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(54) Titre : PROTEINES DE REPETITION DE LIAISON A IL4/IL13 ET LEURS UTILISATIONS
(54) Title: IL4/IL13 BINDING REPEAT PROTEINS AND USES

Fig. 1A

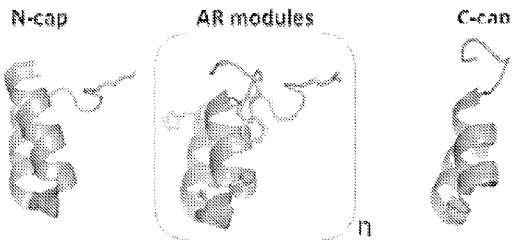
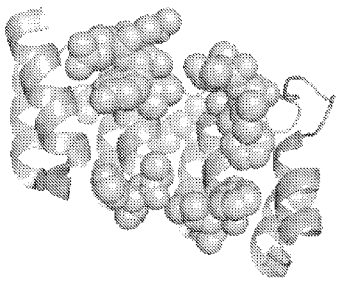


Fig. 1B



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IL4/IL13-binding proteins comprise binding domains, which inhibit IL4/IL13 binding to IL4Ralpaha and common gamma chain complexes (Type 1) and inhibit IL4 binding to IL4Ralpha and IL13Ralpha1 complexes (Type 2), and IL13 binding to IL13Ralpha1 and/or IL13Ralpha2, are useful in the treatment of cancer, inflammatory, and other pathological conditions, such as allergic or fibrotic conditions, especially pulmonary conditions.

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(54) Title: IL4/IL13 BINDING REPEAT PROTEINS AND USES

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Fig. 1A

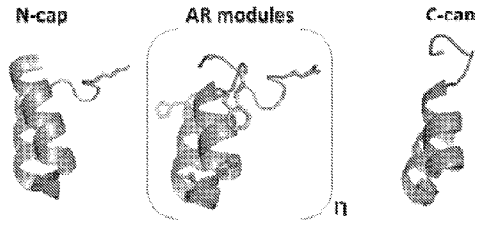
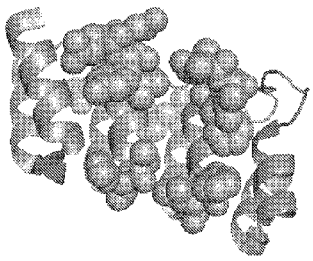


Fig. 1B



WO 2012/149439 A2

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IL4/IL13 BINDING REPEAT PROTEINS AND USES

FIELD OF THE INVENTION

The present invention relates to recombinant binding proteins comprising a binding domain which is a repeat protein comprising designed modular repeat units and selected for the ability to inhibit the binding of IL4 and IL13 to their cognate receptors thereby representing useful and stable therapeutic proteins. More particularly, the present invention is directed to bi-specific IL4/IL13 binding proteins comprising ankyrin repeat modules.

BACKGROUND OF THE INVENTION

Interleukin 4 (human IL4, UniProt P05112) is a 129 amino acid cytokine derived from T cells and mast cells with multiple biological effects on many cell types including B-cells, T-cells and nonlymphoid cells including monocytes, endothelial cells and fibroblasts. IL4 is a pleiotropic cytokine and has been implicated in many of the cellular responses associated with asthma including IgE production, inflammation, airway hypersensitivity, and goblet cell hyperplasia (Perkins, et al., J Allergy Clin Immunol 118: 410-9, 2006; Pene, et al., Proc Natl Acad Sci U S A 85: 6880-4, 1988). Its production by both T-cells and mast cells is regulated by a variety of mediators and cytokines that sustain Th2-mediated responses. IL4 signaling is mediated via two receptor complexes, the Type I receptor complex and the Type II receptor complex. Signaling through the type II receptor complex, composed of one IL-4Rα and one IL13Rα1 chain, is largely responsible for the shared biological effects of IL4 and IL13 and both IL4 and IL13 may contact the components of the complex. The type I receptor complex, comprised of the IL-4Rα and common γ-chain is, exclusively responsive to IL4 and mediates IL4 responses in T-cells which do not express IL13αR1 (Idzerda, et al., J Exp Med 171: 861-73, 1990; Nelms, et al., Annu Rev Immunol 17: 701-38, 1999).

Neutralizing the effects of IL4 using antibodies or as demonstrated by the responses of IL4 deficient mice, inhibits allergen-specific IgE and reduces eosinophilia (Zhu and Paul, Blood 112: 1557-69, 2008), as well as airway hyperresponsiveness (AHR) (Heaton, et al., Lancet 365: 142-9, 2005) in murine models of TH2 inflammation. Similarly, soluble IL4 receptor has been used to inhibit IL4 signaling and has been shown to reduce allergen-induced AHR as well as VCAM-1 expression, mucus production and eosinophil recruitment to the lungs of mice (McKinley, et al., J Immunol 181: 4089-97, 2008). In human cells, IL4 has been shown to drive the differentiation of naïve T helper (Th0) lymphocytes into TH2 lymphocytes (Breekveldt-Postma, et al., Curr Med Res Opin 24: 975-83, 2008; Wraight, et al., Respirology 7: 133-9, 2002). TH2 cells have been shown to secrete IL-4, IL-5, IL-9 and IL13 but do not produce IFNγ, contributing to an imbalance of pro-inflammatory TH2 cytokines (Partridge, Ann Oncol 17: 183-4, 2006). Neutralization of IL4 with antibodies that inhibit receptor binding blocks T-cell differentiation ((Idzerda, et al., J Exp Med 171: 861-73, 1990; Nelms, Keegan et al., Annu Rev

Immunol 17: 701-38, 1999)). Polymorphisms in the genes encoding IL4, IL4Ra, and IL13 have been associated with asthma, in fact, both IL4 and IL4Ra polymorphisms are associated with severe asthma and exacerbations of asthma (Sandford, et al., J Allergy Clin Immunol 106: 135-40, 2000; Wenzel, et al., Am J Respir Crit Care Med 175: 570-6, 2007). Based on the perceived central role of IL4 in asthma, biotherapeutics that inhibit the activity of IL4 were expected to be valuable tools for the treatment of asthma and other Th2-associated pathologies. However the results of clinical studies using a soluble IL4 receptor were disappointing and showed minimal differences in the incidence of asthma exacerbations between placebo and treatment groups (Borish, et al., J. Allergy Clin. Immunology 107: 963-70, 2001).

Like IL4, Interleukin 13 (IL13) is cytokine identified from activated human T lymphocytes. Over the last 10 years, a variety a reports have demonstrated a role for IL13 in many of the cellular responses associated with asthma including IgE production, inflammation, airway hypersensitivity, mucus production and lung fibrosis (Kasaian and Miller, Biochem Pharmacol 76: 147-55, 2008). Its production is regulated by a variety of mediators and cytokines that interact in a positive feedback loop to sustain Th2-mediated immune responses. IL13 signaling is predominantly mediated via the Type 2 receptor, IL13α1 and IL-4Rα complex. The Type 2 complex, when present, is also activated by IL4 binding (Wills-Karp, Immunological Reviews 202: 175-90, 2004; LaPorte, et al., Cell 132: 259-72, 2008). IL13Ralpha2, is a receptor capable of high affinity binding of IL13 and may play a more functional role either by attenuation of the actions of IL13 and IL4 or via induction of TGF-beta and development of lung fibrosis.

A variety of in vivo data supports a role for IL13 in the pathogenesis of asthma. In cynomologus monkey models of allergic respiratory disease, antibodies that block the action of IL13 have been shown to reduce lung inflammation (Kasaian, et al., J Pharmacol Exp Ther 325: 882-92, 2008). In humans, increased IL13 levels can be measured in the bronchial tissue, nasal lavage flurid, and induced sputum from asthmatic patients. Genetic polymorphisms that are associated with asthma have been identified at the IL13 locus (Heinzmann, et al., Hum Mol Genet 9: 549-59, 2000). In addition, IL13 appears to play an important role in other atopic diseases including dermal fibrosis and atopic dermatitis. Antibodies or other protein molecules that inhibit the activity of IL13 may be valuable therapeutics for the treatment of asthma and other atopic diseases (Brightling, et al., Clin Exp Allergy 40: 42-9).

Taken together, the in vivo and in vitro data for IL13 and IL4 suggest that therapeutics that can inhibit the actions of both cytokines may be efficacious agents for the treatment of asthma.

The technical problem underlying the present invention is to identify novel IL-4 and IL-13 antagonists (e.g., neutralizing binders) which can be used alone or in combination for an improved treatment of inflammatory disorders, cancer, atopic diseases and other pathological conditions associated with allergic or atopic responses, e.g., asthma, eosinophilia, and fibrotic conditions and where pulmonary functions are affected, to provide for local delivery of an IL4, IL-13, or an IL4 and IL13, neutralizing molecule.

SUMMARY OF THE INVENTION

The present invention relates to binding protein constructs comprising IL4/IL13-binding ankyrin repeat (AR) proteins capable of binding IL4 and IL13 and that inhibit bioactivity of IL4 and IL13. An IL4 and IL13 inhibiting construct as exemplified herein is comprised of an IL4-binding AR repeat domain linked to an IL13-binding AR repeat domain. Such bispecific AR proteins have application as biotherapeutics for a variety of Th2 mediated diseases, including asthma and other atopic diseases associated with the presence or bioactivity of IL4 and IL13.

The present invention also relates to binding protein constructs comprising IL4 or IL13-binding ankyrin repeat (AR) proteins capable of binding IL4 or IL13 and that inhibit bioactivity of IL4 or IL13. An IL4 or IL13 inhibiting construct as exemplified herein is comprised of an IL4-binding AR repeat domain or an IL13-binding AR repeat domain. Such bispecific AR proteins have application as biotherapeutics for a variety of Th2 mediated diseases, including asthma and other atopic diseases associated with the presence or bioactivity of IL4 or IL13.

The invention further relates to nucleic acid molecules encoding the recombinant binding proteins of the present invention, and to a pharmaceutical composition comprising one or more of the binding proteins or nucleic acid molecules.

The invention further relates to a method of treatment of inflammatory diseases, cancer, atopic diseases and other pathological conditions, especially pulmonary conditions, such as asthma and those conditions leading to pulmonary fibrosis, using the binding proteins of the invention. In a particular embodiment, the binding proteins capable of IL4-binding or IL13-binding, alone or in combination may be used in methods of prophylactic or therapeutic treatment to prevent, ameliorate, reduce or eliminate the symptoms or pathophysiology of IL4 and/or IL13 mediated disease. A particular method of treatment is by local delivery of an IL4-binding protein and/or IL-13-binding protein of the invention. In one embodiment of the method of treatment, the IL4-binding protein and/or IL-13-binding protein is administered as an aerosolized formulation. In one method of local delivery, the aerosolized formulation comprising an IL4-binding protein and/or IL-13-binding protein is administered to pulmonary compartment of the subject in need of treatment. The method of treatment is provided to a subject, as prophylactic or therapeutic treatment comprising the IL4-binding protein and/or IL-13-binding protein where the subject is diagnosed or suspected of having a condition, such as

asthma, an inflammatory disorder, cancer, atopic disease, or other pathological conditions associated with allergic or atopic responses, e.g., eosinophilia, and fibrotic conditions and, especially, where pulmonary functions are affected.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic ribbon diagram of a binding protein showing N- and C-Caps and a binding domain comprising multiple ARs

Figure 1B is a schematic ribbon diagram of a binding protein showing a complete ankyrin repeat domain comprising an N-Cap, two ankyrin repeat modules and a C-Cap.

Figure 2A is a graph representing the neutralization of IL13 and IL4 dependent activities before and after 30 minutes of nebulization. Concentration of aerosolized AR protein or AR protein retained in the cup were assessed by A280 and the activity was measured using an IL13 STAT6 activation assay; pre-nebulized AR protein (shown in squares); aerosolized AR protein (shown in triangles); and retained AR protein (shown in diamonds).

Figure 2B is a graph representing the neutralization of IL13 and IL4 dependent activities before and after 30 minutes of nebulization. Concentration of aerosolized AR protein or AR protein retained in the cup were assessed by A280 and the activity was measured using an IL4 dependent HT2 proliferation assay; pre-nebulized AR protein (shown in squares); aerosolized AR protein (shown in triangles); and retained AR protein (shown in diamonds).

Figure 3 shows the particle size distribution for AR protein 11G11-21H2 as evaluated by cascade impaction using a solution of AR protein 11G11-21H2 prepared at 20 mg/ml in PBS. The MMAD is 2.84 μm and the GSD is 1.66 μm.

Figure 4 shows a plot of data for 11G11-21H2 serum, lung tissue or bronchial lavage fluid (BAL) concentrations over time after dosing via intratracheal instillation groups of mice (n=5) and sacrificed at various timepoints.

Figure 5 shows the effect of repeat protein 11G11 or repeat protein 11G11-21H2 dosed via intratracheal instillation on OVA-induced airway hyperresponsiveness to methacholine in the acute OVA sensitization and challenge model. Non-sensitized, vehicle challenged (NSV) animals (shown in solid squares); Control AR protein (shown in solid diamonds); 11G11 20mg/kg (shown in squares); 21H2 20 mg/kg (shown in solid circles); 11G11-21H2 AR protein, 40 mg/kg (shown in solid triangles).

Figure 6 is a bar graph showing the effect of various AR constructs on ovalbumin induced eosinophil recruitment to the lungs of Balb/C mice in the acute OVA sensitization and challenge

model. The effect of 11G11-21H2 protein (labeled mCNTX413) is significantly different from the either monospecific AR protein 21H2 or 11G11 alone.

Figure 7 shows the effect of various AR constructs (with 11G11-21H2 labeled mCNTX413) on OVA induced eosinophil recruitment to the lungs.

Figure 8 is a ribbon presentation of the complex between Binding protein 6G9 (top) and IL13 (below). Arrows indicate "opening" of the IL13 binding protein upon IL13 binding.

Figure 9 shows interactions at the IL13 binding protein loops. It shows a back view with respect to Figure 8.

Figure 10 shows interactions at the IL13 binding protein groove. It shows a top view with respect to Figure 8.

Figure 11 shows the amino acid sequence of IL13 Binding Protein 6G9 (SEQ ID NO:162) and alignment of ankyrin repeats. Residues involved in binding IL13 are underlined. E114 (italics) may also be involved. Secondary structure elements are indicated by letters "t" (β-turn) and "h" (helix).

Figure 12 shows a sequence alignment of human and cyno IL13. The 6G9 epitope residues are underlined.

Figure 13 shows a superposition of IL-13 structures from complexes with binding protein 6G9 and 3 different IL13 antibodies.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

CCL17 = chemokine (CC-motif) ligand 17; ECD = extracellular domain; IL = interleukin; TARC = Thymus and Activation-Regulated Chemokine; PBS = phosphate buffered saline; AR = ankyrin repeat; MEM = Minimum Essential Media, NEAA = Non-Essential Amino Acids, SPR = surface plasmon resonance.

Definitions

The term "protein" refers to a polypeptide, wherein at least part of the polypeptide has, or is able to; acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its polypeptide chain(s). If a protein comprises two or more polypeptides, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptides. A part of a protein, which individually has, or is able to acquire a defined three-dimensional arrangement by forming secondary or tertiary structures, is termed "protein domain." Such protein domains are well known to the practitioner skilled in the art.

In the context of the present invention, the term "polypeptide" relates to a molecule consisting of multiple, i.e., two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds.

The term "binding protein" refers to a protein comprising one or more binding domains. In various embodiments of the invention, the binding protein comprises two, three, or four binding domains. Furthermore, any such binding protein may comprise additional protein domains that are not binding domains, multimerization moieties, polypeptide tags, polypeptide linkers and/or a single Cys residue. Examples of multimerization moieties are immunoglobulin heavy chain constant regions which pair to provide functional immunoglobulin Fc domains, and leucine zippers or polypeptides comprising a free thiol which forms an intermolecular disulfide bond between two such polypeptides. Free thiol, residing on e.g. a Cys residue, may be used for conjugating other moieties to the polypeptide, for example, by using the maleimide chemistry well known to the person skilled in the art. Preferably, said binding protein is a recombinant binding protein. Also preferably, the binding domains of the binding protein of the invention possess different target specificities. Non-proteinaceous atoms, such as metals; actives, and non-proteinaceous material may be attached or associated with the binding protein of the invention in a useful composition.

The term “binding domain” as used herein, means a protein domain exhibiting the same or substantially the same "fold" (three-dimensional arrangement) as a protein scaffold and having a specified property, such as binding a target molecule. A protein scaffold will have exposed surface areas in which amino acid insertions, substitutions or deletions are highly tolerable which may be modified to provide a binding domain with a selected, specified or determined property. Other specified properties of a binding domain may include: binding to a target, blocking of target binding or target activity, activation of a target-mediated reaction, enzymatic activity, and related further properties. Depending on the type of desired property, one of ordinary skill will be able to identify and perform the necessary steps for screening and/or selection of a binding domain with the desired property. Such a binding domain may be obtained by rational, or most commonly, combinatorial protein engineering techniques, skills which are known in the art (Skerra, A., J. Mol. Recog. *13*, 167-187, 2000; Binz, H. K., Amstutz, P. and Plückthun, A., Nat. Biotechnol. *23*, 1257-1268, 2005). For example, a binding domain having a selected property can be obtained by a method comprising the steps of (a) providing a diverse collection of protein domains exhibiting the same fold as a protein scaffold as defined further below; and (b) screening said diverse collection and/or selecting from said diverse collection to obtain at least one protein domain having said property. The diverse collection of protein domains may be provided by several methods in accordance with the screening and/or selection system being used, and may comprise the use of methods well known to the person skilled in the art, such as phage display or ribosome display libraries.

As described herein, the binding domain is a “repeat domain” or a “designed repeat domain.” Such a repeat domain may comprise one, two, three or more internal repeat modules that will participate in binding to a target or other specified property. Preferably, such a repeat domain further comprises an N-terminal capping module, two to four internal repeat modules, and a C-terminal capping module. Preferably, said binding domain is an ankyrin repeat domain or designed ankyrin repeat domain where the repeat modules sequences are from naturally proteins (repeat units) or are derived from consensus sequences of the natural repeat units (repeat modules). Thus, a repeat domain can be naturally occurring or can be formed, such as those obtained as the result of the inventive procedure explained in patent publication WO 02/20565.

A binding protein according to the invention may be a "repeat protein" or “designed repeat protein” which refers to a protein comprising two or more consecutive repeat units or modules (Figs. 1A and 1B) which are structural units, each having the same fold, and which stack tightly to create a structure having a joint hydrophobic core. The stacked arrangements of the repeat units of a repeat protein, which independently lack the ability to form a stable protein domain or have specific functional activity, assemble within a tandem array of between 2 and 25 or more repeating units (modules) and form a repeat domain having a superhelical structure capable of protein-protein interactions. The term "folding topology" or “fold” refers to the tertiary structure of the repeat units within the repeat protein. Repeat modules or repeat units are of relatively short sequence motifs, typically from 20 to 40 amino acid residues in length. In most cases, repeat units will exhibit a high degree of sequence identity (same amino acid residues at corresponding positions) or sequence similarity (amino acid residues being different, but having similar physicochemical properties), and some of the amino acid residues might be key residues being strongly conserved in the different repeat units found in naturally occurring proteins. However, a high degree of sequence variability by amino acid insertions and/or deletions, and/or substitutions between the different repeat units will be possible as long as the common folding topology is maintained.

The term "repeat unit" refers to amino acid sequences comprising repeat sequence motifs of one or more naturally occurring repeat proteins, wherein said "repeat units" are found in multiple copies, and which exhibit a defined folding topology common to all said motifs determining the fold of the protein. Such repeat units comprise framework residues and interaction residues. Examples of such repeat units are armadillo repeat units, leucine-rich repeat units, ankyrin repeat units, tetratricopeptide repeat units, HEAT repeat units, and leucine-rich variant repeat units. Naturally occurring proteins containing two or more such repeat units are referred to as "naturally occurring repeat proteins.” The amino acid sequences of the individual repeat units of a repeat protein may have a significant number of mutations,

substitutions, additions and/or deletions when compared to each other, while still substantially retaining the general pattern, or motif, of the repeat units.

The term "repeat modules" refers to the repeated amino acid sequences of designed repeat proteins or domains. Each repeat module comprised in a repeat domain is derived from one or more repeat units of one family of naturally occurring repeat proteins where the members of said group comprise similar repeat units. Such "repeat modules" may comprise positions with amino acid residues present in all copies of the repeat module ("fixed positions") and positions with differing or "randomised" amino acid residues ("randomised positions"). Examples of such repeat modules are armadillo repeat modules, leucine-rich repeat modules, ankyrin repeat modules, tetratricopeptide repeat modules, HEAT repeat modules, and leucine-rich variant repeat modules. The amino acid sequences of the individual repeat units/repeat modules of a repeat protein may have a significant number of mutations, substitutions, additions and/or deletions when compared to each other, while still substantially retaining the general pattern, or motif, of the repeat units/repeat modules.

The term "set of repeat modules" refers to the total number of repeat modules present in a repeat domain. Such "set of repeat modules" present in a repeat domain comprises two or more consecutive repeat modules, and may comprise just one type of repeat module in two or more copies, or two or more different types of modules, each present in one or more copies. In the set of repeat modules, the order of the modules determines the composition of the repeat domain and, where a repeat domain has been selected for a specific activity, the repeat domain biological function, such as a binding domain. The repeat units/modules in a repeat domain will herein be numbered consecutively from the N-terminus of the polypeptide to the C-terminus of the polypeptide.

The term "repeat sequence motif" refers to an amino acid sequence, which is deduced from one or more repeat units or repeat modules. Such repeat sequence motifs comprise framework residue positions and target interaction residue positions. Said framework residue positions correspond to the positions of framework residues of the repeat units (or modules). Likewise, said target interaction residue positions correspond to the positions of target interaction residues of the repeat units (or modules). The target interaction residues will generally be positioned along one face of the repeat domain. An example of such a repeat sequence motif is an ankyrin repeat sequence motif, such as shown in SEQ ID NO: 1.

