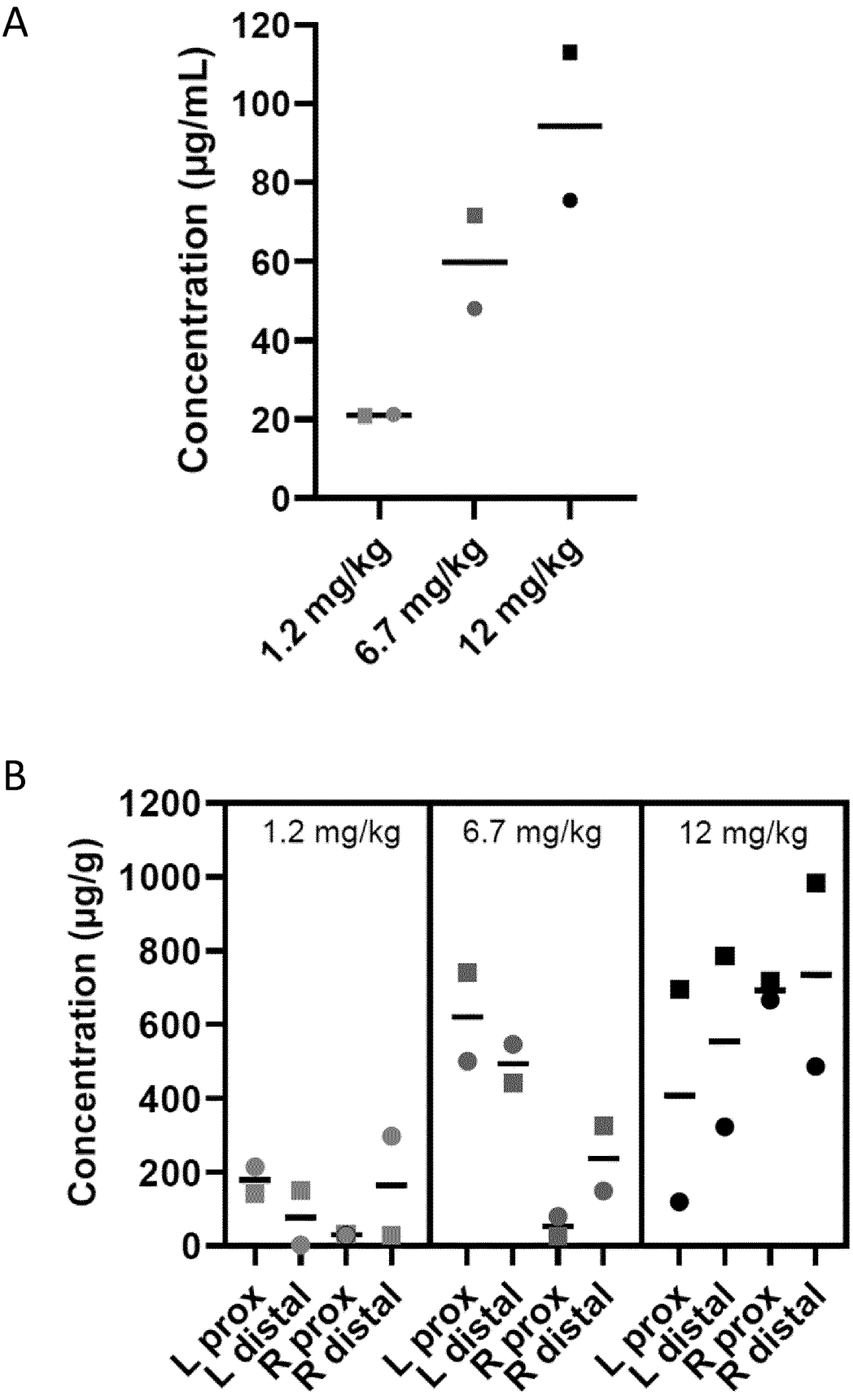

A
Concentration (µg/mL)
120
100
80
60
40
20
0
1.2 mg/kg
6.7 mg/kg
12 mg/kg
B
Concentration (µg/g)
1200
1000
800
600
400
200
0
1.2 mg/kg
6.7 mg/kg
12 mg/kg
L prox
L distal
R prox
R distal