The term "framework residues" relates to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which contribute to the folding topology, i.e., which contribute to the fold of said repeat unit (or module) or which contribute to the interaction with a neighboring unit (or module). Such contribution might be the interaction with other residues in the repeat unit (module), or the influence on the polypeptide backbone

conformation as found in α-helices or β-sheets, or amino acid stretches forming linear polypeptides or loops.

The term "target interaction residues" refers to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which may contribute to the interaction of the repeat unit (or module) with a target substance. Such contribution might be the direct interaction with the target substances, or the influence on other directly interacting residues, e.g., by stabilizing the conformation of the polypeptide of a repeat unit (or module) to allow or enhance the interaction of directly interacting residues with said target. Such framework and target interaction residues may be identified by analysis of the structural data obtained by physicochemical methods, such as X-ray crystallography, NMR and/or CD spectroscopy, or by comparison with known and related structural information well known to practitioners in structural biology and/or bioinformatics.

Preferably, the repeat units/modules used for the deduction of a repeat sequence motif are homologous repeat units, wherein the repeat units comprise the same structural motif and wherein more than 70% of the framework residues of said repeat units are identical to each other. Preferably, more than 80% of the framework residues of said repeat units are identical. Most preferably, more than 90% of the framework residues of said repeat units are identical. Computer programs to determine the percentage of identity between polypeptides, such as Fasta, Blast or Gap, are known to the person skilled in the art. More preferably, the repeat units used for the deduction of a repeat sequence motif are homologous repeat units obtained from repeat domains selected on a target, for example, as described in Example 1, and having the same target-specificity.

Repeat sequence motifs comprise fixed positions and randomized positions. The term "randomized position" refers to an amino acid position in a repeat sequence motif, wherein two or more amino acids are allowed at said amino acid position, for example, wherein any of the usual twenty naturally occurring amino acids are allowed, or wherein most of the twenty naturally occurring amino acids are allowed, such as amino acids other than cysteine, or amino acids other than glycine, cysteine and proline. These amino acids may be in modified form as known in the art. Most often, such randomized positions correspond to the positions of target interaction residues. However, some positions of framework residues may also be randomized.

The term “capping module,” “capping unit” or “N-Cap” (for an N-terminal capping module) or “C-Cap” (for a C-terminal capping module) refers to a polypeptide fused to the N- or C-terminal repeat module of a repeat domain, wherein said capping module forms tight tertiary interactions with the adjacent repeat unit thereby providing a cap that shields the hydrophobic core of said repeat module at the side not in contact with the consecutive repeat module from the solvent. Said N- and/or C-terminal capping module may be, or may be derived from, a

capping unit or other domain found in a naturally occurring repeat protein adjacent to a repeat unit. The N- or C-Cap forms tight tertiary interactions with the adjacent repeat unit. Such capping units may have sequence similarities to the repeat sequence motif. Capping modules and capping repeats are described in WO 02/020565 and exemplified herein.

The term "target" refers to a molecule, polypeptide or protein, carbohydrate, complexes of two or more molecules, which may exist in isolated form or reside in a biological form, such as on or in a cell or a tissue sample and may exist in multiple forms, such as naturally occurring or non-naturally occurring chemical modifications, for example, modified by phosphorylation, acetylation, or methylation, or exhibiting damage or cross-linked residues such as may occur upon reaction with ionizing radiation or reactive oxygen species caused be natural or non-natural processes. In the particular application of the present invention, the target is a soluble protein which is a cytokine.

By IL4, IL-4, or hIL4, is meant a small cytokine, human Interleukin 4 (UniProt P05112, SEQ ID NO: 4) or a species homolog thereof. Where specifically stated, the species homolog sequence is specified, e.g. cynomolgous monkey IL4, cyno IL4, or cIL4 (SEQ ID NO: 5). The protein is also known as B-cell stimulatory factor 1, B-cell growth factor, BCGF1, BCGF-1, BSF1, BSF-1, and Lymphocyte stimulatory factor 1, among other names. The human mature protein is expressed as a 153 amino acid polypeptide (UniProt P05112) with a 24 amino acid signal peptide, a single N-linked glycosylation site, and is cleaved to produce a 129 amino acid mature protein (SEQ ID NO: 1) with three interchain disulfide bonds. Two types of IL4 receptor exist: Type 1 and Type 2. Type 1is a heterodimer consisting of the IL4 R-alpha (IL4 RA, CD124, UniProt P24394 and where SEQ ID NO: 6 represents the ECD thereof) and the common receptor subunit gamma, CD132 (IL2RG, UniProt P31785, SEQ ID NO: 7). The Type 2 receptor is a heterodimer consisting of IL4 R-alpha and IL13R-alpha1 (IL13RA1, CD213a1, UniProt P78552, SEQ ID NO: 8). IL13 (SEQ ID NO: 101) but not IL4 binds the Type 2 receptor by binding the IL13RA protein. In addition, IL13 binds IL13RA2 (SEQ ID NO: 102).

A “consensus amino acid residue” is the amino acid found most frequently at a certain position in a sequence identified by structural and/or sequence aligning of multiple repeat units. If two or more, e.g., three, four or five, amino acid residues are found with a similar probability in said two or more repeat units, the consensus amino acid may be one of the most frequently found amino acids or a combination of said two or more amino acid residues.

As used herein, the term "affinity" of binding between two molecules refers to a biophysical measurement of strength of interaction. The term "K_{dis}" or "K_D” or "K_d” as used herein, is intended to refer to the dissociation rate of a particular composition-target interaction. The “K_D,” is the ratio of the rate of dissociation (k_2), also called the “off-rate (k_{off})” or “k_d”, to the

rate of association (k_1) or “on-rate (k_{on})” or "k_a." Thus, K_D equals k_2/k_1 or k_{off} / k_{on} or k_d / k_a and is expressed as a molar concentration (M). It follows that the smaller K_D, the stronger the binding. Thus, a K_D of 10^{-6}M (or 1 μM) indicates weak binding compared to 10^{-9}M (or 1 nM). The K_D can be determined by surface plasmon resonance or the Kinexa method, as practiced by those of skill in the art. The measured affinity of a particular protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of affinity (e.g., K_D, k_{on}, k_{off}) are preferably made with standardized solutions of protein, and a standardized buffer.

The repeat proteins of the invention, selected for their biological activity resulting from interactions with other proteins or peptides, can be further modified to enhance or impart additional biophysical or biological properties to the molecules such as a polypeptide tag, a radioisotope, a chelator, and a multimerizing domain, which may be of a proteinaceous or a nonproteinaceous nature. For example, the ability to persist in the body can be enhanced by the addition of certain physiologically compatible polymers or the fusion of an immunoglobulin constant domain sequence to the protein. Examples of non-proteinaceous polymer molecules are hydroxyethyl starch (HES), polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylene. Modifications that enhance the ability of the protein to persist in the body through a decrease in clearance or increase in re-uptake are referred to as “half-life extending” modifications.

The term "polypeptide tag" refers to an amino acid sequence attached to a polypeptide/protein, wherein said amino acid sequence is useful for the purification, detection, or targeting of said polypeptide/protein, or wherein said amino acid sequence improves the physicochemical behavior of the polypeptide/protein, or wherein said amino acid sequence possesses an effector function. The individual polypeptide tags, moieties and/or domains of a binding protein may be connected to each other directly or via polypeptide linkers. These polypeptide tags are all well known in the art and are fully available to the person skilled in the art. Examples of polypeptide tags are small polypeptide sequences, for example, His, myc, FLAG, or Strep-tags or moieties, such as enzymes (for example enzymes like alkaline phosphatase), which allow the detection of said polypeptide/protein, or moieties which can be used for targeting (such as immunoglobulins or fragments thereof) and/or as effector molecules.

Examples of multimerization moieties are immunoglobulin heavy chain constant regions which pair to provide functional immunoglobulin Fc domains, and leucine zippers or polypeptides comprising a free thiol which forms an intermolecular disulfide bond between two such polypeptides.

The term "polypeptide linker" refers to an amino acid sequence, which is able to link, for example, two protein domains, a polypeptide tag and a protein domain, a protein domain and a non-polypeptide moiety, such as polyethylene glycol or two sequence tags. Such additional domains, tags, non-polypeptide moieties and linkers are known to the person skilled in the relevant art. A polypeptide linker or any intervening sequence between the repeat modules may be any sequence which does not interfere with the topology or the fold of the module or the ability of the modules to stack. Particular examples of such linkers are flexible glycine-serine-linkers of variable lengths; preferably, said linkers have a length between 2 and 16 amino acids, and Proline-Threonine linkers.

Overview

New IL4 and IL13 binding proteins were identified using libraries of repeat proteins comprising a consensus 33 amino acid ankyrin repeat module containing diversified potential interaction residues (any amino acid except cysteine, glycine or proline). As described herein, the amino acids at randomized positions in stacked repeat modules form an interaction surface that can bind with high affinity to a variety of targets (Figs. 1A and 1B). Binders have been selected from libraries of potential binding domains encompassing two to four AR modules having diversified amino acids at specific residue position and, which repeat domain is flanked by an N-terminal and C-terminal module. A preferred binding domain of the invention is a repeat domain or a designed repeat domain, preferably as described in WO 02/20565; Binz, H. K. et al., 2004, loc. cit.).

In a specific embodiment, the invention relates to a recombinant IL4 binding protein comprising a binding domain with specificity for IL4 selected from a library of repeat proteins comprising one or more repeat modules with the AR sequence motif

$X_1DX_3X_4GX_6TPLHLAAX_{14}X_{15}GHLEIVEVLLKX_{27}GADVNA$ (SEQ ID NO: 1),

wherein X_1, X_3, X_4, X_6, X_{14}, and X_{15} represent, independently of each other, an amino acid residue selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y. X_{27} represents A, H, N, or Y;

an N-terminal capping module of the amino acid sequence:

DLGKKLLEAARAGQDDEVRILMANGADVNA (SEQ ID NO: 2); and

a C-terminal capping module has an amino acid sequence:

QDKFGKTAFDISIDNGNEDLAEILQKLN (SEQ ID NO: 3).

The term “AR” means an ankyrin repeat module and “AR1” means the first tandem AR of an ankyrin repeat domain, the term “AR2” means the second AR of an ankyrin repeat domain, the term “AR3” means the third AR of an ankyrin repeat domain, and the term “AR4”

means the fourth AR of an ankyrin repeat domain. When arranged in tandem, the AR1 module is N-terminus of the AR2 module; the AR2 module is N-terminus of the AR3 module and, as applicable, the AR3 module is N-terminus of the AR4 module such that an AR arrangement is AR1-AR2-AR3-AR4. ARs do not include N-Cap or C-Cap sequences and, preferably, each AR has an N-Cap and C-Cap module. It will be appreciated that SEQ ID NO:2 is an example of an N-Cap sequence and SEQ ID NO:3 is an example of an C-Cap sequence and that these sequences may be modified as needed.

In specific embodiment, the invention relates to a recombinant IL13 binding protein comprising a binding domain with specificity for IL13 selected from a library of repeat proteins comprising one or more repeat modules with the AR sequence motif

$X_1DX_2X_3GX_4TPLHLAAX_5X_6GHLEIVEVLLKX_7GADVNA$ (SEQ ID NO: 1),

wherein X_1, X_2, X_3, X_4, X_5, and X_6 represent, independently of each other, an amino acid residue selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y. X_7 represents A, H, N, and Y;

an N-terminal capping module of the amino acid sequence (wherein bracketed sequences mean alternate amino acids for that position):

DL[D,G]KKLLEAARAGQDDEVRILMANGADVNA (SEQ ID NO: 174); and

a C-terminal capping module has an amino acid sequence:

QDKFGKT[A,P][A,F]DI[A,S][A,I]DNG[H,N]ED[I,L]AE[I,V]LQK[A,L][A,N] (SEQ ID NO: 175).

In addition to substitutions of the residues at the positions diversified in the creation of libraries based on the formula N-Cap-$[AR]_n$-C-Cap; generic binding protein mutations are encompassed by the identified binding protein structures. Generic mutations can be applied to any binding protein of the invention, in that these mutations occur within positions of the sequence that are common to all binding proteins of the above referenced library of binding domains. Common generic changes to specified residues of a binding domain of the invention are as summarized below.

Module	Position	Final Amino Acid Residue
N-Cap	1	G, A
N-Cap	3	D
AR	27	H, Y, A
C-Cap	27	A
C-Cap	28	A

Position 1 of the N-Cap is mutated from Asp to Gly or Ala to aid in the processing of the N-terminal methionine residue for expression in *E. coli* (Hirel et. al. PNAS 86:8247-8251 1989). Position 3 of the N-Cap is mutated from Gly to Asp, as this mutation has been found to stabilize the repeat protein consensus sequence as described in WO2010/060748. As the AR sequence motif (SEQ ID NO: 1) position 28 of the framework is Gly, there is the possibility of isolating AR proteins consisting of the sequence Asn_{27}-Gly_{28}. The Asn-Gly di-peptide is prone to deamidation reactions (Geiger and Clarke J. Biol. Chem. 252:785-794, 1987) and therefore position 27 of isolated Asn-Gly sequences can be mutated to His, Tyr or Ala to avoid potential deamidation. In some cases, the residue at position 27 is changed to Ala to reduce the potential immunogenicity of the region of the protein. Finally, target binding AR proteins selected by ribosome display end with the amino acid sequence Leu-Asn in the C-cap. This sequence is appended onto the AR proteins in order to accommodate a restriction site for sub-cloning into expression vectors for screening. The preferred amino acid sequence of these positions is Ala-Ala.

The invention relates to a binding protein comprising a binding domain, wherein said binding domain inhibits IL13 binding to IL13Ralpha1 or IL13Ralpha2 or IL4 binding to IL4RA and wherein said binding protein and/or binding domain has a midpoint denaturation temperature (Tm) above 40°C upon thermal unfolding and forms less than 5% (w/w) insoluble aggregates at concentrations up to 10 g/L when incubated at 37°C for 1 day in phosphate buffered saline (PBS). In a specific embodiment, the IL13 binding protein is comprised of two or three repeat modules represented by SEQ ID NO: 1 proceeded by an N-terminal capping module such as SEQ ID NO: 2 or 174, and followed by a C-terminal capping module such as SEQ ID NO: 3 or 175.

The invention relates to a binding protein comprising a binding domain, wherein said binding domain inhibits IL4 binding to IL4RA. Preferably, the K_D for the interaction of the binding domain to IL4 is below 10^{-7} M, below 10^{-8} M, below 10^{-9} M, or, in certain embodiments

below 10^{-10} M. Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies, are well known to the person skilled in the art.

The invention relates also to a binding protein comprising a binding domain, wherein said binding domain inhibits IL13 binding to IL13Ralpha1 and/or IL13Ralpha2. Preferably, the K_D for the interaction of the binding domain to IL13 is below 10^{-7} M, below 10^{-8} M, below 10^{-9} M, or, in certain embodiments below 10^{-10} M. Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies, are well known to the person skilled in the art.

Preferably, the binding protein and/or binding domain has a midpoint denaturation temperature (Tm) above 45°C, more preferably above 50°C, more preferably above 55°C, and most preferably above 60°C upon thermal unfolding. A binding protein or a binding domain of the invention possesses a defined secondary and tertiary structure under physiological conditions. Thermal unfolding of such a polypeptide results in a loss of its tertiary and secondary structure, which can be followed, for example, by circular dichroism (CD) measurements. The midpoint denaturation temperature of a binding protein or binding domain upon thermal unfolding corresponds to the temperature at the midpoint of the cooperative transition in physiological buffer upon heat denaturation of said protein or domain by slowly increasing the temperature from 10°C to about 100°C. The determination of a midpoint denaturation temperature upon thermal unfolding is well known to the person skilled in the art. This midpoint denaturation temperature of a binding protein or binding domain upon thermal unfolding is indicative of the thermal stability of said polypeptide.

Also preferred is a binding protein and/or binding domain forming less than 5% (w/w) insoluble aggregates at concentrations up to 20 g/l, preferably up 40 g/L, more preferably up to 60 g/L, even more preferably up to 80 g/L, and most preferably up to 100 g/L when incubated for over 5 days, preferably over 10 days, more preferably over 20 days, more preferably over 40 days, and most preferably over 100 days at 37°C in aqueous solution. The formation of insoluble aggregates can be detected by the appearance of visual precipitations, gel filtration or dynamic light scattering, which strongly increases upon formation of insoluble aggregates. Insoluble aggregates can be removed from a protein sample by centrifugation at 10,000xg for 10 minutes. Preferably, a binding protein and/or binding domain forms less than 2%, 1%, 0.5%, 0.2%, 0.1%, or 0.05% (w/w) insoluble aggregates under the mentioned incubation conditions at 37°C in PBS. Percentages of insoluble aggregates can be determined by separation of the insoluble aggregates from soluble protein, followed by determination of the protein amounts in the soluble and insoluble fraction by standard quantification methods.

Bioactivity

An EC_{50} value is the concentration of a substance, such as a binding protein or binding domain, which is required to produce for 50% of the complete or predetermined maximum effect under a specific set of conditions. When the effect is blocking or inhibiting an activity, the value is termed an inhibitory concentration producing 50% reduction in the effect (IC_{50}). An IC_{50} value may be applied to inhibition *in vitro* of an experimental determined parameter, such as the release of a detectable amount of a pathologic marker, or biomarker, from a cell, tissue, organ or in the body of a subject or animal. Such measurements may be direct measures of the activity of the protein composition or may be surrogates or downstream markers of the biological activity to be modified.

IL4 shares several biological activities with IL13. For example, either IL4 or IL13 can cause IgE isotype switching in B cells (Tomkinson et al. 200l J. Immunol. 166:5792-5800). Additionally, increased levels of cell surface CD23 and serum CD23 (sCD23) have been reported in asthmatic patients (Sanchez- Guererro et al. (1994) Allergy 49:587-92; DiLorenzo et al. (1999) Allergy Asthma Proc. 20.119-25). In addition, either IL4 or IL13 can upregulate the expression of MHC class II and the low-affinity IgE receptor (CD23) on B cells and monocytes, which results in enhanced antigen presentation and regulated macrophage function (Tomkinson et al., supra). Importantly, either IL4 or IL13 can increase the expression of VCAM-I on endothelial cells, which facilitates preferential recruitment of eosinophils (and T cells) to the airway tissues (Tomkinson et al., supra). Either IL4 or IL13 can also increase airway mucus secretion, which can exacerbate airway responsiveness (Tomkinson et al., supra). By acting to block signaling pathways which are different from those of IL13, IL4 inhibitors/antagonists can be used to inhibit differentiation of naïve T-cells to Th2 cells.

The present invention further relates to methods for using a binding protein which has both IL4 and IL13 neutralizing activity as described to inhibit an IL4 and IL13 mediated biological activity including but not limited to: IgE production; CD23 upregulation on B cells or monocytes; upregulation of VCAM-I on endothelial cells, eosinophil recruitment, TGFbeta induction, increased mucus secretion; fibrosis caused by fibroblast proliferation, collagen synthesis, and extracellular-matrix remodeling (Wynn TA et al. Nat Rev Immunol. 2004; 4: 583-94) or by stimulation of TGFbeta; and stimulation of 15-lipoxygenase activity with relaese of leukotrienes (e.g., LTA4, LTB4, LTC4, LTD4, LTE4, and/or LTF4). Therefore, any of IgE production, LTA4 and LTB4 release from blood monocytes, eosinophil recruitment, TGFbeta release, enhanced collagen synthesis, and extracellular-matrix remodeling may be used as measurement of the bioactivity of the effects of the IL4 or IL13 binding protein described herein.

IL13 bioassays also include the proliferation of cancerous or precancerous cell types such as TF-1 erythroleukemic cells. IL13 neutralization can be measured specifically as the ability of the IL13 binding protein to reduce IL13 binding to IL13R-alpha1 or IL13R-alpha2.

An IL13 binding composition of the invention can inhibit IL13 binding in a way that the apparent dissociation constant (K_d) between IL13 and IL13Ralpha2 or IL13Ralpha2 or an IL4 binding composition of the invention can inhibit IL4 binding in a way that the apparent dissociation constant (K_d) between IL4 and IL4RA is increased more than 10^2-fold, preferably more than 10^3-fold, more preferably more than 10^4-fold, more preferably more than 10^5-fold, and most preferably more than 10^6-fold. Preferred for IL13-binding is a binding protein and/or binding domain that inhibits IL13 or the human IL13 R130Q protein variant (IL13 R130Q – Vladich et al. "IL13 R130Q, a common variant associated with allergy and asthma, enhances effector mechanisms essential for human allergic inflammation" *J Clin Invest.* 2005; 115(3):747–754) binding to IL13Ralpha2 under specified in vitro conditions with an IC_{50} value below 100 nM, preferably below 10 nM, and more preferably below 1.0 nM.

IL4 neutralization can be measured specifically as the ability of the IL4 binding protein to reduce IL4 binding to IL4 RA. The IL4 binding proteins of the invention are characterized by the ability to inhibit IL4 dependent phosphorylation of STAT6 in a cell expressing a Type 2 IL4 receptor complex, such as a recombinant HEK cell line expressing a STAT6-*bla* reporter. The IL4 binding proteins are further characterized as having the additional property of being able to block or reduce signaling in a cell having the Type 1 IL4 receptor complex, such as demonstrated by inhbiting naive T-cell differentiation to the Th2 phenotype. The IL4 binding protein may block or reduce stimulation of IL-4 dependent TARC production from cells, such as A549 cells in the presence of 67 pM IL4. The IL4 binding protein of the invention binds to human and to Macaque spp. monkey IL4 homolog protein.

When an IL4 binding protein of the invention is coupled to an IL13 binding protein, the composition can inhibit IL13 binding in a way that the apparent dissociation constant (K_d) between IL13 and IL13Ralpha1 or IL13Ralpha2 is increased more than 10^2-fold, preferably more than 10^3-fold, more preferably more than 10^4-fold, more preferably more than 10^5-fold, and most preferably more than 10^6-fold. Preferred is a binding protein and/or binding domain that inhibits IL13 or the human IL13 R130Q protein variant (IL13 R130Q, Vladich et al. *J Clin Invest.* 2005; 115(3):747–754) binding to IL13Ralpha2 under specified in vitro conditions with an IC_{50} value below 100 nM, preferably below 10 nM, and more preferably below 1.0 nM.

One embodiment of the invention is a binding protein comprising a repeat module capable of blocking human IL4 or IL4 and IL13 activation of STAT6 phosphorylation in HEK-Blue STAT-6 cells which display the IL13Ralpha1 and IL4 RA proteins and, which when activated by IL4 or IL13, induces secretion of a reporter protein which is an active enzyme

alkaline phosphatase capable of transforming substrate to a chromophor. The binding protein of the invention inhibits IL4 or IL4 and IL13 activation of STAT6 with an IC_{50} of 1 nM or less, and preferably, 100 pM or less, and more preferably 10 pM or less in an *in vitro* assay. In addition, the binding protein of the invention inhibits cyno IL4 or IL13 from binding to the same cells with an IC_{50} which is 5 nM or less, and preferably 1 nM or less and, in addition, where the ratio between the IC_{50} for human IL4 or IL13 and the cynomolgous homologue IL4 or IL13 IC_{50} inhibition of STAT6 in engineered HEK-blue cells is 10 or less in an *in vitro* assay. Representative assays are described herein an known to those in the art.

Whereas, thymus and activation-regulation chemokine (TARC) is upregulated by IL13 (Imai et al. (1999) Int. Immunol. 11:81-88), induces the migration of TH2 cells (Hijnen et al. (2004) J. All.Clin. Immun. 113(2):334-40) and is upregulated in the airways of asthmatic patients (Leung et al. (2004) J. All. Clin. Immun. 114(1): 199-202); an embodiment of the binding protein and/or binding domain of the invention will inhibit TARC production by A549 cells with an IC_{50} value below 500 pM, preferably below 100 pM, and more preferably below 50 pM in the presence of 67pM IL4.

Compositions

The IL4-binding AR compositions of the invention conform to the formula of a binding protein (N-Cap-$[AR]_n$-C-Cap (**I**)) having two or three repeat modules which have affinity for binding to IL13 measured as a K_D of 10^{-6} M or less, a K_D of 10^{-7} M or less, a K_D of 10^{-8} M or less, or a K_D of 10^{-9} M or less, which binding protein molecules are comprised of a repeat module of SEQ ID NO: 1. In one embodiment of the IL4-binding protein, the AR domain comprises a repeat module with the sequence selected from any of SEQ ID NOS: 31 – 81.

In a particular embodiment of the invention, the IL4-binding protein, the AR1 sequence is selected from the group consisting of SEQ ID NOS: 31 – 46; followed by a second designed ankyrin repeat domain (AR2) selected from the group consisting of SEQ ID NOS: 47 – 61; and, optionally, where the second designed ankyrin repeat unit is followed by a third designed ankyrin repeat (AR3) unit selected from the group consisting of SEQ ID NOS: 62-78; and, optionally, the AR3 repeat unit is followed by an AR4 unit selected from the group consisting of SEQ ID NO: 79-81.

In a particular embodiment, the IL4 binding protein comprises an ankyrin repeat module with the ankyrin repeat sequence of SEQ ID NO: 53, wherein said repeat module is preceded by a repeat module with the ankyrin repeat sequence motif of SEQ ID NO: 36 and/or followed by a repeat module with the ankyrin repeat sequence motif of SEQ ID NO: 68.

In a particular embodiment, the IL4 binding protein comprises an ankyrin repeat module with the ankyrin repeat sequence of SEQ ID NO: 56, wherein said repeat module is

preceded by a repeat module with the ankyrin repeat sequence motif of SEQ ID NO: 39 and/or followed by a repeat module with the ankyrin repeat sequence motif of SEQ ID NO: 71.

In a particular embodiment, the IL4 binding protein comprises an AR unit with the sequence of SEQ ID NO: 59, wherein said repeat module is preceded by a repeat module with the AR sequence motif of SEQ ID NO: 43 and/or followed by a repeat module with the AR sequence motif of SEQ ID NO: 74.

In further embodiments exemplified herein, the AR units tandem arrangement is as specified in Table 3 by the designated SEQ ID NO: corresponding to the AR sequence motif at the specified position in the binding protein.

In one embodiment, the invention is an IL4 binding protein, wherein one AR unit selected from SEQ ID NOS: 31-81 is preceded by an N-Cap comprising SEQ ID NO: 2 and variants thereof. The variants comprise SEQ ID NO: 1 and molecules having 75% or greater identity to any of the molecules of SEQ ID NOS: 31-81 that bind to IL4 protein. In another embodiment, the invention is an IL4 binding protein, wherein one AR unit selected from SEQ ID NOS: 31-81 is followed by a C-cap comprising SEQ ID NO: 3 and variants thereof.

The IL4 binding protein having a binding domain with binding specificity for IL4 comprising the AR unit sequence selected from SEQ ID NOS: 31-81, may have its sequence modified for the purpose of: improving expression in a host cell, reducing the potential for one or more residues to undergo oxidation, reducing the potential for residues to undergo chemical deamidation, reducing the potential for a host to which the binding protein is administered to mount an immunological response, and/or where one or more residues is added or modified for the purpose of joining the IL13 binding protein with another protein or moiety. The binding protein will retain the binding specificity, affinity, and biophysical characteristics of solubility in aqueous solutions and lack of tendency to self-aggregate, and have a melting temperature greater than 45°C.

The invention more specifically encompasses an IL4 binding protein derived from a consensus sequence (motif) or observed frequency of identity of a particular amino acid at a diversified position obtained from a multi-sequence alignment of repeat units. For example, the IL4 binding protein may comprise ankyrin repeat modules AR1, AR2, and AR3 arranged in tandem having sequence motif of SEQ ID NO: 1, and wherein the AR1 module has an amino acid according to the formula (wherein bracketed sequences mean alternate amino acids for that position):

X_1D-[DW]-GX_4TPLHLAA-[TD]-GHLEIVEVLLKX_7GADVNA, wherein X_1 is selected from T, V, I, L, S, A, E, F, H, K, and Y; X_4 is selected from D, F, L, I, N, E, S, Y, and T; and X_7 is selected from H, N, and Y (SEQ ID NO: 82);

the AR2 module having an amino acid sequence represented by the formula:

X_1D-X_2X_3-G X_4TPLHLAA-[X_5X_6]-GHLEIVEVLLKX_7GADVNA, wherein X_1 is selected from S, I, D, Q, A, E, H, K, L, M, N, and V; X_2X_3 is selected from AM, RM, AI, AS, NF, NI, NL, MN, WN, SQ, DD, DT, ET, and LF; X_4 is selected from D, M, L, F, I, N, W, and Y; X_5X_6 is selected from VY, VE, FF, FV, AD, AT, DF, FD, VD, LY, YY, and WT and X_7 is selected from H, N, and Y (SEQ ID NO: 83); and

the AR3 module having an amino acid sequence represented by the formula:

X_1D-X_2X_3-G F-TPLHLAA-X_5X_6-GHLEIVEVLLKX_7GADVNA, wherein X_1 is selected from M, K, V, E, N, Q, T, S,and Y; X_2X_3 is selected from FS, QT, LA, HH, SH, IL, IS, NL, MI, SN, RT, and LH; X_5X_6 is selected from FY, FS, NF, VD, EF, FA, FF, FW, SY, YN, and YY; and X_7 is selected from H, N, and Y (SEQ ID NO: 84).

In another embodiment, IL4 binding protein may comprise ankyrin repeat modules AR1, AR2, and AR3 arranged in tandem having sequence motif of SEQ ID NO: 1, and wherein the AR1 module has an amino acid according to the formula:

TD-[DW]-GX_4TPLHLAA-[TD]-GHLEIVEVLLKX_7GADVNA, wherein X_4 is selected from D, F, L, I, N, E, Y, and T and X_7 is selected from H, N, and Y (AR1-F, SEQ ID NO:85);

the AR2 module has an amino acid sequence represented by the formula:
X_1D-[AM]-GX_4TPLHLAA-[VY]-GHLEIVEVLLKX_7GADVNA, wherein X_1 is selected from S, I, D, Q, A, E, H, K, N, and V; X_4 is selected from D, M, L, F, I, and Y; and X_7 is selected from H, N, and Y (AR2-F, SEQ ID NO: 86); and

the AR3 module has an amino acid sequence represented by the formula:

X_1D-[X_2X_3]-G-F-TPLHLAA-[FY]-GHLEIVEVLLKX_7GADVNA, wherein X_1 is selected from M, K, V, E, N, T, S, and Y; X_2X_3 is selected from FS, QT, LA, HH, SH, IL, IS, NL, MI, SN, RT, LH, and VH; and X_7 is selected from H, N, and Y (AR3-F, SEQ ID NO: 87).

Based on optimized, active IL4 binding protein sequences, an IL4 binding protein of the invention may comprise ankyrin repeat modules AR1, AR2, and AR3 arranged in tandem having sequence motif of SEQ ID NO: 1, and wherein the AR1 module has an amino acid according to the formula:

[A,L,T]-DD-[S,W]-G-[D,I,Y]-TPLHLAA-[E,T]-DGHLEIVEVLLK-[A,H]-GADVNA (AR1-O) (SEQ ID NO: 88), followed by an AR2 module according to the formula:

[A,N,Q]-D-[NL,RL,AI]-GDTPLHLAA-[WT,FV,LY]-GHLEIVEVLLK-[A,Y]-GADVNA (AR2-O) (SEQ ID NO: 89), followed by an AR3 module according to the formula:

[T,V,Y]-D-[IS, LA, LH]-G-[F,I,V]-TPLHLAAF-[W,Y]-GHLEIVEVLLK-[A,H]-GADVNA (AR3-O) (SEQ ID NO: 90); where the bracketed entries represent the alternative amino acid residue or pair of residues.

In a preferred embodiment, the binding domains of the IL4 binding proteins include an N-capping module and a C-capping module as described and exemplified herein.

The IL13-binding AR compositions of the invention conform to the formula of a binding protein (N-Cap-$[AR]_n$-C-Cap (**I**)) having two or three repeat modules which have affinity for binding to IL13 measured as a K_D of 10^{-6} M or less, a K_D of 10^{-7} M or less, a K_D of 10^{-8} M or less, or a K_D of 10^{-9} M or less, which binding protein molecules are comprised of a repeat module of SEQ ID NO: 1. In one embodiment of the IL4-binding protein, the AR domain comprises a repeat module with the sequence selected from any of SEQ ID NOS: 108-155.

In a particular embodiment of the invention, the IL13-binding protein, the AR1 repeat sequence is selected from the group consisting of SEQ ID NOS: 108-125; followed by a second designed ankyrin repeat domain (AR2) selected from the group consisting of SEQ ID NOS: 109-143; and, optionally, where the second designed ankyrin repeat unit is, optionally, followed by a third designed ankyrin repeat doman (AR3) selected from the group consisting of SEQ ID NOS: 144-155.

The invention more specifically encompasses an IL13 binding protein derived from a consensus sequence (motif) or observed frequency of identity of a particular amino acid at a diversified position obtained from a multi-sequence alignment of repeat units. For example, the IL13 binding protein may comprise ankyrin repeat modules AR1, AR2, and AR3 arranged in tandem having sequence motif of SEQ ID NO: 1, and wherein the AR1 module has an amino acid sequence according to the formula:

X_1DX_2-X_3-GSTPLHLAA-RH-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from T, A, F, E, I, K, M, S, R, V, W; X_2 is selected from D, E, H, I, K, M, S, T,and V; X_3 is F or Y; and X_7 may be H, N, or Y (Formula AR1-C, SEQ ID NO: 156).

the AR2 module has an amino acid sequence according to the formula;

X_1DFIGDTPLHLAAY-X_4-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from N, T, A, D, K, E, H, M, and F; X_4 may be H or R; and X_7 may be H, N, or Y (Formula AR2-C, SEQ ID NO: 158); and

the AR3 module has an amino acid sequence according to the formula:

X_1D-X_2TGETPLHLAA-X_5X_6-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from D, S, T, K, E, and M; X_2 is chosen from A, I, T, and V or is absent; X_5X_6 are a pair of residues chosen from SM, HL, YH; and X_7 may be H, N, or Y (Formuala AR3-C, SEQ ID NO: 160); wherein the residues appearing in brackets are used in the alternative.

In one embodiment of the IL13-binding protein, the AR domain comprises a repeat module with the sequence selected from any of SEQ ID NOS: 108-155. In another embodiment, a binding protein with an AR domain, N-Cap and C-cap modules, may be constructed using the formulas provided herein for the tandem repeat modules such as N-Cap-[AR1-C:AR2-C:AR3-C]-C-cap where the repeat modules are specified by SEQ ID NO: 156, 158 and 160; or N-Cap-[AR1-F:AR2-F:AR3-F]-C-cap where the repeat modules are specified by SEQ ID NO: 157, 159, and 161; or N-Cap-[AR1-O:AR2-O:AR3-O]-C-cap where the repeat modules are specified by SEQ ID NO: 168, 169, and 170; and the N-cap and C-cap are specified by SEQ ID NO: 2 or 171 and SEQ ID NO: 3 and 172, respectively, or modification as described herein or as required for further chemical linkage, biological processing, and the like.

The IL13 binding protein comprising at least one repeat domain with binding specificity for IL13 comprising the repeat unit sequence selected from SEQ ID NOS: 108-155, may have its sequence modified for the purpose of: improving expression in a host cell, reducing the potential for one or more residues to undergo oxidation, reducing the potential for residues to undergo chemical deamidation, reducing the potential for a host to which the binding protein is administered to mount an immunological response, and/or where one or more residues is added or modified for the purpose of joining the IL13 binding protein with another protein or moiety. The binding protein will retain the binding specificity, affinity, and biophysical characteristics of solubility in aqueous solutions and lack of tendency to self-aggregate, and have a melting temperature greater than 45°C.

In one embodiment, the invention is an IL13 binding protein, wherein one ankyrin repeat module selected from SEQ ID NOS: 108-155 is preceded by an N-Cap comprising SEQ ID NO: 2 and variants thereof. The variants comprise SEQ ID NO:1 and molecules having 75% or greater identity to any of the molecules of SEQ ID NOS: 162-167 that bind to IL13 protein and/or IL13 R130Q. In another embodiment, the invention is an IL13 binding protein, wherein one ankyrin repeat domain selected from SEQ ID NOS: 162-167 is followed by a C-cap comprising SEQ ID NO: 3 and variants thereof. In further embodiments exemplified herein, the AR units tandem arrangement is as specified in Table 4 by the designated SEQ ID NO: corresponding to the AR sequence motif at the specified position in the binding protein.

In one embodiment, the invention is an IL13 binding protein, wherein one AR unit selected from SEQ ID NOS: 108-155 is preceded by an N-Cap comprising SEQ ID NO: 2 and variants thereof. The variants comprise SEQ ID NO: 1 and molecules having 75% or greater

identity to any of the molecules of SEQ ID NOS: 108-155 that bind to IL13 protein. In another embodiment, the invention is an IL13 binding protein, wherein one AR unit selected from SEQ ID NOS: 108-155 is followed by a C-cap comprising SEQ ID NO: 3 and variants thereof.

A binding protein that competes with IL13Ralpha2 for binding to IL13 with a selected repeat domain can be identified by methods well known to the person skilled in the art, such as a competition Enzyme-Linked ImmunoSorbent Assay (ELISA). Further, a modified binding protein having one or more modified repeat unit sequences may be tested for activity using a competition binding to IL13 with a binding protein known to compete with IL13Ralpha2 for binding to IL13.

Functional Properties

An IL4 binding protein that competes with IL4RA for binding to IL4 or an IL13 binding protein that competes with IL13Ralpha1 and IL13Ralpha2 for binding to IL13 with a selected repeat domain can be identified by methods well known to the person skilled in the art, such as a competition Enzyme-Linked ImmunoSorbent Assay (ELISA). Further, where a modified IL4 or IL13 neutralizing binding protein having one or more modified repeat unit sequences is desired to be produced, the activity of the modified binding protein may be tested for activity using a competition binding to of the modified binding protein with the unmodified protein. A modified IL4 binding protein may be tested in competition with a known IL4 binding protein for binding to IL4 and a modified IL13 binding protein may be tested in competition with an IL13 binding protein known to compete with IL13Ralpha1 and IL13Ralpha2 for binding to IL13.

In one embodiment of a modified binding protein, one or more of the amino acid residues of the repeat modules of said repeat domain are exchanged by an amino acid residue found at the corresponding position on alignment of a repeat unit. In one aspect, up to 30% of the amino acid residues are exchanged, more frequently, up to 20%, and even more frequently, up to 10% of the amino acid residues are exchanged. Most preferably, the source of the exchanged residue is a repeat unit which is a naturally occurring repeat unit. In still another particular embodiment, the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units.

In further embodiments, any of the IL4 and/or IL13 binding proteins or domains described herein may be covalently bound to one or more additional moieties, including, for example, a moiety that improves persistence in the circulation or decreases elimination from the body (i.e., improves pharmacokinetics), a labeling moiety (e.g., a fluorescent label, such as fluorescein, or a radioactive tracer), a moiety that facilitates protein purification (e.g., a small peptide tag, such as a His- or strep-tag), a moiety that provides effector functions for improved therapeutic efficacy (e.g., the Fc part of an antibody to provide antibody-dependent cell-mediated cytotoxicity), a toxic protein moiety, such as *Pseudomonas aeruginosa* exotoxin A

(ETA) or a small molecular toxic agent such as a maytansinoid, calicheamicin, or platinum containing DNA alkylating agents. Improved pharmacokinetics may be assessed according to the perceived therapeutic need. Often it is desirable to increase bioavailability and/or increase the time between doses, possibly by increasing the time that a protein remains available in the serum after dosing. In some instances, it is desirable to improve the continuity of the serum concentration of the protein over time (e.g., decrease the difference in serum concentration of the protein shortly after administration and shortly before the next administration). Moieties that slow clearance of a protein from the blood include hydroxyethyl starch (HES), polyethylene glycol (PEG), sugars (e.g., sialic acid), well-tolerated protein moieties (e.g., Fc fragment or serum albumin), and binding domains or peptides with specificity and affinity for abundant serum proteins, such as those capable of binding to serum albumin.

Nucleic Acids and Uses

In a further embodiment, the invention relates to nucleic acid molecules encoding the particular IL4 and/or IL13 binding proteins and, further, a vector comprising the nucleic acid molecule. In general, a bacterial expression vector will contain (1) regulatory elements, usually in the form of viral promoter or enhancer sequences and characterized by a broad host and tissue range; (2) a sequence, facilitating the insertion of a DNA fragment within the vector; and (3) the sequences encoding the final protein. The vector will likely also contain (4) a selectable marker gene(s) (e.g., the beta-lactamase gene), often conferring resistance to an antibiotic (such as ampicillin), allowing selection of initial positive transformants; and (5) sequences facilitating the replication of the vector in bacterial and mammalian host cells, or sequences promoting stable insertion into the genome of the host. A plasmid origin of replication are included for propagation of the expression construct in bacteria such as E. coli and for transient expression in Cos cells, the SV40 origin of replication is included in the expression plasmid. In addition, a suitable mammalian cell line may be used having the properties addressed above.

Therefore, the invention contemplates host cells used in the recombinant expression of the IL4 and IL13 binding protein repeat domains and proteins and more complex constructs comprising the IL4 and IL13 binding repeat proteins, which host cells will comprise the nucleic acids encoding such proteins. The IL4 and IL13 binding proteins may be purified from cultures in which such host cells are maintained as batch or continuous cultures by methods known in the art. The isolated proteins may be expressed with appended moieties, such as tags, that facilitate purification and which can be subsequently removed prior to final formulation and packaging of the protein for its intended use.

Formulations and Uses of IL4 and IL13 Binding Repeat Proteins

A pharmaceutical composition of the invention comprises one or more of the above mentioned binding proteins, in particular, binding proteins comprising repeat domains, or

nucleic acid molecules encoding the particular binding proteins and, optionally, a pharmaceutically acceptable carrier and/or diluent. Pharmaceutically acceptable carriers and/or diluents are known to the person skilled in the art and are explained in more detail below. Even further, the invention comprises a diagnostic composition comprising one or more of the above mentioned binding proteins, in particular, binding proteins comprising repeat domains. Where delivery of a nucleic acid encoding the IL4 and IL13 binding protein is performed, the pharmaceutical preparation of the therapy vector can include the vector in an acceptable diluent, or can comprise a slow release matrix in which the vector is imbedded. Alternatively, where the complete vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the nucleic acid delivery system.

A pharmaceutical composition of the invention is a stable formulation comprising the IL4 and IL13 binding protein, which may be an aqueous phosphate buffered saline or mixed salt solution or, alternatively, preserved solutions and formulations, multi-use preserved formulations suitable for pharmaceutical or veterinary use in a pharmaceutically acceptable formulation. Suitable vehicles and their formulation, inclusive of other proteins, e.g., human serum albumin, are described, for example, in e.g. Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, See especially pp. 958-989. The formulations to be used for in vivo administration may be aseptic or sterile. This is readily accomplished by filtration through sterile filtration membranes but other methods may be applied, such as heat, gas or chemical sterilization, or by the use of ionizing radiation to some or all of the components of the formulation.

The pharmaceutical composition may be administered by any suitable method within the knowledge of the skilled practitioner, wherein the administration may be performed by another or self-administered. The route of administration may be selected from a variety of delivery methods including but not limited to: intravenous (I.V.); intramusclular (I.M.); subcutaneous (S.C.); transdermal; pulmonary; transmucosal (oral, intranasal, intravaginal, rectal); using a formulation in a tablet, capsule, solution, powder, gel, particle; and contained in a syringe, an implanted device, osmotic pump, cartridge, micropump; or other means appreciated by the skilled artisan, as well-known in the art.

For example, site specific administration may be to body compartment or cavity such as intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intracardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial,

intrathoracic, intrauterine, intravascular, intravesical, intralesional, vaginal, rectal, buccal, sublingual, intranasal, or transdermal means.