Figure 13

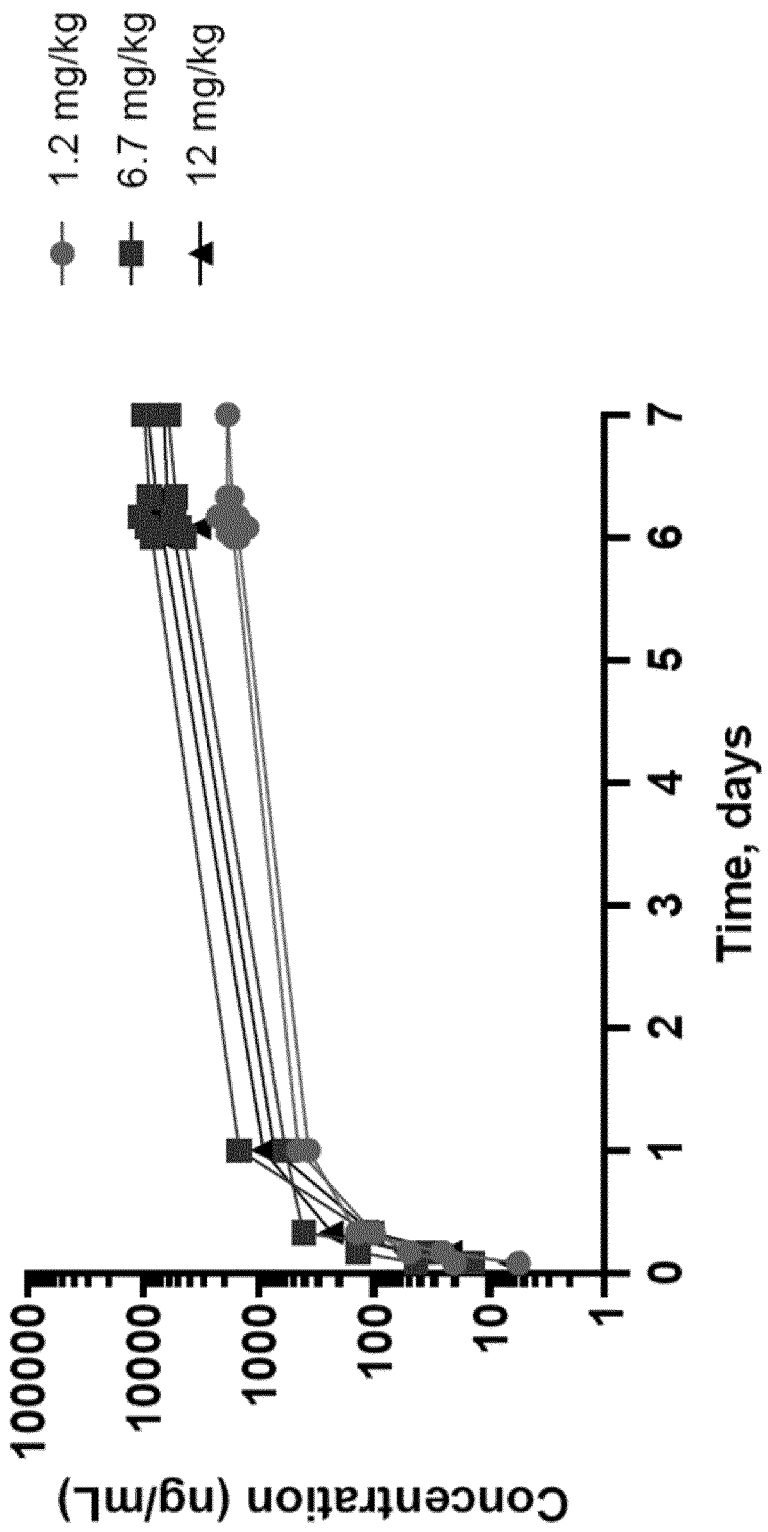
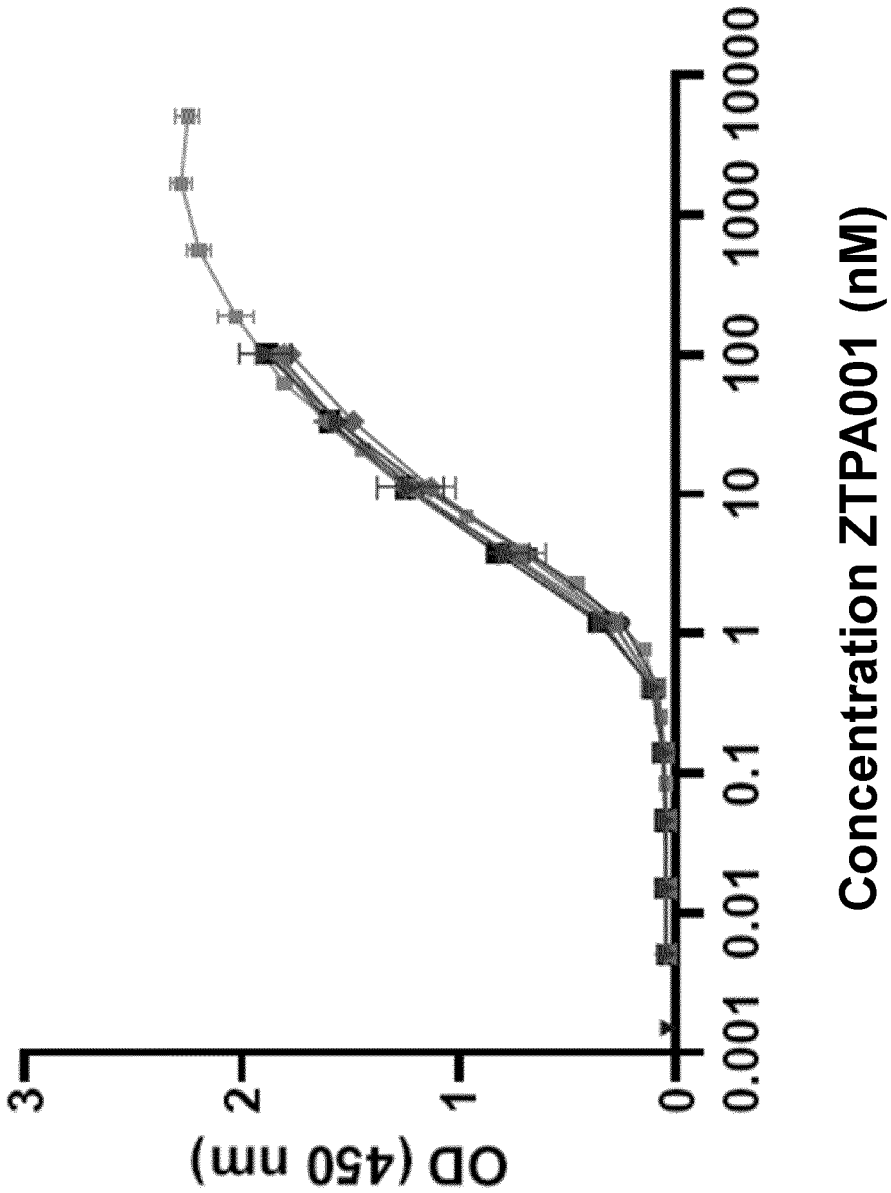

1.2 mg/kg
6.7 mg/kg
12 mg/kg
Concentration (ng/mL)
100000
10000
1000
100
10
1
0
1
2
3
4
5
6
7
Time, days