The IL4 and IL13 binding protein can be administered directly to the respiratory tract by a nebulizer, such as jet nebulizer or an ultrasonic nebulizer. Typically, in a jet nebulizer, a compressed air source is used to create a high-velocity air jet through an orifice. As the gas expands beyond the nozzle, a low-pressure region is created, which draws a solution of protein through a capillary tube connected to a liquid reservoir. The liquid stream from the capillary tube is sheared into unstable filaments and droplets as it exits the tube, creating the aerosol. A range of configurations, flow rates, and baffle types can be employed to achieve the desired performance characteristics from a given jet nebulizer. In an ultrasonic nebulizer, high-frequency electrical energy is used to create vibrational, mechanical energy, typically employing a piezoelectric transducer. This energy is transmitted to the formulation of protein either directly or through a coupling fluid, creating an aerosol including the protein. Advantageously, particles of protein delivered by a nebulizer have a particle size less than about 10 μm, preferably in the range of about 1 μ m to about 5 μ m, and most preferably about 2 μm to about 3 μm.

Indications

The invention further provides novel methods of treatment of IL4 and IL13 mediated diseases and conditions, in particular, respiratory conditions, such as asthma and pulmonary fibrosis, cardiovascular conditions, cancer, dermatological, and fibrotic conditions. The present invention provides high affinity IL4/IL13 bispecific binding proteins, and nucleic acids encoding them, capable of blocking the biological activity of IL4, *in vitro*, *in vivo*, or *in situ* including, but not limited to, inhibiting wild-type or natural variants of human IL4 binding to IL-4RA or fragments thereof, inhibiting IL4 RA complexes on cells from signaling in the presence of IL4 , and preventing or suppressing IL4 -dependent differentation of naïve T-cells to Th2 cells.

Binding proteins capable of binding and neutralizing IL13 have one or more properties selected from: inhibiting binding of IL13 to human IL13 receptor alpha 1 (IL13Ralpha1) (UniProt. P78552) or fragments thereof, inhibiting human IL13 or natural variants of human IL13 binding to the human IL13 receptor alpha 2 (IL13Ralpha2) (UniProt. Q14627) or fragments thereof, preventing or suppressing human IL13-dependent proliferation of human tumor cells, inhibiting human IL13-dependent IgE production, reducing eosinophilic infiltration of tissues, and which protein has a specific binding site on human IL13.

In one aspect, a method of treating a human patient afflicted with a disease or disorder selected from the group consisting of a form of asthma which can be named allergic asthma, severe asthma, difficult asthma, brittle asthma, nocturnal asthma, premenstrual asthma, steroid resistant asthma, steroid dependent asthma, aspirin induced asthma, adult-onset asthma,

pediatric asthma, or exercise induced asthma; atopic disease such as atopic dermatitis; allergic rhinitis; Crohn's disease; COPD; fibrotic diseases or disorders such as idiopathic pulmonary fibrosis, progressive systemic sclerosis, hepatic fibrosis, radiation-induced fibrosis, chemotherapy-induced fibrosis; hepatic granulomas; schistosomiasis, leishmaniasis, diseases of cell cycle regulation such as Hodgkins disease; B cell chronic lymphocytic leukaemia; which method comprises administering a therapeutically effective amount of an IL4/IL13 binding protein.

Examples of IL4/IL13-associated disorders or conditions include, but are not limited to, disorders chosen from one or more of: IgE-related disorders, including but not limited to, atopic disorders, e.g., resulting from an increased sensitivity to IL4/IL13 (e.g., atopic dermatitis, urticaria, eczema, and allergic conditions such as allergic rhinitis and allergic enterogastritis); respiratory disorders, e.g., asthma (e.g., allergic and nonallergic asthma (e.g., asthma due to infection with, e.g., respiratory syncytial virus (RSV), e.g., in younger children)), chronic obstructive pulmonary disease (COPD), and other conditions involving airway inflammation, eosinophilia, fibrosis and excess mucus production, e.g., cystic fibrosis and pulmonary fibrosis, systemic sclerosis (SSc), and idiopathic pulmonary fibrosis (IPF) and sarcoidosis, desquamative interstitial pneumonia, acute interstitial pneumonia, respiratory bronchiolitis-associated interstitial lung disease, idiopathic bronchiolitis obliterans with organizing pneumonia; lymphocytic interstitial pneumonitis; Langerhans' cell granulomatosis; idiopathic pulmonary hemosiderosis; acute bronchitis; pulmonary alveolar proteinosis; bronchiectasis; atelectasis; cystic fibrosis; inflammatory and/or autoimmune disorders or conditions, gastrointestinal disorders or conditions (e.g., inflammatory bowel diseases (IBD) and eosinophilic esophagitis (EE), and eosinophilic-mediated gastrointestinal disease, ulcerative colitis and/or Crohn's disease), liver disorders or conditions (e.g., cirrhosis, hepatocellular carcinoma), and scleroderma; tumors or cancers (e.g., soft tissue or solid tumors), such as leukemia, glioblastoma, and lymphoma, e.g., Hodgkin's lymphoma; viral infections (e.g., from HTLV-I); fibrosis of other organs, e.g., fibrosis of the liver (e.g., fibrosis caused by a hepatitis B and/or C virus); and suppression of expression of protective type 1 immune responses, (e.g., during vaccination).

The method of treating a subject includes administering a high affinity IL4 and IL13 bispecific binding protein to the subject, in an amount effective to reduce one or more symptoms of the disorder or condition (e.g., in an amount effective to reduce one or more of: a respiratory symptom (e.g., bronchoconstriction), IgE levels, release or levels of histamine or leukotriene, or eotaxin levels in the subject). In the case of prophylactic use (e.g., to prevent, reduce or delay onset or recurrence of one or more symptoms of the disorder or condition), the subject may or may not have one or more symptoms of the disorder or condition.

In one embodiment, the high affinity IL4 and IL13 bispecific binding protein inhibits or reduces one or more symptoms associated with an early phase of the IL4 or IL13 associated disorder, e.g., an "early asthmatic response" or "EAR." For example, the IL4 and IL13 bispecific binding protein reduces one or more symptoms associated with an EAR, at about 0.25 to 3 hours after an insult (e.g., allergen exposure) until about 3 hours after insult (e.g., allergen exposure). The IL4 and IL13 bispecific binding protein can decrease or prevent one or more symptoms of the EAR as compared to the level or degree of the symptom in the subject in the absence of the IL4 and IL13 bispecific binding protein. Alternatively, the IL4 and IL13 bispecific binding protein can prevent as large of an increase in the symptom, e.g., as compared to the level or degree of the symptom in the subject in the absence of the IL4 and IL13 bispecific binding protein) including, but not limited to, one or more of: a release of at least one allergic mediator such as a leukotriene and/or histamine, e.g., from airway mast or basophil cells; an increase in the levels of at least one allergic mediator, such as a leukotriene and/or histamine; bronchoconstriction; and/or airway edema.

In other embodiments, the IL4 and IL13 bispecific binding protein inhibits or reduces one or more symptoms associated with a late phase of an IL4 or IL13 associated disorder, e.g., a "late asthmatic response" or "LAR." For example, the IL13 binding protein reduces one or more symptoms associated with an LAR, e.g., at about 3 hours and up to about 24 hours after an insult (e.g., allergen exposure). For example, the IL4 and IL13 bispecific binding protein can decrease or prevent one or more symptoms of the LAR (e.g., as compared to the level or degree of the symptom in the subject in the absence of the binding protein), e.g., one or more of: airway reactivity and/or an influx and/or activation of inflammatory cells, such as lymphocytes, eosinophils and/or macrophages, e.g., in the airways and/or bronchial mucosa. Alternatively, the IL4 and IL13 bispecific binding protein can prevent as large of an increase in the symptom, e.g., as compared to the level or degree of the symptom in the subject in the absence of the IL4 and IL13 bispecific binding protein.

The IL4 and IL13 bispecific binding protein can be administered prior to the onset or recurrence of one or more symptoms associated with the IL4/IL13-disorder or condition, but before a full manifestation of the symptoms associated with the disorder or condition. In certain embodiments, the IL4 and IL13 bispecific binding protein is administered to the subject prior to exposure to an agent that triggers or exacerbates an IL4/IL13-associated disorder or condition, e.g., an allergen, a pollutant, a toxic agent, an infection and/or stress. In some embodiments, the IL4 and IL13 bispecific binding protein is administered prior to, during, or shortly after exposure to the agent that triggers and/or exacerbates the IL13-associated disorder or condition. For example, the IL4 and IL13 bispecific binding protein can be administered 1, 5, 10, 25, or 24 hours; 2, 3, 4, 5, 10, 15, 20, or 30 days; or 4, 5, 6, 7 or 8 weeks, or more before or after exposure to the triggering or exacerbating agent. Typically, the IL4 and IL13 bispecific

binding protein can be administered anywhere between 24 hours and 2 days before or after exposure to the triggering or exacerbating agent.

In another embodiment of the invention, an IL4 and IL13 bispecific binding protein inhibiting the activity of human IL4 or IL13 or naturally occurring variant, as described above, can be used in combination with a second binding protein or with an active that is a small molecule which can act additively or synergistically with the IL4 and IL13 bispecific binding protein or can act through a complementary mechanism to ameliorate one or more disease symptoms or sequelae. For example, an IL4 binding protein that is an IL4 antagonist could be administered with an IL13 binding protein. Since many disease pathologies are multi-factorial, efficacy may be improved by combining agents that inhibit multiple targets on one pathway or multiple targets on different pathways. One advantage of the IL4 and IL13 bispecific binding proteins of the invention is the ability to genetically link them together so that one binding protein inhibits one target and a second binding protein inhibits a different target or multiple targets. Alternatively, a specific cysteine residue could be introduced into a unique position on the binding protein that does not interfere with binding and used to directly couple a small molecule therapeutic. Coadministration of an IL4 and IL13 bispecific binding protein with a second therapeutic agent is also possible.

An additional embodiment is the use of a binding protein according to the present invention for manufacture of a medicament for treating IL4/IL13-associated disorders or conditions, including, without limitation, cardiovascular conditions, respiratory conditions, cancer, dermatological conditions, and fibrotic conditions, and, more specifically, for treating asthma and/or pulmonary fibrosis.

Examples of preferred additional therapeutic agents that can be coadministered and/or coformulated with an IL4 and IL13 bispecific binding protein include: inhaled steroids; beta-agonists, e.g., short-acting or long-acting beta-agonists; antagonists of leukotrienes or leukotriene receptors; combination drugs such as ADVAIR®; IgE inhibitors, e.g., anti-IgE antibodies (e.g., XOLAIR®); phosphodiesterase inhibitors (e.g., PDE4 inhibitors); xanthines; anticholinergic drugs; mast cell-stabilizing agents such as cromolyn; IL4 inhibitors (e.g., an IL4 inhibitor antibody, IL4 receptor fusion or an IL4 mutein); IL-5 inhibitors; eotaxin/CCR3 inhibitors; and antihistamines. Such combinations can be used to treat asthma and other respiratory disorders. Additional examples of therapeutic agents that can be co-administered and/or co-formulated with an IL4 and IL13 bispecific binding protein include one or more of: TNF antagonists (e.g., a soluble fragment of a TNF receptor, e.g., p55 or p75 human TNF receptor or derivatives thereof, e.g., 75 kd TNFR-IgG (75 kD TNF receptor- IgG fusion protein, ENBREL®)); TNF enzyme antagonists, e.g., TNFalpha converting enzyme (TACE) inhibitors; muscarinic receptor antagonists; TGFbeta antagonists; interferon gamma; perfenidone; chemotherapeutic agents, e.g., methotrexate, leflunomide, or a sirolimus (rapamycin) or an

analog thereof, e.g., CCI-779; COX2 and cPLA2 inhibitors; NSAIDs; immunomodulators; and NFkB inhibitors, among others.

Method of Producing the IL4 and IL13 binding protein

The IL4 and IL13 bispecific binding protein according to the invention may be obtained and/or further evolved by several methods, such as ribosomal display (WO 98/48008), display on the surface of bacteriophages (WO 90/02809, WO 07/006665) (a different signal sequence that allows export of folded proteins may be required; Steiner, D. et al. JMB 2008 382(5) 1211-1227) or bacterial cells (WO 93/10214), display on plasmids (WO 93/08278) or by using covalent RNA-repeat protein hybrid constructs (WO 00/32823), or intracellular expression and selection or screening such as by protein complementation assay (WO 98/341120). Such methods are known to the person skilled in the art.

A library of ankyrin repeat proteins used for the selection, screening, and characterization of a binding protein according to the invention may be obtained according to protocols known to the person skilled in the art (WO 02/020565, Binz, H.K. et al., JMB, *332*, 489-503, 2003, and Binz et al., 2004, loc. cit). The use of such a library for the selection of human IL4 and IL13 specific binding proteins is given in Example 1. In analogy, the ankyrin repeat sequence motifs as presented above can used to build libraries of ankyrin repeat proteins that may be used for the selection or screening of human IL4 and/or IL13 binding proteins. Furthermore, repeat domains of the present invention may be modularly assembled from repeat modules according the current inventions and appropriate capping modules (Forrer, P., et al., FEBS letters *539*, 2-6, 2003) using standard recombinant DNA technologies (e.g. WO 02/020565, Binz et al., 2003, loc. cit. and Binz et al., 2004, loc. cit).

As the nucleic acids encoding the desired IL4 and/or IL13 binding repeat modules are identified from, for example, the libraries described herein comprising designed repeat modules coded in tandem repeats to form binding domains; they are isolated and used to form expression vectors for use as therapeutics or for construction of host cells for the purpose of preparing and purifying the IL4 and IL13 bispecific binding domains. The host cells may be bacterial, insect, plant, or mammalian and or may be selected from COS-1, COS-7, HEK293, BHK21, CHO, BSC-1, Hep G2, 653, SP2/0, 293, HeLa, myeloma, lymphoma, yeast, insect or plant cells; or may be any derivative, subline, immortalized or transformed cell related to the aforementioned cell types or cell lines.

The invention is not restricted to the particular embodiments described in the Examples. Other sources may be used and processed following the general outline described below.

EXAMPLES

All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Materials

Chemicals were purchased from Fluka (Switzerland). Oligonucleotides were from Microsynth (Switzerland). Unless stated otherwise, DNA polymerases, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas (Lithuania). The cloning and protein production strain was *Escherichia coli* XL1-blue (Stratagene, USA). The PBS used contained 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl at pH 7.4

Designed ankyrin repeat protein libraries

The N2C and N3C designed ankyrin repeat protein libraries are described (WO 02/20565; Binz et al. 2003, loc. cit.; Binz et al. 2004, Nat Biotechnol 22: 575-82, 2004; Binz, et al., J Mol Biol 332: 489-503, 2003). The digit in N2C (e.g., 2 ankyrin repeat modules) and N3C (e.g., 3 ankyrin repeat modules) describes the number of randomized ankyrin repeat modules present between the N-terminal and C-terminal capping modules. The nomenclature used to define the positions inside the repeat units and modules is based on Binz et al. 2004, loc. cit. with the modification that borders of the repeat modules and repeat units are shifted by one amino acid position. For example, position 1 of a repeat module of Binz et al. 2004 (loc. cit.) corresponds to position 2 of a repeat module of the current disclosure (SEQ ID NO: 1) and consequently position 33 of a repeat module or of the N-cap module of Binz et al. 2004, loc. cit. corresponds to position 1 of a following repeat module as presently described. All the DNA sequences were confirmed by sequencing.

EXAMPLE 1: SELECTION OF BINDING PROTEINS COMPRISING A REPEAT DOMAIN WITH BINDING SPECIFICITY FOR IL4 AND IL13

The selection of IL4- and IL13- binding specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using a recombinant human IL4 target protein (UniProt Accession No: P05112, SEQ ID NO: 4) and IL13 protein.

Selection and Screening of human IL4 binding proteins:

In total, nine ribosome display selection rounds on biotinylated human IL4 (Peprotech #200-04, mature protein produced in E. coli) were performed with the N2C and N3C AR protein libraries. The first four rounds were standard ribosome display selection rounds according to previously published protocols, using decreasing target concentration and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz, Amstutz, Kohl, Stumpp, Briand, Forrer, Grutter and Pluckthun, Nat Biotechnol 22: 575-82, 2004; Zahnd, et al., Nat Methods 4: 269-79, 2007). The pools after these four initial rounds were screened for

binders to human IL4 by crude extract ELISA and a crude extract cellular HEK/STAT6 functional assay. The selected binders were of nanomolar affinity (K_D), as revealed by SPR measurements of single clones (data not shown).

To specifically enrich higher affinity AR proteins, two off-rate selection rounds with increased selection stringency, each followed by one or two standard selection rounds, were performed after the first four rounds (Zahnd, et al., J Biol Chem 281: 35167-75, 2006).

Following this sixth round of ribosome display, single clones obtained from these rounds were screened by crude extract cellular HEK/STAT6 functional assay, to identify the most potent candidates. The pool of selected AR proteins was subcloned into a T5 promoter based vector for expression. Following expression, crude lysates from 200 individual AR proteins were assessed for binding to recombinant IL4 by ELISA and inhibition of IL4 dependent STAT6 phosphorylation in HEK-STAT6 cells. Lysates were prepared by transforming plasmids encoding specific AR proteins into *E. coli* XL-1 blue cells. A 1.2 ml starter culture in Luria Bertani medium (LB) containing 50 ug/ml ampicillin and 1% glucose was inoculated with a single colony. The starter cultures were incubated overnight at 37°C, shaking at 220 rpm. On the next day, a part of the overnight culture was used as inoculum of 0.9 ml LB. Protein expression was induced using 500 uM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were incubated 4 hours at 37°C, shaking at 220 rpm. Cell pellets were harvested by centrifugation and lysed with 50 µl B-Per solution (Pierce). These lysates were diluted with PBS before using them in subsequent screening assays.

In order to assess the binding to IL4, each crude extract of the lysates containing a binding protein was added to Maxisorp ELISA plate pre-coated with neutravidin and biotinylated IL4 and incubated for 1 hour. After extensive washing, bound AR proteins were detected using an anti-RGS-His6-HRP conjugate (34450, Qiagen).

In parallel, the same 200 single clone *E. coli* lysates were subjected to a cellular inhibition assay. The activity of each crude extract sample was assayed for their ability to inhibit IL4 dependent activation of STAT6 using HEK-Blue STAT-6 cells (Invivogen™, SanDiego, CA). Stimulation of HEK-Blue STAT-6 cells was carried out as follows: on Day 1, cells were plated in 96-well cell culture plates at a density of $2.5x10^5$/ml in 100 µl of cell culture media (DMEM with 4.5 g/L Glucose (11995, Gibco/Invitrogen, Carlsbad, CA), 10% Heat Inactivated FBS (10082, Gibco/Invitrogen, Carlsbad, CA), 10 µg/mL Blasticidin S, a peptidyl nucleoside antibiotic active (Invivogen), and 100 µg/mL Zeocin™, a copper-chelated glycopeptide antibiotic produced by Streptomyces CL990 (Invivogen) for 8 hours. On the same day, 100 µl of cell culture media containing the diluted AR protein crude extracts premixed with 50 pg/ml (3.3 pM) human IL4 (Peprotech) were added. The plates were incubated overnight at 37 °C and 5% CO_2. To measure secreted embryonic alkaline™

phosphatase, 30 μl of each cell supernatant was mixed with 80 μl of Quanti-Blue™ (Invivogen) in a clear 96-well plate. The plate was incubated for 1 hour at 37 °C and absorbance at 620 nm was read using a plate reader.

As the initial screen of 200 clones produced only a few AR proteins that bound with high affinity to IL4 and effectively inhibited signaling, single clone crude extracts of 5100 more AR proteins obtained following additional rounds of ribosome display with off-rate selection (rounds 7, 8, and 9) were tested for their ability to inhibit IL4 dependent STAT6 phosphorylation as described above. The activity of these clones were compared in this assay to a benchmark AR protein, clone C06_28E5, found in the first round screen to bind to IL4 with an apparent affinity of 50 pM as revealed by SPR and inhibit STAT6 production with an IC50 of 3 pM in the presence of 3 pM IL4. Thus, comparing subsequently selected clones to the test values of the benchmark allowed for expedient selection of additional high potency candidates.

Based on the results of the STAT6 phosphorylation screen, 22 AR proteins (SEQ ID NO: 9-30) that showed inhibition of IL4 with better or equal activity compared to that of the benchmark were selected for further characterization.

The selection of IL13-binding specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using a human IL13 variant (R130Q) target protein. The IL13 R130Q variant (R110Q of SEQ ID NO: 1) is a variant of human IL13 that has been linked to atopic patients (Arima et al. J. Aller. Clin. Immunol. 109:980-987, 2002).

Selection and Screening of human IL13 binding proteins:

In total, 6 ribosome display selection rounds on human IL13 R130Q (Peprotech) were performed in solution with both the N2C and N3C AR libraries. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentration and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. Nature Biotech 22:575-582, 2004). After four rounds of panning, the pools were screened for binders to human IL13 in the crude extract using an ELISA format. The selected binders were of nanomolar affinity, as revealed by BIAcore measurements of single clones (data not shown).

Following the fourth round of ribosome display selection, the pool of selected binding proteins were cloned into a T5 promoter based expression vector. Following expression, 200 individual binding proteins were assessed for binding as crude extracts to human L-13 R130Q captured on neutravidin plates. Of these, 32 binding proteins with the highest binding signal by ELISA were expressed and purified by immobilized metal ion affinity chromatography (IMAC) and screened for their ability to inhibit IL13R13Q dependent binding to human IL13Rα2 –Fc fusion using an ELISA format: binding protein candidates (10 nM or 100 nM final

concentration) were pre-incubated with 10 nM biotinylated human IL13 R130Q for 30 minutes, the binding protein-IL13 mixture was added to a Maxisorp ELISA plate pre-coated with IL13Rα2-Fc (R&D Systems) and incubated for 15 minutes to capture free biotinylated IL13 and detected using streptavidin-horse radish peroxidase. The relative amount of inhibition produced by the binding protein was assessed by comparing the signal measured for 10 nM biotinylated IL13 with no binding protein added. Based on the results of this screening assay at 10 nM binding protein, which was equimolar to the IL13R130Q, IL13 binding protein 2F1 was chosen as a benchmark for all further screenings.

To identify higher affinity human IL13 binders, the output from the fourth round of standard ribosome display screening (above) was subjected to an off-rate selection round with increased selection stringency. A final standard selection round was performed to amplify and recover the off-rate selected binding proteins. Again, crude extracts were screened for binding to IL13 as described above and the signal relative to 2F1 binding protein was assessed. To enable differentiation between high and low affinity binding proteins, the dilution of the crude extracts of 1:10 was chosen because it allowed clear differentiation of binding proteins with stronger binding to IL13 than the benchmark binding protein 2F1. About 700 binding protein clones were assayed in this manner.

In parallel, crude extracts screened for binding were evaluated for their ability to inhibit IL13 dependent activation of STAT6 using HEK-Blue STAT-6 cells (InVivogen, SanDiego, CA). To enable differentiation between high and low affinity binding proteins, an optimal dilution of the crude extract (1:5200) was selected. In the HEK-Blue STAT-6 cells, IL13 activates the IL13Rα1: IL4R complex (Type 2 receptor) to induce secretion of an embryonic alkaline phosphatase (SEAP) reporter gene via the STAT-6 signaling pathway. Stimulation of HEK-Blue STAT-6 cells using crude protein extracts was carried out as follows: on Day 1, cells were plated in 96-well cell culture plates at a density of $2.5X10^5$/ml in 100 uL of cell culture media (DMEM with 4.5 g/L Glucose (11995, Gibco/Invitrogen, Carlsbad, CA) with 10% Heat Inactivated FBS (10082, Gibco/Invitrogen, Carlsbad, CA), 10 μg/mL Blasticidin (Invivogen), and 100 μg/mL Zeocin (Invivogen)) for 24 hours. On Day 2, 100 μL of cell culture media containing the appropriate concentration of AR protein premixed with 1 ng/mL (80 pM) human IL13 (Peprotech) was added to the cells. The plates were incubated for 24 hours at 37°C and 5% CO_2. To measure secreted embryonic alkaline phosphatase, 40 μL of each cell supernatant was mixed with 160 μL of Quanti-Blue (Invivogen) in a clear 96-well plate. The plate was incubated for 2 hours at 37°C and absorbance at 650 nm was read using a plate reader.

By evaluating 700 tested crude extracts, 94 binding proteins were identified as showing higher binding and inhibition activity than the benchmark binding protein 2F1. HEK/STAT6 and ELISA screening data were plotted against each other to identify binding proteins that

performed better than the benchmark binding protein 2F1 in both screening and activity assays (Figs. 2A and 2B). In this plot, a lower HEK/STAT6 signal indicates higher inhibition of IL13 dependent signaling through IL13Ralpha1 and increasing ELISA signal indicates increasing affinity for IL13. The benchmark binding protein 2F1 is indicated with a filled triangle at the intersection of the crosshairs. All binding proteins lying in the lower right quadrant (94) indicated by grey shading were identified for further characterization.

EXAMPLE 2: CHARACTERIZATION OF IL4 AND IL13 BINDING PROTEINS

The 22 AR proteins selected for further characterization were expressed using a T5-promoter based system in the cytoplasm of *E. coli* and purified via immobilized metal ion affinity chromatography (IMAC). Briefly, AR proteins were transformed in *E. coli* XL-1 blue cells and used to inoculate a 5 ml starter culture in Luria Bertani medium (LB) containing 50 μg/ml ampicillin and 1% glucose. The starter cultures were incubated overnight at 37°C, shaking at 220 rpm. On the next day, the overnight culture was used as inoculum of 50 ml LB. At a cell density of OD_{600} = 0.7, protein expression was induced using 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were incubated 4 hours at 37°C, shaking at 220 rpm. Cell pellets were harvested by centrifugation. Cells were ruptured by the addition of 1 mg/ml lysozyme, 50 KU/ml DNAse I and sonification for 30 minutes on ice. The insoluble fraction was removed by centrifugation. The clarified supernatant was filtered using 0.22 μM filters. These supernatants were loaded on columns packed with 250 μl Ni-NTA superflow resin (Qiagen). Purification was carried out following the instructions of the manufacturer. 20 ml Tris buffered saline (TBS) containing 20 mM imidazole and 10% glycerol was used as wash buffer, and 600 μl TBS containing 250 mM imidazole was used to elute AR proteins from the column.

SEC of selected human IL4 binding AR proteins

The 22 purified AR protein samples were analyzed for aggregation by size exclusion chromatography (SEC) using a Superdex 75 5/150 column (GE healthcare) and a PBS pH 7.4 mobile phase. 10 uL of each sample was injected per run with a flow rate of 0.3 mL/min. The column was calibrated using conalbumin, ovalbumin, carbonic anhydrase, ribonuclease A, and aprotinin protein standards. Elution of the AR proteins from the column was monitored by absorbance at 214 nm. The elution profiles of the samples were evaluated to identify AR protein candidates that eluted predominantly as monomers as evidenced by a single peak eluting at the appropriate volume for a 15 kDa protein (for N2C library) (18 kDA protein for N3C library) determined using MW standards. The results of biophysical properties of characterized IL4-binding AR proteins are summarized in Table 1.

Affinity Determination of purified Hit AR proteins

Purified binders selected as "hits" were ranked by their affinity on a ProteOn XPR-36 instrument (Bio-Rad). ProteOn is an optical biosensor instrument that measures protein-protein interactions in real time, based on Surface Plasmon Resonance technology similar to Biacore (GE). A rapid experimental protocol was performed as follows: On a GLC sensor chip (Bio-Rad), Neutravidin (Thermo Scientific) was covalently immobilized to a density of >5000 RU using amine coupling chemistry as described by the manufacturer. On one flow cell, biotinylated IL4 (Peprotech) was immobilized to a level of 250 RU, while another flow cell was used as reference, with neutravidin immobilized only. From each of the purified AR proteins, three different concentrations (25, 12.5, 6.25 nM) were analyzed, and kinetic parameters were calculated by fitting using a Langmuir 1:1 model. The k_a, k_d, and K_D obtained for each AR protein from these measurements are presented in Table 1, where E is base 10. The retrieved values were used to rank the AR proteins by their affinity.

Table 1

AR protein	SEC	ka (M-1S-1)	kd (S-1)	K_D (pM)	SEQ ID NO:
C06_6E9	broad monomer	9.56E+05	7.16E-05	74.9	27
C06_28E5	monomer	9.22E+05	4.61E-05	50	16
C06_19C3	monomer	9.62E+05	9.73E-05	101	11
C06_17A11	monomer	1.03E+06	1.40E-04	136	10
C06_20B8	monomer	2.02E+06	1.63E-04	80.9	13
C06_13A10	monomer	2.52E+06	1.49E-04	59.2	9
C06_19F8	monomer	3.66E+04	5.18E-05	1410	12
C06_26H2	dimer shoulder	3.47E+06	2.47E-04	71.2	14
C06_28D4	multiple peaks	1.62E+06	1.38E-04	85.6	15
C06_42A11	monomer	7.27E+05	5.43E-06	7.5	17
C06_42C7	monomer	1.83E+06	1.79E-04	97.8	18
C06_43G2	monomer	1.40E+06	5.98E-05	42.7	19
C06_44C12	monomer	8.64E+05	3.77E-05	43.6	20
C06_44F6	monomer	2.07E+06	2.00E-04	96.7	21
C06_48F3	monomer	8.72E+05	1.51E-04	174	22
C06_50E5	monomer	7.25E+05	1.26E-04	174	23
C06_53E9	monomer	8.62E+05	2.81E-04	326	24
C06_53G6	monomer	6.96E+05	5.11E-05	73.4	25
C06_54C2	monomer	4.03E+05	1.61E-04	400	26
C06_14A4	broad monomer	3.18E+05	2.73E-05	85.8	30
C06_24H1	broad peak	n.a.	n.a.	n.a.	28
C06_4A7	monomer	n.a.	n.a.	n.a.	29

AR Protein Composition

The compositions of the 22 AR proteins represented as expressed proteins are given in SEQ ID NO: 9-30. It was found that the 22 AR proteins represented 51 unique AR modules as given by SEQ ID NO: 31-81. In some instances, mutations in the N-cap module occurred including (based on SEQ ID NO: 2) D1N, K5E, R11S, A12V, R19H, V28A, and A30V alone or in combination. One AR protein, C06_26H2 was found to have G16R in the C-cap (SEQ ID NO: 3).

The specific sequences of the AR units are shown in the sequence tables for each of the modules and all 22 IL4 binding proteins.

The compositions of each of the AR protein binding domains are listed below in Table 2 as the corresponding SEQ ID NO: according to the formula AR1–AR2–AR3 or AR1–AR2–AR3–AR4.

Table 2.

AR Protein	AR1 SEQ ID NO:	AR2 SEQ ID NO:	AR3 SEQ ID NO:	AR4 SEQ ID NO:
C06_13A10	31	47	62	
C06_17A11	32	48	63	
C06_19C3	33	49	63	
C06_19F8	33	50	64	
C06_20B8	34	51	65	
C06_26H2	35	52	66	81
C06_28D4	34	53	67	
C06_28E5	36	53	68	
C06_42A11	37	54	69	
C06_42C7	34	51	64	
C06_43G2	38	55	70	80
C06_44C12	39	56	71	
C06_44F6	39	57	72	
C06_48F3	40	58	71	
C06_50E5	33	49	63	
C06_53E9	42	58	73	
C06_53G6	43	59	74	
C06_54C2	33	49	75	
C06_6E9	44	60	76	79
C06_24H1	45	56	77	
C06_4A7	46	61	78	
C06_14A4	37	54	78	

In comparing the 22 AR1 modules represented by 15 unique sequences (SEQ ID NO: 31 - 46), there was a preference for T at X_1, for D at X_2 of the AR sequence motif, for W at X_3, and D at X_6. The usage of pairs of amino acids at adjacent variable positions (X_2X_3 and X_5X_6) was also tabulated as shown below (Table 3). DW was the most frequently occurring doublet

for X_2X_3 and TD was the most frequently occurring doublet for X_5X_6. Thus, the AR1 module can be represented by the amino acid sequence

X_1D-[DW]-GX_4TPLHLAA-[TD]-GHLEIVEVLLKX_7GADVNA, wherein X_1 and X_4, are chosen from residues as shown in Table 4 and X_7 may be H, N, or Y (C-AR1, SEQ ID NO: 82). Alternatively, the AR1 motif may be chosen from an amino acid sequence represented by the formula:

TD-[DW]-GX_4TPLHLAA-[TD]-GHLEIVEVLLKX_7GADVNA, wherein X_4 is chosen from the residues listed in Table 4 and X_7 may be H, N, or Y (F-AR1, SEQ ID NO: 85).

Table 3.

IL4 Binding AR1	Variants and Frequency
X_1	T x7, V x3, I x2, L x2, S x2, A, E, F, H, K, Y
X_2X_3	DW x6, DS x4, HD x3, AW x2, EW, SD, SS, VT, NS, KD, RI
X_4	D x7, F x4, L x4, Y x2, I, N, E, S, T
X_5X_6	TD x5, ED x3, AD x3, DD, MD, ID, EI, IE, VY x2, LL x2, WE, ML
X_7	H > Y > N

In comparing the 22 AR2 modules which represented by 15 unique sequences (SEQ ID NO: 47-61), there was no dominant residue (more than 50% frequency) at any of X_1, X_2, X_3, X_4, X_5, or X_6 of the AR sequence motif. The usage of pairs of amino acids at adjacent variable positions (X_2X_3 and X_5X_6) was also tabulated as shown below (Table 4). AM was the most frequently occurring doublet for X_2X_3 and VY was the most frequently occurring doublet for X_5X_6. Thus, the AR2 module can be represented by the sequence:

X_1D-[X_2X_3]-G X_4TPLHLAA-[X_5X_6]-GHLEIVEVLLKX_7GADVNA wherein X_1, X_2X_3, X_4, and X_5X_6 are chosen from the residues shown in Table 4 and X_7 may be H, N, or Y (C-AR2, SEQ ID NO: 83). Alternatively, the AR2 motif may be chosen from a sequence represented by the formula:

X_1D-[AM]-GX_4TPLHLAA-[VY]-GHLEIVEVLLKX_7GADVNA where X_1 and X_4 are chosen from the residues in Table 4 and X_7 may be H, N, or Y (F-AR2, SEQ ID NO: 86).

Table 4.

IL4 Binding AR2	Variants and Frequency
X_1	S x3, I x3, Dx3, A x3, H x2, Q x2, E, L, K, M, N, V
X_2X_3	AM x4, RM x3, AI, AS, NF x3, NI, NL, MN, WN, SQ, DD, DT, ET x2, LF

X_4	D x7, M x4, F x3, W x3, L x2, I, N, Y
X_5X_6	VY x4, VE x3, FF x3, FV x3, ADx2, AT, DF, FD, VD, LY, YY, WT
X_7	Y > N > H

In comparing the 22 AR3 modules which represented by 17 unique sequences (SEQ ID NO: 62-78), there was no dominant residue (more than 50% frequency) at any of X_1, X_2, X_3, or X_6 of the AR sequence motif, however, X_4 and X_5 was most frequently F and at least one of X_5 or X_6 was frequent F or Y. The usage of pairs of amino acids at adjacent variable positions (X_2X_3 and X_5X_6) was also tabulated as shown below (Table 5). FY was the most frequently occurring doublet for X_5X_6. Thus, the IL4-binding AR3 module can be represented by the amino acid sequence

X_1D-[X_2X_3]-G-F-TPLHLAA-[X_5X_6]-GHLEIVEVLLKX_7GADVNA, wherein X_1 and X_2X_3, and X_5X_6 are chosen from the residues in Table 5 and X_7 may be H, N, or Y (C-AR3, SEQ ID NO: 84). Alternatively, the AR3 motif may be chosen from an amino acid sequence represented by the formula

X_1D-[X_2X_3]-G-F-TPLHLAA-[FY]-GHLEIVEVLLKX_7GADVNA, wherein X_1 and X_2X_3 are chosen from the residues listed in Table 5 and X_7 may be H, N, or Y (F-AR3, SEQ ID NO: 87).

Table 5.

IL4 Binding AR3	Variants and Frequency
X_1	M x4, K x4, V x4, S x3,T x3, E, N, Q, Y
X_2X_3	FS x4, QT x3, LA X4, HH, SH, IL x3, IS, NL, MI, SN, RT, LH
X_4	F x6, V x4, I x3, L x3, R x3, W x2, T, D
X_5X_6	FY x5, FS x4, NF x3, VD x3, EF, FA, FF, FW, SY, YN, YY
X_7	Y> H > N

There were only 3 AR4 modules (SEQ ID NO: 79-81) and no consensus or focused sequence formula was adopted for this set.

Neutralization of IL4 dependent STAT6 phosphorylation by purified AR proteins

The activity of each purified AR protein was assayed for their ability to inhibit IL4 dependent activation of STAT6 using HEK-Blue STAT-6 cells as described above. Full inhibition curves were assessed for each candidate and absorbance data were plotted as a

function of AR protein concentration to a sigmoidal dose response using the PRISM software (GraphPad PRISM) to determine IC_{50} values with 3.3 pM IL4. IC_{50} values obtained for these AR proteins ranged from 1.3 to 235 pM as are presented in Table 6.

Competition binding with IL4RA for IL4 binding

For biotinylation of IL4, recombinant human IL4 (Peprotech) was biotinylated at a 4:1 ratio using EZ-Link NHS-LC-Biotin (Pierce, #21336) for 2 hours at RT. The protein was dialyzed in PBS overnight to remove excess biotinylation reagent. Competition binding for cynomologous IL4 (SEQ ID NO: 5) was performed using the isolated protein biotinylated in the same manner.

AR protein inhibition of IL4 binding to IL4 RA was assessed using IL4 RA-Fc (R&D Systems), comprising the Gly24-His232 which represents the ECD (SEQ ID NO: 5) fused to a human IgG1-Fc and therefor a disulfide linked homodimer. The protein was conjugated to carboxylated Luminex microspheres according to the manufacturer's protocol. For neutralization experiments, 5000 IL4RA-Fc conjugated beads in 50 μl were added to each well of a 96-well filter plate (Millipore). Biotinylated human IL4 was mixed with an appropriate dilution of AR protein in Luminex Assay Buffer (PBS, 1% BSA, pH 7.4) to give a final concentration of 67 pM IL4. The plate was incubated for 1 hour at RT in the dark on a plate shaker, set to shake vigorously to avoid bead aggregation. The plate was washed twice with 150 μl of wash buffer (PBS, 0.1% BSA, pH 7.4., 0.05% Tween-20) using a vacuum manifold followed by the addition of 50 μl of Streptavidin PE at 25 μg/ml and incubated at RT for 20 minutes. The plates were washed again and 150 μl of sheath fluid was added and the plate was placed on the shaker for 5 minute. Plates were read using a Luminex® 100 system; data were plotted as a function of AR protein concentration. IC_{50} values were determined by fitting the data to the equation for sigmoidal dose response using PRISM software (GraphPAD PRISM) (Table 6).

IL4 -dependent TARC production

TARC is a key regulator of Th2-mediated inflammation in allergic asthma. Stimulation of A549 cells by IL4 *in vitro* leads to the production of TARC. This assay complements the HEK-STAT6 assay described above as it demonstrates the ability of an inhibitor to block IL4 signaling in primary cells. Each AR protein was assayed for inhibition of IL4 dependent TARC production in A549 cells as follows: on Day 1 cells were plated overnight in 96-well culture plates at a density of 2.5 $X10^5$ /ml in 100 μl of cell culture media (alphaMEM with GlutaMax, +10% heat-inactivated FBS, 1X Sodium Pyruvate, and 1X MEM NEAA (Gibco). This media also serves as the assay media. On Day 2 cells were washed once with 200 μl of culture media and stimulated with 200 μl of culture media containing 200 ng/ml (11nM) recombinant

human TNF-alpha and 67 pM recombinant IL4 premixed with appropriate concentration of AR protein. The plates were incubated for 24 hours at 37°C and 5% CO_2. Supernatants were harvested and stored at -80 °C for further analysis. CCL17/TARC Duo Set ELISA kit (RandDSystems) was used to quantify the amount of TARC in the samples using the manufacturer's protocol and a 1:5 dilution of the samples. Data were plotted as a function of AR protein concentration and fit to a sigmoidal dose response using the PRIZM software (GraphPad PRISM) to determine IC_{50} values (Table 6).

STAT6 Signaling in RA-1 cells

Each AR protein was assayed for inhibition of IL4 induced STAT6 signaling in STAT6-*bla* RA-1 cells. The CellSensor® STAT6-bla RA-1 Cell Line contains a beta-lactamase reporter gene under control of the STAT6 response element stably integrated into Ramos-1 (RA-1) cells. In contrast to the HEK-Blue STAT6 cells described above which signal through Type II complexes, RA-1 cells signal through Type I complexes and can be used to confirm the inhibition of IL4 stimulation through Type I complexes.

The assays were performed as recommended by the manufacturer, Invitrogen (Cat. No. K1243). On Day 1, RA-1 cells were plated in black 96-half area well cell culture plates (with clear bottom) at a density of 937,500 cells/ml in 32 μl of Assay Buffer. For cell-free control wells, 32 μl of assay buffer was added. A CD40 solution was prepared (50 μl of stock at 100 μg/ml to 950 assay buffer) and 4 μl was added to each well (final concentration was 556 ng/mL) to ensure that cells repond to IL4. The cells were spun at 14x*g* for 30 sec and placed in 37°C, 5% CO_2 for 16 hours. On Day 2, 4 μl of assay buffer was added to the cells containing a 10x concentration for the range of hIL4 to obtain the EC_{50}. For inhibition studies, a 10x inhibition solution containing the AR protein was premixed with a 10x hIL4 at the EC_{80} and then added to the cells. The concentration of hIL4 was 20.8 pM. The plates were spun at 14x*g* for 30 sec and placed in 37°C, 5% CO_2 for 5 hours. Thereafter, 8 μl of the Live BLAzer-FRET B/G (CCF4-AM) solution was added to each well (composed of 6 μl of solution A, 60 μl of Solution B, and 934 μl of solution C) and spun at 14x*g* for 30 sec. The plates were protected from light and incubated at room temperature for 2.5 hours. Plates were measured on the Envision Machine with bottom read capabilities using an excitation filter at 409/20 nm and two emission filters: one at 460/40nm and one at 530/30nm. A dual mirror was also used. To analyze the fluorescence reading, the background was subtracted (values from the cell-free wells) from both 460nm and 530nm and a 460/530 ratio was determined. The ratio was then plotted against concentration in the GraphPad PRIZM software to obtain an IC_{50} value.