Figure 14


Test sample 1
Test sample 2
Test sample 3
Test sample 4
Negative control
Spike low
Spike high
OD (450 nm)
0
1
2
3
0.001
0.01
0.1
1
10
100
1000
10000
Concentration ZTPA001 (nM)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/053030

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/52 A61K38/00 A61P35/00 A61P37/00 C07K14/31
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61P A61K C12R

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CORREN JONATHAN ET AL: "TSLP: from allergy to cancer", NATURE IMMULOGY, NATURE PUBLISHING GROUP US, NEW YORK, vol. 20, no. 12, 19 November 2019 (2019-11-19), pages 1603-1609, XP036928236, ISSN: 1529-2908, DOI: 10.1038/S41590-019-0524-9 [retrieved on 2019-11-19] abstract page 1607, column 2, paragraph 1 - paragraph 2 ----- -/--	1-15

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2023	12/04/2023

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAUVREAU GAIL M. ET AL: "Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma", EXPERT OPINION ON THERAPEUTIC TARGETS, vol. 24, no. 8, 27 June 2020 (2020-06-27), pages 777-792, XP055950106, UK ISSN: 1472-8222, DOI: 10.1080/14728222.2020.1783242 abstract table 2 page 788, column 1 - column 2	1-15
Y	WO 2016/113246 A1 (AFFIBODY AB [SE]) 21 July 2016 (2016-07-21) sequences 1244, 1245 claim 1 examples 4-9 -& DATABASE Geneseq [Online] 8 September 2016 (2016-09-08), "Interleukin (IL)-17A binding polypeptide (Z-ABD-Z) SEQ ID NO:1245.", XP002807299, retrieved from EBI accession no. GSP:BDC60668 Database accession no. BDC60668 sequence -& DATABASE Geneseq [Online] 8 September 2016 (2016-09-08), "Interleukin (IL)-17A binding polypeptide (Z-ABD-Z) SEQ ID NO:1244.", XP002807300, retrieved from EBI accession no. GSP:BDC60667 Database accession no. BDC60667 sequence	1-15
Y	WO 2021/089695 A1 (AFFIBODY AB [SE]) 14 May 2021 (2021-05-14) claim 1 examples 7-10 sequence 37 -& DATABASE Geneseq [Online] 24 June 2021 (2021-06-24), "B-box binding peptide-albumin binding peptide fusion, SEQ ID 37.", XP002807301, retrieved from EBI accession no. GSP:BJI28802 Database accession no. BJI28802 sequence	1-15

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/053030

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LÖFBLOM J ET AL: "Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 584, no. 12, 11 April 2010 (2010-04-11), pages 2670-2680, XP002807295, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2010.04.014 the whole document ----- 	1-15

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/053030

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/053030

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016113246 A1	21-07-2016	AU 2016208161 A1	13-07-2017
		BR 112017014559 A2	15-05-2018
		CA 2972683 A1	21-07-2016
		CN 108064235 A	22-05-2018
		DK 3245224 T3	21-09-2020
		EP 3245224 A1	22-11-2017
		EP 3738976 A1	18-11-2020
		ES 2822431 T3	04-05-2021
		HU E052121 T2	28-04-2021
		JP 6943764 B2	06-10-2021
		JP 7208963 B2	19-01-2023
		JP 2018508186 A	29-03-2018
		JP 2021073174 A	13-05-2021
		KR 20170116023 A	18-10-2017
		MY 188784 A	31-12-2021
		NZ 732978 A	28-01-2022
		PL 3245224 T3	25-01-2021
		PT 3245224 T	08-10-2020
		RU 2017127012 A	14-02-2019
		US 2017362290 A1	21-12-2017
		US 2021253659 A1	19-08-2021
		WO 2016113246 A1	21-07-2016
		ZA 201704366 B	28-09-2022
WO 2021089695 A1	14-05-2021	US 2022389066 A1	08-12-2022
		WO 2021089695 A1	14-05-2021