Table 6. Characterized Bioactivity for the IL4-binding AR proteins

AR protein	IL4RA Binding Inhibition IC_{50} (pM)	HEK Stat6 Inhibition IC_{50} (pM)	A549 TARC Inhibition IC_{50} (pM)	Type 1 Complex inhibition IC_{50} (pM)
C06_6E9 (SEQ ID NO: 27)	7.6 ± 0.8	0.6 ± 0.2	130.9 ± 7.4	0.4 ± 0.1
C06_28E5 (SEQ ID NO: 16)	10.2 ± 1.8	1.4 ± 0.6	174.4 ± 6.0	1.5 ± 1.0
C06_19C3 (SEQ ID NO: 11)	6.3 ± 0.3	1.5 ± 0.3	Nd	nd
C06_17A11(SEQ ID NO: 10)	74.4 ± 17.6	3.0 ± 1.3	Nd	nd
C06_20B8 (SEQ ID NO: 13)	20.5 ± 6.3	4.0 ± 0.5	288.5 ± 37.4	3.8 ± 1.6
C06_13A10(SEQ ID NO: 9)	30.6 ± 18.9	5.4 ± 1.3	621.1 ± 79.3	6.6 ± 5.9
C06_19F8 (SEQ ID NO: 12)	17.6 ± 3.4	15.2 ± 3.8	Nd	nd
C06_26H2 (SEQ ID NO: 14)	31.4 ± 0.0	3.3 ± 1.4	Nd	nd
C06_28D4 (SEQ ID NO: 15)	35.3 ± 0.0	3.2 ± 2.6	Nd	nd
C06_42A11 (SEQ ID NO: 17)	65.6 ± 0.0	19.3 ± 7.2	324.7 ± 34.6	3.2 ± 3.0
C06_42C7 (SEQ ID NO: 18)	45.9 ± 0.0	6.8 ± 5.3	Nd	nd
C06_43G2 (SEQ ID NO: 19)	23.5 ± 0.0	1.6 ± 1.4	261.9 ± 18.2	0.8 ± 0.5
C06_44C12 (SEQ ID NO: 20)	20.3 ± 0.0	1.1 ± 1.2	231.1 ± 9.3	1.7 ± 1.4
C06_44F6 (SEQ ID NO: 21)	15.8 ± 0.0	6.4 ± 6.6	142.1 ± 5.4	24.9 ± 16.5
C06_48F3 (SEQ ID NO: 22)	19.7 ± 0.0	4.7 ± 4.9	Nd	nd
C06_50E5 (SEQ ID NO: 23)	14.8 ± 0.0	3.5 ± 1.0	Nd	nd
C06_53E9 (SEQ ID NO: 24)	17.8 ± 0.0	14.3 ± 7.1	Nd	nd
C06_53G6 (SEQ ID NO: 25)	16.1 ± 0.0	2.1 ± 2.1	135.1 ± 9.3	0.9 ± 0.6
C06_54C2 (SEQ ID NO: 26)	16.8 ± 0.0	3.2 ± 0.7	Nd	nd
C06_24H1 (SEQ ID NO: 28)	71.7 ± 0.0	241.3 ± 58.8	Nd	nd
C06_4A7 (SEQ ID	44.3 ± 0.0	443.6 ± 181.4	Nd	nd

AR protein	IL4RA Binding Inhibition IC_{50} (pM)	HEK Stat6 Inhibition IC_{50} (pM)	A549 TARC Inhibition IC_{50} (pM)	Type 1 Complex inhibition IC_{50} (pM)
NO: 29)				

The composite of biophysical and biochemical data described in Example 2 (Table 1) were used to select 9 AR protein molecules, C06_13A10, C06_20B8, C06_28E5, C06_42A11, C06_44C12, C06_44F6, C06_53G6, C06_43G2, and C06_6E9 for optimization. These AR proteins were chosen because each was found to be monomeric by SEC, able to bind to recombinant IL4 with a K_D < 9.7E-11, inhibit IL4 -dependent signaling in HEK-STAT6 cells with a potency > 67 pM, and inhibit the binding of recombinant IL4 to the IL4 receptor with an IC_{50} < 66 pM. The ten lead AR proteins were subjected to further cell based assays to confirm the inhibition of IL4 -dependent signaling in additional cell based assays.

The AR proteins C06_44C12, C06_53G6, and C06_28E5 were selected for optimization.

Expression and purification of binding protein candidates

The 94 binding protein candidates selected for further characterization were expressed using a T5-promotor based system which allows for E. coli cytoplasmic expression and purified via immobilized metal ion affinity chromatography (IMAC). Briefly, E. coli XL-1 Blue cells were transformed with binding protein expression plasmids and used to inoculate a 5 ml starter culture in Luria Bertani medium (LB) containing 50 μg/mL ampicillin and 1% glucose. The starter cultures were incubated overnight at 37°C, shaking at 220 rpm. Overnight cultures were used to inoculate 50 mL LB containing 50 μg/ml ampicillin. At a cell density of OD_{600} = 0.7, protein expression was induced using 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were incubated 4 hours at 37°C, shaking at 220 rpm. Cell pellets were harvested by centrifugation. Cells were ruptured by the addition of 1 mg/mL lysozyme and sonification for 30 minutes on ice. The insoluble fraction was removed by centrifugation. The clarified supernatant was filtered using 0.22 μM filters. These supernatants were loaded on columns packed with 250 μL Ni-NTA superfow resin (QIAgen). Purification was carried out following the instructions of the manufacturer. 20 mL Tris buffered saline (TBS) containing 20 mM imidazole and 10% glycerol was used as wash buffer, and 600 μL TBS containing 250 mM imidazole and 10% glycerol was used to elute the binding proteins.

SEC of selected human IL13 binding proteins

The 94 binding protein samples were analyzed for aggregation by size exclusion chromatography (SEC) using a TOSOH G2000SWXL column and a PBS pH 7.4 mobile phase. 20 μL of each sample was injected per run with a flow rate of 0.2 mL/min. The column was

calibrated using conalbumin, ovalbumin, carbonic anhydrase, ribonuclease A, and aprotinin protein standards. Elution of the binding proteins from the column was monitored by absorbance at 214 nm. The elution profiles of the samples were evaluated to identify binding protein candidates that eluted predominantly as monomers.

Thermal stability of IL13 binding proteins:

The melting temperatures of the selected IL13-binding protein samples were measured using Thermofluor technology (Pantoliano et. al. J Biomol Screening: 6:429-440, 2001). Thermofluor is a high throughput kinetic measurement of protein unfolding as a function of heat. As samples are heated, ANS in the sample buffer binds to hydrophobic regions generally buried in the folded molecule inducing an increase in dye fluorescence. After purification (above), each sample was exchanged into PBS buffer pH 7.4 using PD Multi-trap G25 resin (GE Healthcare) and the concentration estimated using the absorbance at 280 nm. Sample concentrations ranged from 1- 50 μM. Binding protein unfolding was monitored between 37-95°C with fluorescence measured every 0.5°C in continuous ramp mode. Melting temperatures measured ranged from 54°C to > 95°C for these samples. No melt was detected for several samples, indicating either that the stability is greater than 95°C or that the protein concentration was too low to accurately measure the fluorescence (data not shown).

Neutralization of IL13 dependent STAT6 phosphorylation

The activity of each purified binding protein was assayed for their ability to inhibit IL13 dependent activation of STAT6 using HEK-Blue STAT-6 cells as described above. Full inhibition curves were assessed for each candidate and absorbance data were plotted as a function of binding protein concentration to a sigmoidal dose response using the PRIZM software (GraphPad PRIZM) to determine IC_{50} values (data not shown).

Single Point Affinity Screening

The affinity of all purified binding proteins was assessed by ProteOn (BioRad) using a rapid affinity screening protocol as follows. On a GLC sensor chip (Biorad), neutravidin was covalently immobilized to a density of >5000 RU using amine coupling chemistry as described by the manufacturer. On one flow cell, biotinylated IL13 R130Q (Peprotech) was immobilized to a level of 250 RU; a second flowcell was used as reference with only neutravidin immobilized. From each of the purified binding proteins, a concentration of 50 nM was analyzed, and kinetic parameters were estimated by fitting using a Langmuir 1:1 model. The retrieved values were used to rank the binding proteins in terms of apparent affinity. These binding proteins had an on-rate (k_a) of between 1.7 and 9.6 X 10^5 $1/M^{*s}$ and an off-rate (k_d) ranging from 1.3 X 10^{-5} to 1.1. x 10^{-4} 1/s providing a K_D of 2.1 x 10^{-11} to 1.7 x 10^{-8} M.

Based on the initial screens,16 lead molecules were chosen for further characterization. A panel of 16 lead binding proteins which exhibited largely monomeric elution from an SEC, had an affinity (K_D) <1.5 nM, inhibited IL13 dependent STAT6 phosphorylation with an IC_{50} better than 100 pM and had a Tm of greater than 50°C by Thermofluor analysis was selected for larger scale expression, purification and characterization as described below.

Expression

E coli XL-1Blue cells were transformed with binding protein expression plasmids. A single colony was picked and grown at 37°C in 500 mL TB media containing carbenicillin. When the culture density reached an A_{600} of between 0.7 and 1.0 unit, expression was induced with 0.4 mM IPTG and incubated for an additional 4h at 37°C. Bacterial pellets were recovered by centrifugation and stored frozen until use. Frozen bacterial pellets were thawed and lysed in 50 mM sodium phosphate pH 7.5, 500 mM sodium chloride, 20 mM imidazole and containing an EDTA-free protease inhibitor cocktail. Resuspended pellets were sonicated and bacterial debris was collected by centrifugation in a JA-17 rotor at 17,000xg for 30 min. Soluble lysates were filtered and 2 mL of Ni-NTA resin (Qiagen) was added to each lysate followed by slow stirring for at least 1h at 4°C to capture the His-tagged binding proteins. The resin-containing lysate was poured into a column and washed with 8 column volumes of 50 mM sodium phosphate pH 7.5, 500 mM sodium chloride and 20 mM imidazole. The His-tagged protein was eluted from the resin with 8 column volumes of 50 mM sodium phosphate pH 7.5, 500 mM sodium chloride containing 500 mM imidazole. Further purification was achieved by size exclusion chromatography using a Superdex 200 26/60 column equilibrated in PBS pH 7.0.

Thermal stability of binding protein leads

The thermal stabilities of the 16 binding protein candidates were measured by differential scanning calorimetry (DSC). For Tm measurements, DSC is a more precise analytical method than the Thermoflour analysis used for high throughput screening. Each sample was dialyzed extensively against PBS pH 7.4 and diluted to a concentration of 1 mg/mL. Melting temperatures were measured for these samples using a model VP DSC instrument equipped with an autosampler (Microcal). Samples were heated from 10°C to 95°C at a rate of 1°C per minute. A buffer only scan collected between each sample scan was subtracted from the sample scan to allow calculation a baseline for integration. Data were fit to a two state unfolding model and results are presented in Table 7. The binding proteins analyzed expressed a wide range of melting temperatures from 48°C to 85°C.

Binding affinity for human IL13

Recombinant human IL13 (Peprotech) was minimally biotinylated on ice using sulfo-NHS-LCLC-Biotin and desalted into the experimental running buffer containing 10 mM HEPES, 150 mM NaCl, pH 7.4, 0.01% Tween-20, and 0.1 mg/mL BSA. Biotinylated IL13 was captured

at three different surface densities (from about 150, 50, and 25 RU) onto three different BIAcore SA (streptavidin) sensor chips. Each binding protein sample was tested at 40 nM as the highest concentration in a 3-fold dilution series over the three different density IL13 surfaces. The dissociation phase for the highest concentration of the binding protein sample was monitored for one hour. The response data from each of the different density surfaces was globally fitted in order to extract estimates of the kinetic and affinity constants which are provided in Table 7 below.

Table 7 - Biophysical Characterization of binding proteins

Binding protein	SEC Pattern	Tm (DSC)	k_a (M-1s-1) X 10^-6	k_d (s-1) X 10^5	K_D (pM)
7H3	monomer, small shoulder	53.15	4.26	56.0	131.4
7G11	monomer	50.87	1.62	5.30	32.8
7D2	monomer	82.62	3.06	29.2	95.5
5H7	monomer, broad	61.3	3.40	89.4e	263
5D12	monomer	73.53	1.099	18.44	167.8
5D3	monomer	61.89	0.877	14.36	164
5D2	monomer, shoulder	55.26	1.506	21.79	144.7
5B9	monomer + aggregates	77.11	1.060	17.87	168.6
6D4	monomer + aggregates	76.39	1.171	9.08e	77.6
6G9	monomer	85.22	0.995	5.741	57.7
6G11	Dimer	61.35	0.754	8.00	106
9E11	monomer/dimer	n.d.	0.640	1.3	21
10A6	aggregate	48.36	2.76	20.1	72.9
7C6	aggregate	81.54	4.82	22.4	46.4
7D7	multiple peaks	64.59	0.3.1	6.25	204

9F8	monomer, broad	n.d.	0.93	9.9	110

Neutralization of IL13 dependent activities

The activity of each binding protein sample was assayed for inhibition of IL13 dependent STAT6 phosphorylation as described above using 80 pM IL13. Data are shown in Table 7. Likewise, each binding protein sample was assayed for the ability to inhibit STAT6 phosphorylation stimulated by IL13 from cynomologous monkey in order to verify cross reactivity with this species for future toxicology and pharmacokinetic studies. Recombinant cyno IL13 was expressed and purified from *E. coli* as a SUMO-tag fusion protein. The SUMO-tag was subsequently enzymatically cleaved from IL13 in preparation for inhibition assays. Neutralization of cyno IL13 was assayed as follows: on Day 1, cells were plated in 96-well cell culture plates at a density of $2.5X10^5$ per ml in 100 uL of cell culture media (DMEM with 4.5 g/L Glucose (11995, Gibco/Invitrogen, Carlsbad, CA) with 10% Heat Inactivated FBS (10082, Gibco/Invitrogen, Carlsbad, CA), 10 μg/mL Blasticidin (Invivogen), and 100 μg/mL Zeocin (Invivogen)) for 24 hours. On Day 2, 100 μL of cell culture media containing the appropriate concentration of AR protein premixed with 1 ng/mL (80 pM) recombinant cyno IL13 was added to the cells. The plates were incubated for 24 hours at 37 °C and 5% CO_2. To measure secreted embryonic alkaline phosphatase, 40 μL of each cell supernatant was mixed with 160 μL of Quanti-Blue (Invivogen) in a clear 96-well plate. The plate was incubated for 2 hours at 37°C and absorbance at 650 nm was read using a plate reader. Results of cyno IL13 inhibition are presented in Table 8 below.

IL13 dependent TARC production

TARC (CCL17) release from A549 cells (a human lung carcinoma-derived cell line) can be stimulated by IL13.

Each binding protein was assayed for inhibition of IL13 dependent TARC production in A549 cells as follows: on Day 1 cells were plated overnight in 96-well culture plates at a density of $1.0 X10^6$ /ml in 200 μL of cell culture media (alphaMEM with GlutaMax, +10% heat-inactivated FBS, 1X Sodium Pyruvate, and 1X MEM NEAA (Gibco)). This media also serves as the assay media. On Day 2 cells were washed once with 200 μL of culture media and stimulated with 200 μL of culture media containing 200 ng/mL (11 nM) recombinant human TNF-alpha and 1 ng/mL (80 pM) recombinant IL13 premixed with appropriate concentration of binding protein. The plates were incubated for 24 hours at 37°C and 5% CO_2. Supernatants were harvested and stored at -80°C for further analysis. A kit was used to measure human CCL17/TARC Duo Set ELISA (R&D Systems) in the samples according to the manufacturer's protocol and where the samples were used at a 1:5 dilution. Data were plotted as a function of

binding protein concentration and fit to a sigmoidal dose response using the PRIZM software (GraphPad PRIZM) to determine IC_{50} values (Table 8 below).

IL13:IL13Rα2 Binding

Binding protein inhibition of IL13 binding to Rα2 was assessed using IL13Rα2-Fc (R&D Systems) conjugated to carboxylated Luminex microspheres according to the manufacturer's protocol. For biotinylation of IL13, recombinant human IL13 R130Q (Peprotech) was biotinylated at a 4:1 ratio using EZ-Link NHS-LC-Biotin (Pierce, #21336) for 2 hours at RT. The protein was dialyzed in PBS overnight to remove excess biotinylation reagent. For neutralization experiments, 5000 IL13 Rα2-Fc conjugated beads in 50 μl were added to each well of a 96-well filter plate (Millipore). 50 μl of biotinylated human IL13 at 1 ng/ml (80pM) was mixed with an appropriate dilution of binding protein in Luminex Assay Buffer (PBS, 1% BSA, pH 7.4). The plate was incubated for 1 hour at RT in the dark on a plate shaker, set to shake vigorously to avoid bead aggregation. The plate was washed 3 times with 150 μl of wash buffer (PBS, 1% BSA, pH 7.4., 0.05% Tween-20) using a vacuum manifold followed by the addition of 50 μl of Streptavidin PE at 25 μg/ml and incubated at RT for 20 minutes. The plates were washed again and 100 μl of sheath fluid was added and the plate was placed on the shaker for 1 minute. Plates were read using a Luminex® 100 system; data were plotted as a function of binding protein concentration. IC_{50} values were determined by fitting the data to the equation for sigmoidal dose response using PRIZM software (GraphPAD PRIZM). The inhibition constants for the lead binding proteins are listed in Table below.

Table 8 - Neutralization of IL13 Dependent Activity

Binding protein	STAT6 Phospho IC50 (pM)	TARC expression IC50 (pM)	IL13: IL13Ra2 binding IC50 (pM)	Cyno IL13 STAT6 IC50 (pM)
7H3	25.3	69.6	66.9	387.8
7G11	2.5	19.6	3.4	98.0
7D2	4.8	14.6	8.3	307.0
5H7	42.9	132.3	157.9	526.9
5D12	5.4	34.1	10.2	159.1
5D3	16.9	88.3	37.5	3022.1
5D2	20.3	71.4	32.2	223.6
5B9	16.5	372.3	13.4	653.5

6D4	4.2	83.3	4.8	51.3
6G9	17.4	64.0	31.0	96.4
6G11	19.8	281.8	10.1	1207.8
9E11	0.9	109.1	5.8	33.4
10A6	8.0	123.3	7.5	1929.9
7C6	2.7	158.7	10.6	48.0
7D7	10.5	353.6	58.0	161.5
9F8	55	n.d.	n.d.	n.d.

AR Protein Compositions

The sequences of the ankyrin repeat domains of the 2F1 and 16 lead anti human IL13 binding proteins where each binding protein follows the format of (N-Cap)-(AR)n-(C-Cap) where n=2 or 3 were analyzed.

It was found that the 2F1 and the additional 16 binding proteins represented 46 distinct AR modules as listed in the sequence tables below where a dot indicates that the amino acid present at its position for a certain AR corresponds to the corresponding amino acid of the AR repeat motif (SEQ ID NO: 1). In a few cases, where framework mutations were observed in the selected binding protein sequence they are noted. In a few cases, deletions arose during the ribosome display selection process; these deletions are noted with a dash (-). Binding protein 10A6 contains only 2 ARs. In all cases, the C-Cap sequence starts immediately after residue 33 of the last AR.

The compostion of each of the binding domain tandem AR units (AR1-AR2-AR3) of each binding protein are listed below (Table 9 below)

Table 9 Binding Protein Composition

IL13 Binding Protein	AR1 SEQ ID NO:	AR2 SEQ ID NO:	AR3 SEQ ID NO:
6G9	109	127	144
7G11	110	128	145
9F8	111	129	145
10A6	112	130	Absent
5B9	113	131	146
7D2	114	132	147

6G11	115	133	148
7D7	116	134	149
5D12	117	135	150
5D2	118	136	145
7H3	119	137	145
5D3	120	138	145
5H7	121	139	151
9E11	122	140	152
6D4	123	141	153
7C6	124	142	154
2F1	125	143	155

In comparing the AR1 modules which represented by 17 unique sequences (SEQ ID NO: 9 - 25) (Table 10 below), there was a preference for Y or F at position 4 (X_3) of the motif, S at position 6 (X_4), R at position 14 (X_5), H at position 15 (X_6), and at position 27 (X_7) H or Y.

Thus, the IL13 binding AR1 module can be represented by the formula

X_1DX_2-[F,Y]-GSTPLHLAA-RH-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from residues as shown below and X_7 may be H, N, or Y (AR1-C, SEQ ID NO: 156). Alternatively, the AR1 motif may be chosen from an amino acid sequence represented by the formula:

TDYGSTPLHLAARHGHLEIVEVLLKX_7GADVNA, wherein X_7 may be H, N, or Y (AR1-F, SEQ ID NO: 157).

Table 10

IL13 Binding AR1	Variants and Frequency (17)
X_1	T x4, A x2, F x2, E, I, K, M, S x2, R, V, W
X_2X_3	X_2X_3 = DY x2, SY x2, DF x2, EF, IF, LF, IL, IY, MY x2, HF, VF, KY, TY X2 = D, E, H, I, K, M, S, T, V X3 = Y x9, F x7, L
X_4	S x14, D, I, T
X_5X_6	X_5X_6= RH x8, RE x3, RS x3, RQ, RT, HH X5 = R x16, always basic side chain X6 = H x9, E x3, S x3, Q, T

X_7	H, Y, N

In comparing the 17 AR2 modules which represented by 17 unique sequences (SEQ ID NO: 127-143) (Table 11 below), there was no dominant residue (more than 50% frequency) at X_1, however, at the randomized positions X_3, X_4, X_5 and X_6 of the AR sequence motif there was a most frequently used amino acid. The usage of pairs of amino acids at adjacent variable positions (X_2X_3 and X_5X_6) was also tabulated as shown below. FI was the most frequently occurring doublet for X_2X_3. Thus, the IL13-binding AR2 module can be represented by the formula (wherein the bracketed residues are alternate amino acids for that position):

X_1DFIG DTPLHLAAY- X_6-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from N, T, A, D, K, E, H, M, and F; X_6 may be H or R; and X_7 may be H, N, or Y (AR2-C, SEQ ID NO: 158). Alternatively, the AR2 seqence may be chosen from an amino acid sequence represented by the formula

[A, D, N, T, K]-DFIG DTPLHLAAY-[H,R]-GHLEIVEVLLK- [H,N,Y]-GADVNA (AR2, SEQ ID NO: 159).

Table 11

IL13 Binding AR2	Variants and Frequency (17)
X_1	N x4, T x3, A x2, D x2, K x2, E, H, M, F
X_2X_3	X_2X_3 = FI x9, MI x4, FA, FL x2, II X_2 = F x12, M x4, I X_3 = I x14, L x2, A
X_4	D x15, Y, N
X_5X_6	X_5X_6 = YH x6, YR x6, FK, FR, VY, WH, YN X_5 = Y x13, F 2x, V, W, Y (always hydrophobic side chain) X_6 = H x7, R x7, K, N, Y
X_7	H, Y, N

In comparing the 16 AR3 modules which represented by 12 unique sequences (SEQ ID NO: 144-155), there was no dominant residue (more than 50% frequency) at any of X_1, X_2, X_5, or X_6 of the AR sequence motif, however, X_3 was most frequently T, and X_4 was most frequently E. The usage of pairs of amino acids at adjacent variable positions (X_2X_3 and X_5X_6) was also tabulated as shown below. IT was the most frequently occurring doublet for X_2X_3. SM was the most frequently occurring doublet for X_5X_6. Thus, the IL13-binding AR3 module can be represented by the amino acid sequence

X_1D- X_2 TG-E-TPLHLAA-[X_5X_6]-GHLEIVEVLLKX_7GADVNA, wherein X_1 and X_2, are chosen from the residues in Table 12 below, and X_5X_6 are selected from the pair SM, HL, and YH; and X_7 may be H, N, or Y (AR3-C, SEQ ID NO: 160). Alternatively, the AR3 motif may be chosen from an amino acid sequence represented by the formula

X_1D-IT-G-E-TPLHLAA-SM-GHLEIVEVLLKX_7GADVNA, wherein X_1 is chosen from the residues listed in Table 12 below and X_7 may be H, N, or Y (AR3-F, SEQ ID NO: 161).

Table 12

IL13 Binding AR3	Variants and Frequency (16)
X_1	D x5, S x5, T x2, K x2, E, M
X_2X_3	X_2X_3 = IT x7, -H x5, -N, AW, TS, VT X_2 = I x7, - x6, A, T, V X_3 = T x8, H x5, N, S, T, W
X_4	E x10, D x5, T
X_5X_6	X_5X_6 = SM x5, HL x2, YH x2, HN, QI, YT, TA, DS, ER, ES X_5 = S x5, H x3, Y x3, D, E x2, Q, T X_6 = M x5, L x2, H x2, S x2, A, I, N, R, T
X_7	N, H, Y

EXAMPLE 3: OPTIMIZATION OF LEAD MOLECULES

To optimize selected IL4-binding AR proteins for large scale manufacturing, formulation, and stability, it was necessary to mutate several residues that were found in the variable residues, $X_1 – X_6$ of SEQ ID NO: 1. For example, oxidation of purified recombinant proteins can lead to product heterogeneity and loss of activity. In order to reduce the risk of these modifications, Met residues found in the variable sequences were mutated to amino acids of similar chemical makeup or to those found in other sequence related IL4 binding AR proteins. In addition, several mutations were made to eliminate potential sites of immunogenicity as assessed by the presence of potential T-cell epitopes. Finally, random amino acid changes to the ankyrin repeat framework occasionally arose during the PCR steps used for ribosome display selection. Such residues were reverted to the consensus designed ankyrin repeat sequence.

AR protein C06_44C12 was engineered by mutating AR2 postion 4 (X_3) from Met to Leu in order to avoid potential oxidation in the IL4 binding site. Position 27 (X_7) of AR2 and AR3 were mutated from Tyr to Ala in order to eliminate potential T-cell epitopes based on an analysis of neighboring upstream and downstream residues, and remove potential sites of deamidation. Similar mutations were made to C06_28E5, changing position 27 of AR1 and AR2 from Tyr to Ala. For C06_53G6, position 1 of the N-cap was changed from Asn to Asp to

restore the ankyrin consensus sequence and position 27 of AR1 was mutated from Tyr to Ala as described above.

After optimization, the AR modules based on the motif formula (SEQ ID NO: 1) for these three proteins were:

Table 13

Motif Designation	Position Number	AR1	AR2	AR3
X_1	1	A, L, T	A, N, Q	T, V, Y
X_2X_3	3-4	DS, DW	NL, RL, AI	IS, LA, LH
X_4	6	D, I, Y	D	F, I, V
X_5X_6	14-15	ED, TD	WT, FV, LY	FY, FW
X_7	27	A, H	A, Y	A, H

The mutations described here were made in a singular or combinatorial manner, in order determine the effect each mutation had on activity. Engineered AR proteins of the sequences designated, were assayed for binding to recombinant IL4, inhibition of IL4 dependent signaling, solubility by SEC, and determination of the melting temperature by DSC (Table 14).

Table 14. Activity of Optimized anti IL4 AR proteins

AR protein	SEQ ID	k_a (×10^-6)	k_d (×10^6)	K_D (pM)	IC_{50} STAT6 (pM)	T_m (°C)
C06_44C12v2	91	5.14	8.24	1.6	6.9	68.2
C06_28E5v1	92	0.657	5.50	8.38	3.1	72.4
C06_53G6	93	3.37	6.07	1.8	1.4	73.7

Positional tandem AR units for optimized IL4 binding proteins are represented by the AR formulas:

[A,L,T]-DD-[S,W]-G-[D,I,Y]-TPLHLAA-[E,T]-DGHLEIVEVLLK-[A,H]-GADVNA (AR1-O, SEQ ID NO: 88),

[A,N,Q]-D-[NL,RL,AI]-GDTPLHLAA-[WT,FV,LY]-GHLEIVEVLLK-[A,Y]-GADVNA (AR2-O, SEQ ID NO: 89), and

[T,V,Y]-D-[IS, LA, LH]-G-[F,I,V]-TPLHLAAF-[W,Y]-GHLEIVEVLLK-[A,H]-GADVNA (AR3-O, SEQ ID NO: 90);

where the bracketed entries represent the alternative amino acid residue or pair of residues in the three optimized binding proteins which exhibit the desired biologic activities. Therefore, IL4 binding proteins may be constructed from these AR motifs by tandem positioning in the order specified.

IL13 Binding Proteins

Three IL13-binding candidate binding proteins; 7G11, 6G9 and 9F8, were selected for protein optimization for potential large scale manufacturing, formulation, and stability. Each candidate was modified by multiple rounds of site directed mutagenesis.

Mutations were designed that have been found to generally increase the stability of binding proteins, decrease potential immunogenicity, remove the N-terminal HIS tag, enhance the processing of the N-terminal methionine, or remove potential sites of oxidation or deamidation in the putative antigen binding site. The mutations introduced into all final molecules included: N-Cap, position 3 (G to D) to improve the biophysical behavior was introduced to all lead molecule candidates and the terminal residues of the C-cap changed to a di-alanine. In addition, candidates 6G9 and 9F8 were found to contain the 27Asn-Gly28 dipeptide in the modules and therefore, residue 27 was substituted.

Besides the mutations described above which can be applied generically to all binding proteins, a number of mutations specific to the activity of binding proteins 6G9, 7G11, and 9F8 were introduced. Oxidation of purified recombinant proteins can lead to product heterogeneity and loss of activity. In order to reduce the risk of these modifications, Met and Cys residues found in the ankyrin repeat modules of 6G9, 9F8 and 7G11 were mutated to amino acids of similar chemical makeup or to those found in other IL13 binding proteins. Additionally, a number of mutations in the ankyrin repeats of these binding proteins were made to eliminate potential sites of immunogenicity suggested by screening in T-cell activation assays. Finally, for proteins expressed in *E. coli*, processing of the N-terminal methionine residue can be affected by the amino acid immediately following the N-terminal methionine Hirel et. al. PNAS 86:8247-8251 1989. Total processing of this methionine residue is desirable to increase the homogeneity of the purified product. In the N-Cap, position 1 was changed from aspartic acid to glycine or alanine in order to determine if the N-terminal methionine residue could be efficiently processed when expressed without the HIS tag. A summary of the binding protein specific mutations in specific repeat module positions examined for 6G9, 9F8 and 7G11 is shown in Table 15.

The generic and specific mutations described above were made in singular, or in a combinatorial manner, in order determine the results of each change on activity. Engineered

binding proteins were assayed for binding to recombinant IL13, inhibition of IL13 dependent signaling, and determination of the melting temperature by DSC. All of the candidates remained monomeric as determined by SEC. In most cases, the activity and affinity of the mutant were not significantly different from the parent molecule. The properties of each of the three parent and the final optimized lead candidates are shown in Table 15.

Table 15.

Binding protein	Module	Position	Replacement Residues	Purpose
6G9	AR2	1	A	Reduce immune response potential
	AR2	3	F,I,A,L	Reduce oxidation
	AR3	27	H	Reduce immune response potential
7G11	AR2	27	Y	Reduce oxidation
	AR2	29	A	Reduce immune response potential
	AR3	14	T, L, I, V	Reduce oxidation
9F8	AR2	1	F	Reduce oxidation
	AR3	13	Q	Reduce immune response potential
	AR3	14	I	Reduce oxidation

Affinity Analysis of Lead Candidates

Complete kinetic data describing binding of various engineered binding protein molecules to human IL13 was measured using a method similar to that described above for the single point affinity analysis. Briefly, streptavidin (Pierce) was immobilized to similar levels (~ 1800 RUs) on all six channels of a ProteOn GLC chip via amine coupling (pH 5.0). Biotinylated hIL13 was captured at different surface densities (600 ~ 100 RUs) on different channels. Protein binding was tested starting at 40 nM diluted in a 3-fold concentration series over the different density IL13 surfaces; a buffer sample was injected to monitor the baseline stability. Dissociation phases for all concentrations of each binding protein sample were monitored for one hour at a flow rate of 100 μl/min. Response data for all concentration series from the different density surfaces were globally fit to a 1:1 simple langmuir binding model to extract values of the kinetic (k_{on}, k_{off}) and affinity (K_D) constants provided in Table 16.

Purification of untagged binding proteins

Binding proteins without a HIS tag were subcloned into a pET27 vector modified to include a ligase independent cloning site by standard PCR methods and expressed in BL21-GOLD(DE3) *E. coli* strain (Stratagene). Expression was performed in terrific broth after inducing expression by the addition of 1 mM IPTG at 30°C. Cells were harvested 5 hours after induction by centrifugation and frozen at -20°C. Frozen cell pellets were resuspended in lysis buffer composed of 20 mM histidine pH 6.4 at a concentration of 0.1 g of pellet per mL of buffer. Cell lysis was accomplished by sonication and the lysate cleared by centrifugation at > 15,000 x g followed by filtration through a 0.45 um filter. Cleared lysates were loaded onto a 5 mL HiTrap Q FF column (GE Healthcare) in lysis buffer. A linear gradient from lysis buffer to buffer B, 20 mM Histidine pH 6.4 with 600 mM NaCl, over 20 column volumes eluted the binding proteins from the Q column. Fractions were analyzed by SDS-PAGE and those containing binding protein were pooled and heated to 70°C for 20 minutes and then placed on ice for 30 minutes. Precipitated, contaminating proteins were removed by centrifugation. The supernatant of the precipitation step containing the binding protein was then concentrated by ultrafiltration and purified on a Superdex 75 16/60 column (GE Healthcare) with PBS as the mobile phase. The heat step after the initial ion exchange chromatography step was omitted for binding proteins with lower melting temperatures, such as 9F8r3. The biophysical properties and bioactivity measurements for each construct is summarized in Table 16.

Table 16.

Binding protein	SEQ ID NO:	Mutations compared to Parent (Generic)	Mutations compared to parent (Specific)	K_D (M)	T_m (°C)	HEK STAT6 IC_{50} (pM)
6G9	162			2.49E-11	86.8	17.4
6G9r13	163	Ncap D1A Ncap G3D AR2 N27Y Ccap L27A Ccap N28A	AR2 K1A AR2 M3F	2.51E-11	82.7	5.6
7G11	166			1.68E-11	50.9	2.5
7G11r7	167	Ncap D1G NcapG3D AR3 N26H Ccap L27A Ccap N28A	AR2 C27Y AR3 M14T	2.42E-11	53.5	85

9F8	164			1.64E-12	-	N.D.
9F8r3	165	Ncap D1G Ncap G3D AR3 N26H Ccap L27A Ccap N28A	AR2 M1F AR3 S13Q AR3 M14I	6.56E-11	49.6	201.9

Positional tandem AR units for optimized IL13 binding proteins are represented by the AR formulas:

[R,S,T]-D-[S,W]- [Y,F]-[D,G]-STPLHLAAR-[E,H]-GHLEIVEVLLKYGADVNA (AR1-O, SEQ ID NO: 168);

[A, F]-DFIGDTPLHLAAYRGHLEIVEVLLKYGADVNA (AR2-O, SEQ ID NO: 169), and

[S,D]-D-[HG, IT]-G-[S, D]-TPLHLAA-[QI,ST]-GHLEIVEVLLKHGADVNA (AR3-O, SEQ ID NO: 170);

wherein the bracketed entries represent the alternative amino acid residue or pair of residues in the three optimized binding proteins which exhibit the desired biologic activities. Therefore, IL13 binding proteins may be constructed from these AR motifs by tandem positioning in the order specified. The IL13 binding protein according to the formula N-Cap-[AR]n-C-Cap additionally comprises an N-Cap selected from SEQ ID NO: 2 or a variant thereof, such as those exemplified by the formula of SEQ ID NO: 171, and a C-Cap such as SEQ ID NO: 3 or 172 or variants thereof.

EXAMPLE 4: BIVALENT AR PROTEIN CONSTRUCTS

The three IL4-binding AR protein molecules described in Example 3 were combined with a previously discovered anti-IL13 AR protein designated 6G9_V1 (SEQ ID NO: 94) in order to produce a bispecific molecule that could block signaling of both human IL13 and IL4. Nucleic acid sequences for each AR protein synthesized to include a sequence encoding for a $(GGGGS)_4$ linker between the 2 AR proteins. Alternative GS linkers with the formula $(GGGGS)_n$ may be used to join AR proteins. The length of the linker can be varied to control binding domain availabilty, steric and other properties of the molecule A total of 6 bispecific AR proteins were synthesized representing the 6G9 linked either at N-terminal to the IL4 binding protein or C-terminal to the IL4 binding protein (SEQ ID NO: 95 – 100) to examine the effects of the orientation of the AR proteins relative to one another on activity. Each coding nucleic acid sequence was cloned into the expression vector and purified by IMAC chromatography as described above for monospecific AR proteins.

The bispecific AR proteins were evaluated for binding to hIL13 and IL4 as well as the ability to inhibit IL13 and IL4 dependent signaling.

In the HEK-Blue STAT-6 cells, IL13 activates the IL13RA1: IL4R complex (Type 2 receptor) to induce secretion of an embryonic alkaline phosphatase (SEAP) reporter gene via the STAT-6 signaling pathway. An assay for IL13 dependent activation of STAT6 using HEK-Blue STAT-6 cells is commercially available (InVivogen, SanDiego, CA). To enable differentiation between high and low affinity binding proteins, an optimal dilution of the crude extract (1:5200) was selected. Stimulation of HEK-Blue STAT-6 cells using crude protein extracts was carried out as follows: on Day 1, cells were plated in 96-well cell culture plates at a density of $2.5X10^5$/ml in 100 uL of cell culture media (DMEM with 4.5 g/L Glucose (11995, Gibco/Invitrogen, Carlsbad, CA) with 10% Heat Inactivated FBS (10082, Gibco/Invitrogen, Carlsbad, CA), 10 microgm /mL Blasticidin (Invivogen), and 100 microgm per mL Zeocin (Invivogen)) for 24 hours. On Day 2, 100 microL of cell culture media containing the appropriate concentration of AR protein premixed with 1 ng/mL (80 pM) human IL13 (Peprotech) or cyno IL13 was added to the cells. The plates were incubated for 24 hours at 37 °C and 5% CO_2. To measure secreted embryonic alkaline phosphatase, 40 microL of each cell supernatant was mixed with 160 microL of Quanti-Blue (Invivogen) in a clear 96-well plate. The plate was incubated for 2 hours at 37 °C and absorbance at 650 nm was read using a plate reader.

All of the bispecific AR proteins retained their ability to bind with high affinity to hIL13 and IL4 (Table 17) irrespective of the orientation of the construct as well as the ability to inhibit IL13 and IL4 dependent signaling (Tables 18 and 19).

Table 17: Biophysical properties of bispecific AR proteins

Bispecific AR protein	SEQ ID No.	IL13 Ra2 Binding IC_{50} (pM)	Affinity to IL13 (pM)	IL-4RA Binding IC_{50} (pM)	Affinity to IL4 (pM)	Tm (°C)
C01_6G9_V1_C06_28E5_V1	95	32 ± 19	16.6	24 ± 9	3.1	72.4/83.6
C06_28E5_V1_C01_6G9_V1	96	36 ± 19	10.6	25 ± 10	21.7	76.8/80.8
C01_6G9_V1_C06_44C12_V2	97	23 ± 15	9.1	25 ± 11	2.0	70.3/83.3
C06_44C12_V2_C01_6G9_V1	98	28 ± 14	11.6	17 ± 5	1.7	72.6/82.6
C01_6G9_V1_C06_53G6_V1	99	99 ± 15	9.3	15 ± 7	3.1	76.0/79.8
C06_53G6_V1_C01_6G9_V1	100	199 ± 27	38.4	34 ± 28	10.9	76.8/80.0

Table 18: Neutralization of IL13 dependent activity by bispecific AR proteins

	SEQ ID No:	IL13 Ra2 Binding IC_{50} (pM)	HEK Stat6 human IL13 IC_{50} (pM)	A549/TARC IL13 IC_{50} (pM)
C01_6G9_V1_C06_28E5_V1	95	32 ± 19	19 ± 9	92 ± 103
C06_28E5_V1_C01_6G9_V1	96	36 ± 19	19 ± 5	84 ± 80
C01_6G9_V1_C06_44C12_V2	97	23 ± 15	15 ± 9	128 ± 155
C06_44C12_V2_C01_6G9_V1	98	28 ± 14	14 ± 7	63 ± 76
C01_6G9_V1_C06_53G6_V1	99	99 ± 15	15 ± 8	75 ± 83
C06_53G6_V1_C01_6G9_V1	100	199 ± 27	27 ± 13	131 ± 70

Table 19: Neutralization of IL4 dependent activity by bispecific AR proteins

Construct Composition	SEQ ID No:	IL4R Binding IC_{50} (pM)	HEK Stat6 hIL4 IC_{50} (pM)	A549/TARC IL4 IC_{50} (pM)	Ramos Assay IC_{50} (pM)
C01_6G9_V1_C06_28E5_V1	95	24 ± 9	2.0 ± 0.6	33 ± 14	6.9 ± 2.8
C06_28E5_V1_C01_6G9_V1	96	25 ± 10	1.1 ± 0.4	39 ± 13	5.4 ± 2.5
C01_6G9_V1_C06_44C12_V2	97	25 ± 11	7.2 ± 2.8	55 ± 3	8.6 ± 5.3

C06_44C12_V2_C01_6G9_V1	98	17 ± 5	0.7 ± 0.3	16 ± 9	5.8 ± 6.1
C01_6G9_V1_C06_53G6_V1	99	15 ± 7	0.5 ± 0.2	24 ± 10	4.8 ± 5.6
C06_53G6_V1_C01_6G9_V1	100	34 ± 28	3.9 ± 1.7	50 ± 7	8.0 ± 9.4

EXAMPLE 5: OPTIMIZATION OF BISPECIFIC AR PROTEINS

In addition to substitutions of the residues at the positions diversified in the creation of libraries based on the formula N-cap-[AR]n-C-cap as well as those mutations described above, generic AR protein mutations may be incorporated. These mutations can be applied to any AR protein molecule, in that these mutations occur within positions of the sequence that are common to all AR proteins as summarized in Table 20 below.

Table 20: Protein Mutations

Module	Position	Possible Residues	Rationale
N-Cap	1	G, A	Process N-terminal methionine
N-Cap	3	D	Stabilize AR proteins
AR	27	Y, H	Reduce deamidation
C-Cap	27	A	Remove restriction site/restore AR module
C-Cap	28	A	Remover restriction site/restore AR module

For proteins expressed in E. coli, processing of the N-terminal methionine residue can be affected by the amino acid immediately following the N-terminal methionine (Hirel, et al., Proc Natl Acad Sci U S A 86: 8247-51,1989). Total processing of this methionine residue is desirable to increase the homogeneity of the purified product. In the N-Cap, position 1 was changed from aspartic acid to glycine or alanine in order to determine if the N-terminal methionine residue could be efficiently processed when expressed without the HIS tag. Position 3 of the N-cap is mutated from Gly to Asp, as this mutation has been found to stabilize the AR protein consensus sequence as described in WO2 01/0060748. Position 27 of the AR modules is restricted in diversity to Asn, Tyr, or His in the AR protein library design (Binz et al. Nature Biotech 22:575-582, 2004). As position 28 of the framework is Gly, there is the possibility of isolating AR proteins consisting of the sequence 27Asn-Gly28. The Asn-Gly di-peptide is prone to deamidation reactions (Geiger and Clarke, J Biol Chem 262: 785-94, 1987). As such, position 27 of isolated Asn-Gly sequences can generally be mutated to either Tyr or His. In addition, IL4-binding AR proteins selected by ribosome display end with the amino acid sequence Leu-Asn in the C-cap. This sequence is appended onto the AR proteins in order to

accommodate a restriction site for sub-cloning into expression vectors for screening. The preferred amino acid sequence of these positions is Ala-Ala. The C-cap has been further mutated for stabilty and optimized expression characteristics (SEQ ID NO: 103).

An examplary, optimized bispecific IL4/IL13 binding protein is that given in SEQ ID NO: 104.

EXAMPLE 6: GENERATION OF SURROGATE ANTI-MURINE IL4 AR PROTEINS

As human and murine IL4 share only 41% sequence identity, it is unlikely that AR proteins selected against human IL4 cross react with mouse IL4. Thus, to enable studies in mouse models where murine IL4 has been demonstrated to play a role in asthma pathologies, it was necessary to select a AR protein that specifically binds to murine IL4 with subnanomolar affinity. Five rounds of ribosome display selection were completed with the N2C and N3C AR protein libraries (Binz, et al., Nat Biotechnol 22: 575-82, 2004) using biotinylated murine IL4 (Peprotech) followed by capture on neutravidin beads. To identify high affinity binders, an off rate selection strategy was performed as follows: biotinylated mIL4 (5 nM) was bound to ribosome displayed AR proteins for either 2 or 6 hours followed by incubation with 2.1 mM unbiotinylated IL4 as a competitor for 4 or 16 hours. AR proteins with a slow off-rate remaining attached to the biotinylated mIL4 were captured on neutravidin particles. An additional round of ribosome display selection was performed under standard conditions to enrich for the high affinity binders. Selected AR proteins were screened using purified AR protein for inhibition of mIL4 dependent HT2 proliferation. HT2 cells, T-lymphocytes isolated from murine spleens (ATCC, CRL-1841™) were cultured using the manufacturer's recommendations (RPMI 1640, 2mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5 g/L glucose, 10mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 100-200 IU/ml IL-2, and 10% FBS). For the proliferation assay, the cells were removed from the flask and washed 4 times in assay buffer consisting of culture media without IL-2 and plated in 96-well opaque-bottom plates at a density of 5.0X104 cells/ml in 50 µl. Cells were treated with 74 pM IL4 and appropriate concentrations of AR protein and incubated at 37°C, 5% CO2 for 48 hours. Cell Titer Glo (Promega G7571) was added to the assay plate (100 µL), covered and placed on a shaker for 40 minutes at room temperature. Luminescence was measured from a top read using the SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Based on affinity for mIL4, neutralization of mIL4 binding to IL4R, neutralization of mIL4 dependent HT2 proliferation, thermal stability and monodispersity by size exclusion chromatography, AR protein C06_21H2 (SEQ ID NO: 105) was chosen as the surrogate mIL4 binding AR protein for in vivo work.

Bispecific Activity

In order to test the effects of simultaneous inhibition of both IL4 and IL13 inhibition in murine models of asthma, a bispecific AR protein linking C06_21H2 and C02_11G11 (SEQ ID

NO: 106), a potent murine IL13 inhibitor was engineered to link the N-terminus of C06_21H2 to the C-terminus of C02_11G11 via a (GGGGS)4 polypeptide linker. An N-terminal histidine tag was appended to the N-terminus in order to aide purification, as described above.

EXAMPLE 7: NEBULIZATION OF 11G11-21H2

In order to evaluate the potential to deliver a AR protein via nebulization using a rodent inhalation system, nebulization stability studies were performed with the surrogate bispecific AR protein (11G11-21H2). Aerosols were generated with a Pari LC Plus jet nebulizer connected to compressed air with an inlet pressure of 20 psi. This resulted in an output flow of ~ 5 L/min. Solution formulations of 11G1-21H2 were prepared at 20 mg/mL in PBS. Aerosols were directed through approximately 24 in. of a 1.58-cm (diameter) delivery line. The delivery line was fitted with forced air dilution flow of approximately 10 L/min. Aerosols transited into a flow-past 24-port nose-only rodent exposure chamber. The chamber exhaust flow rate was adjusted to a volumetric flow rate of approximately 20 L/min, resulting in the chamber being slightly negative to ambient conditions. Aerosols were collected on 47-mm Zefluor filters at a nominal volumetric flow rate of 1.0 L/min. Samples recovered from filters were analyzed by SEC and absorbance at 280 nm to assess potential aggregates and AR protein concentration.

Particle size distribution was measured by a Mercer-style, seven-stage cascade impactor (IN-TOX Products, Inc., Albuquerque, NM). Impactor samples were collected for between 1 and 2 min, as aerosol concentration required, at a nominal flow rate of 2 L/min. Impactor data were analyzed to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). In order to extract the samples from the filters they were rolled and placed in glass vials. Four milliliters of PBS was added to each vial. The vials were sealed and placed on a rotator for 45 minutes at 40 rpm. Samples were transferred into HPLC vials for analysis. The samples were then analyzed by size exclusion chromatography (SEC). The analysis showed that nebulized AR protein 11G11-21H2, collected as condensed aerosol at 30 minutes and 1 hour, the main peaks eluted at 6.09 minute. In the sample of 11G11-21H2 remaining in the sample cup post nebulization; the main peak eluted at 5.9 minutes. The sample increase in sample concentration with longer nebulization times was evidenced by an increase in peak intensity.

The samples were also tested for activity in the IL13 and IL4 dependent Stat6 and IL4 dependent HT2 proliferation assay respectively as previously described. Prior to testing, the concentration of aerosolized AR protein or AR protein retained in the cup were assessed by A280 and the activity was measured using the IL13 STAT6 activation assay (Fig. 2A) or the IL4 dependent HT2 proliferation assay (Fig. 2B); pre-nebulized AR protein, squares; aerosolized AR protein, triangles; retained AR protein, diamonds. These experiments showed that the bispecific AR protein construct, 11G11-21H2, was monodisperse by SEC and retains both

activities. Cascade impaction data shown in Figure 3 indicated that the nebulized AR protein 11G11-21H2 has an MMAD of 2.84 μm with a GSD of 1.66 appropriate for rodent exposures.

EXAMPLE 8: PHARMACOKINETIC PROFILE OF 11G11-21H2

The pharmacokinetic profile of 11G11-21H2 was determined for protein delivered via intratracheal instillation at 4 mg/kg, in healthy or mice sensitized and challenged with ovalbumin to mimic the asthmatic lung. Animals were anesthetized with 3-5% isoflurane until they failed to respond to toe pinch and did not respond to having the catheter inserted into the trachea. A 20 gauge catheter or smaller was inserted into the trachea and the compound instilled into the lungs in a smooth motion. The volume of solution inserted during a single instillation was approximately 50 μl. Data were collected at multiple times points with n=5 mice/time point. The concentration of the 11G11-21H2 construct was determined from bronchoaveolar lavage (BAL), lung homogenate and serum samples by ELISA using AR protein specific antibodies (Figure 4). There was no significant difference in PK profiles obtained in normal vs diseased mice and the predicted terminal half life in the mouse BAL and lungs is ~6-8 hrs. The overall systemic exposure, as assessed by serum concentration, was significantly lower than the exposure achieved in the lung. At t_{max}, BAL levels were on average greater than 40 μg/ml while serum levels were less than 100 ng/mL (approximately 400-fold lower relative to BAL concentration). AR protein concentration assessed by ELISA in BAL samples, lung homogenates or serum. Serum concentrations beyond 24 hours were below the level of detection for the assay.

For comparison, due to their small size, AR proteins that are dosed systemically by intravenous injection clear from circulation with a half-life of less than 30 minutes (data not shown).

EXAMPLE 9: IN VIVO DATA FOR 11G11-21H2

In order to evaluate the ability of 11G11-21H2 to inhibit both IL4 and IL13 dependent outcomes in vivo, the murine acute OVA sensitization and challenge model was used. Briefly, 8-10 wk old female BALB/c mice were immunized with an intraperitoneal injection of a mixture of ovalbumin (OVA, 10 microgram) and aluminum hydroxide (Alum, 2 mg) in sterile water on day 0 and 7. Mice were then challenged intranasally with ovalbumin for 2 days starting on day 14 and sacrificed for analysis on day 16. For the non-sensitized group, mice were immunized with sterile water only and treated with PBS by inhalation (vehicle control). For all other groups, mice were sensitized with OVA and treated with 11G11 (anti-IL13 AR protein), 21H2 (anti-IL4 AR protein), 1G11-21H2 (anti-IL4; anti-IL13 bispecific AR protein) or E3_5 (a non-binding control AR protein) at 20 mg/kg (monospecifics) or 40 mg/kg (bispecific). 11G11-21H2 or E3_5 was delivered via intratracheal instillation 1X per day beginning the day before OVA challenge and up to the day before sacrifice (day 13-16). Forty eight hours following the last OVA

challenge, mice were anesthetized and their pulmonary function (response to methacholine challenge) tested by whole body plethysmography (Buxco) and then immediately sacrificed. BAL (1 mL PBS) was performed post mortem to collect cells and fluid from all animals, cell number and differentials were calculated and BAL supernatant was frozen at -80°C.

As shown in Figure 5, both 11G11 and 11G11-21H2 inhibited OVA induced airway hyperresponsiveness in response to methacholine. In addition blockade of both IL4 and IL13 using mCNTX413 11G11-21H2 inhibited OVA induced eosinophil recruitment to the lungs, a hallmark of allergen associated inflammatory diseases, of mice as shown in Figure 6. In this model, eosinophil recruitment is largely TH2 cytokine (i.e. IL4, IL13) dependent. Data is shown as eosinophil percentages of the total cellular infiltrate.

EXAMPLE 10: CRYSTAL STRUCTURE OF IL13 BINDING PROTEIN AND CYNO IL-13

The crystal structure of the complex between IL13 binding protein 6G9 (SEQ ID NOS: 162 formed from ARs in SEQ ID NOS: 109, 127 and 144) and found in bi-specific IL4/IL13 binding protein (SEQ ID NOS: 41, 104 and 177) and cyno IL13 was determined at 1.6 Å resolution. The conformational epitope has been identified, as well as the binding protein residues involved in target recognition.

The following abbreviations are used: HEPES: N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid; MES: 2-(N-morpholino)ethanesulfonic acid; PBS: phosphate buffered saline; PEG: polyethylene glycol; RMSD: root-mean-square deviation; SEC: size exclusion chromatography; binding protein 6G9 was developed to bind IL13 with high affinity and block signaling through its receptor IL13Rα1/IL4Rα.

6G9 binds human IL13 with a K_D in the picomolar range and exhibits cross-reactivity towards cyno IL13. For insight into the mechanism of action, binding protein 6G9 was crystallized in complex with cyno IL13. The structure was determined at 1.6 Å resolution.

Proteins

Cyno IL13 with the N-terminal SUMO tag was expressed in *E. coli* and purified by HisTrap, SUMO tag cleavage, and SEC in a final PBS buffer, pH 7.2; Lot No. 081126-CP00721y.

Complex preparation

Binding protein 6G9 was further purified on a MonoQ HR 5/5 column (GE Healthcare) equilibrated with 20 mM MES, pH 6.5 (buffer A). Elution was performed with an 11-29% gradient of 20 mM MES, pH 6.5, 1 M NaCl (buffer B) in 40 column volumes. The main peak fractions were concentrated and used for complex formation.

Binding protein:IL13 complex was prepared by mixing 6G9 with excess IL13 at a molar ratio of 1:1.1 and incubated for 2 hours at 4°C. SEC on a Superdex 200 column separated the unbound species. The complex was concentrated using an Amicon-Ultra 5 kDa device to 13.75 mg/mL in 20 mM HEPES pH 7.5, 100 mM NaCl.

Crystallization

Crystallization of the complex was carried out by the vapor-diffusion method at 20°C using an Oryx4 robot (Douglas Instruments). The experiments were composed of equal volumes of protein and reservoir solution in a sitting drop format in 96-well Corning 3550 plates. The initial screening was performed with the PEGs suite (Qiagen) and in-house screens IH1 and IH2, and protein complex solution at 13.75 mg/mL. Plate-shaped stacked crystals appeared from IH2 conditions A1-A4 with 0.1 M Na acetate buffer, pH 4.5, 18-25% PEG 3350, and either 0.2 M lithium sulfate or 0.2 M ammonium sulfate. These crystals were used to prepare seeds for microseed matrix screening in a stabilizing solution of 0.1 M Na acetate buffer, pH 4.5, 25% PEG 3350, and 0.2 M lithium sulfate. Seeding was performed using 0.2 µL protein, 0.05 µL seeds, and 0.15 µL reservoir. Diluted protein complex (4.8 mg/mL) and 50-fold diluted seeds were used for optimization of conditions. X-ray quality crystals were obtained from 0.1 M Na acetate, pH 4.5, 11% PEG 3350, 0.2 M Li_2SO_4. The crystal data are given in Table 21.

X-ray data collection and structure determination

For X-ray data collection, one crystal was soaked for a few seconds in a cryo-protectant solution containing 0.1 M Na acetate, pH 4.5, 20% PEG 3350, 0.2 M LiCl, 20% glycerol and was frozen in liquid nitrogen. Diffraction data were collected at the Swiss Light Source synchrotron over a 180° crystal rotation with 0.25-sec exposures per 0.25°-image and were processed with the program XDS. X-ray data statistics are given in Table 21.

Table 21. Crystal data, X-ray data, and refinement statistics.

Crystal data		
Space group	C2221	
Unit cell axes (Å)	52.41, 78.49, 119.44	
Molecules/asym.unit	1 complex	
Vm (Å3/Da)	2.0	
Solvent content (%)	39	
X-ray data		
Resolution (Å)	30–1.6	(1.64–1.60)
No.measured reflections	210,223	(14,049)
No.unique reflections	32,998	(2,329)
Completeness (%)	99.6	(96.4)
Redundancy	6.4	(6.0)
Rmerge (I)	0.065	(0.542)
<I/σ>	17.1	(3.7)
B-factor (Wilson) (Å2)	25.7	
Refinement		
Resolution (Å)	15–1.6	
No. refls used in refinement	31,810	
Completeness (%)	96.4	
No. all atoms	2,129	
No water molecules	218	
R-factor (%)	0.169	
R-free (%)	0.196	
RMSD bond lengths (Å)	0.008	
RMSD bond angles (°)	1.1	
RMSD B-factor main-chain (Å2)	2.5	
Mean B-factor (Å2)	23.2	

The structure was solved by molecular replacement. The crystal structures of binding protein 6G9 (DAR6G9XP01) and human IL-13 (I130062G02) were used as search models. All crystallographic calculations were performed with the CCP4 suite of programs. Model

adjustments were carried out using the program COOT. The refinement statistics are given in Table 21.

Binding Protein/IL13 interface

The crystal structure of the complex is shown in Fig. 8. Binding protein 6G9 binds helices A and D of IL13 so that helix D fits in the major groove of the binding protein molecule. The ridge formed by four β-turns of the ankyrin repeats fits into the space between helices A and D. Target recognition involves all 4 β-turns and 4 out of 5 helices forming the groove. The interface is extensive and covers nearly 1,000 Å2 on each molecule.

Comparison of the binding protein structures in complex with IL13 and alone indicates that the binding protein molecule is relatively rigid. Upon binding the target (IL13), binding protein opens by ~3.5° as shown in Fig. 8.

Intermolecular interactions at the ridge are mostly hydrophobic (Fig. 9). They involve binding protein residues Ser45-Tyr46 (β-turn 1), Phe78-Ile79 (β-turn 2), Ile111-Val112 (β-turn 3), Lys144-Phe145 (β-turn 4).

Interactions at the groove of binding protein involve both hydrophobic and charged residues (Fig. 10). In total 20 binding protein residues are involved in binding IL13, based on the 4-Å cut-off distance (Fig. 11). The binding protein epitope on IL13 includes 17 residues, 10 from helix D and 7 from helix A (Fig. 12). The sequences shown in Fig. 12 have a 1 residue difference from SEQ ID NO:101 as a result of a leader sequence Pro residue at the start of the sequence (not in SEQ ID NO:101).

Three residues at the end of helix D seem to contribute a good portion of the binding energy: F107, R108 and N113 (each are 1 amino acid different in position than in SEQ ID NO: 101 for which it is F106, R107 and N112). The latter provides the C-terminal carboxyl group, which forms 3 salt bridges, to R23 (two) and R56 (Fig. 10). The side chain of N113 makes 3 H-bonds (to D44, S46, R56) and a van-der-Waals contact to Y46. Obviously, this binding protein must be very sensitive to the presence of N113 as the C-terminal residue of IL13. One residue shorter or longer will most likely limit binding.

The neutralization effect of 6G9 is due to blocking the IL13 interaction with the receptor chain IL13Rα1. 6G9 does not interfere with IL4Rα as can be judged from the crystal structure of IL13: IL13Rα1: IL4Rα complex.

Electrostatic interactions

Although charged residues play a significant role in the interactions, their distribution is quite unexpected. The binding surface of IL13 formed by helices A and D is positively charged due to a number of basic residues. The groove of binding protein, however, also bears a positive charge in the left (N-terminal) half, i.e. exactly where it binds IL13. Somehow, the

positive charge of the central cluster (R23, R56, R90) is balanced by the IL13 C-terminal charge and the dipole of helix D. The acidic patch in the groove that includes D77, D81, D110, E114, D143, D151 and D155, does not contribute much to the interactions.

Cross reactivity

Human and cyno IL13 differ in only 6 positions (Fig. 12). One of them, position 11, happens to be in the 6G9 epitope. However, this residue (Arg in human, Lys in cyno) contacts the binding protein through the aliphatic part of the side chain. Therefore, no difference in binding is expected between human and cyno IL13.

Also, the R/Q substitution in position 111 should not affect binding. Curiously, residue 111 is the only residue in the C-terminal portion of helix D that is not involved in the interactions (Fig. 12). Gln in this position is observed in cyno IL13 and in the natural variant of human IL13 (in the old literature, it is referred as Q130). In conclusion, binding protein 6G9 should bind cyno IL13 and both variants of human IL13 equally well.

IL13 structure

The IL13 structure is available for comparisons from the antibody complexes determined previously. All these antibodies bind IL13 at the surface formed by helices A and D. Superposition of the structures show that the arrangement of helices in the 4-helical bundle is essentially the same in all structures (Fig. 13). Some differences at the N- and C-termini may be due to interactions with the corresponding receptor molecules. Note that there is no difference between two of the IL13 antibodies, which share the same CDRs. Helix D in the binding protein complex is straight, whereas it is noticeably bent in the antibody complexes. The angular deviation is about 15°, which translates into a 3.3 Å shift at residue 110.

In contrast to the helical core, loops AB and CD connecting the helices exhibit substantial variability. In the present structure, loop CD is completely disordered. Given their flexibility, the observed conformations of the loops are most likely affected by crystal packing since the loops are not involved in contacts with antibodies.

The crystal structure of the IL13:Binding protein 6G9 complex has revealed that this binding protein recognizes helices A and D of IL13. The epitope is virtually the same as that of one of the IL13 antibodies. The C-terminal carboxyl group of N113 is a key element of the epitope. Target recognition involves all 4 β-turns and 4 out of 5 helices forming the groove. R23 from the N-terminal cap is an essential recognition residue. Binding protein–IL13 interactions at the ridge formed by β-turns are predominantly hydrophobic. Interactions at the groove are both hydrophobic and electrostatic. Upon binding IL13, the binding protein molecule opens by ~3.5°.

Sequence alignment indicates that the binding protein epitope identified here for cyno IL13 is preserved in wt human IL13 and R111Q (aka R130Q) human variant. Therefore, binding protein 6G9 is expected to be cross-reactive towards these species. The neutralization effect of binding protein 6G9 is due to blocking the IL13 interaction with the receptor chain IL13Rα1. 6G9 does not interfere with IL4Rα. The acetate ion bound to R108 of IL13 is located at the binding protein –IL13 interface. This suggests that not only acetate but possibly other anions, e.g. phosphate, may have a negative impact on binding by the binding protein.

Sequence Tables

SEQ ID NO:	Name	Sequence	Features
1	AR Consensus	X_1DX$_3$X$_4$GX$_6$TPLH LAAX$_{14}$X$_{15}$GHLEI VEVLLKX$_{27}$GAD VNA	X_1, X_3, X_4, X_6, X_{14}, and X_{15} = A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W or Y X_{27} = H, N, or Y
2	N-Cap	DLGKKLLEAA RAGQDDEVRI LMANGADVNA	
3	C-Cap, library	QDKFGKTAFD ISIDNGNEDL AEILQKLN	
4	IL4, Human, P05112, mature chain	HKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI MREKYSKCSS	
5	IL4, cynomolgous (UniProt P79339, 25-153)	HKCDITLQEIIKTLNSLTEQKTLCTKLTITDILAA SKNTTEKETFCRAATVLRQFYSHHEKDTRCLGATA QQFHRHKQLIRFLKRLDRNLWGLAGLNSCPVKEAN QSTLENFLERLKTIMREKYSKCSS	
6	IL4RA ECD (P24394, 24-232) receptor	GN MKVLQEPTCVSDYMSISTCEWKMNGPTNCSTELRL LYQLVFLLSEAHTCIPENNGGAGCV	

	for IL4	CHLLMDDVVSADNYTLDLWAGQQLLWKGSFKPSEH VKPRAPGNLTVHTNVSDTLLLTWSN PYPPDNYLYNHLTYAVNIWSENDPADFRIYNVTYL EPSLRIAASTLKSGISYRARVRAWA QCYNTTWSEWSPSTKWHNSYREPFEQH	
7	IL2RG, CD132, cytokine receptor common subunit gamma (P31785, 23-262) receptor for IL4 & IL13	LNTTILTPNGNEDTTADFFLTTMPTDSLSVSTLPL PEVQCFVFNVEYMNCTWNSSSEPQP TNLTLHYWYKNSDNDKVQKCSHYLFSEEITSGCQL QKKEIHLYQTFVVQLQDPREPRRQA TQMLKLQNLVIPWAPENLTLHKLSESQLELNWNNR FLNHCLEHLVQYRTDWDHSWTEQSV DYRHKFSLPSVDGQKRYTFRVRSRFNPLCGSAQHW SEWSHPIHWGSNTSKENPFLFALEA	
8	IL13Ralpha1, CD213a1; P78552, A27-T343	GGGGAAPTETQPPVTNLSVSVENLCTVIWTWNPPE GASSNCSLWYFSHFGDKQDKKIAPE TRRSIEVPLNERICLQVGSQCSTNESEKPSILVEK CISPPEGDPESAVTELQCIWHNLSY MKCSWLPGRNTSPDTNYTLYYWHRSLEKIHQCENI FREGQYFGCSFDLTKVKDSSFEQHS VQIMVKDNAGKIKPSFNIVPLTSRVKPDPPHIKNL SFHNDDLYVQWENPQNFISRCLFYE VEVNNSQTETHNVFYVQEAKCENPEFERNVENTSC FMVPGVLPDTLNTVRIRVKTNKLCY EDDKLWSNWSQEMSIGKKRNST	
9	C06 13A10	DLGKKLLEAARAGQDDEVRILMANGADVNVSDRIG NTPLHLAAVYVHLEIVEVLLKNGADVNALDDDGLT PLHLAAADGHLEIVEVLLKHGADVNATDSHVWTPL HLAAFFGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
10	C06 17A11	DLGKKLLEAARAGQDDEVRILMANGADVNAIDEWG DTPLHLAAIEGHLEIVEVLLKYGADVNASDAMGMT PLHLAAVYGYLEIVEVLLKNGADVNAMDFSGFTPL HLAAFSGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
11	C06 19C3	DLGKKLLEAARAGQDDEVRILMANGADVNATDDWG DTLLHLAATDGHLEIVEVLLKNGADVNAIDAMGMT PLHLAAVYGYLEIVEVLLKNGADVNAMDFSGFTPL HLAAFSGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	

12	C06 19F8	DLGKKLLEAARAGQDDEVRILMANGADVNATDDWG DTLLHLAATDGHLEIVEVLLKNGADVNASDSQGLT PLHLAAYYGHLEIVEVLLKYGADVNANDHHGITPL HLAAFAGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
13	C06 20B8	DLGKKLLEAASAGQDDEVHILMANGADVNAVDHDG FTPLHLAAADGHLEIVEVLLKHGADVNADDNFGWT PLHLAAFFGHLEIVEVLLKHGADVNAKDQTGLTPL HLAAVDGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
14	C06 26H2	DLGKKLLEAARAGQDDEVRILMANGADVNAYDSSG DTPLHVAAIDGHLEIVEVLLKHGADVNASDASGDT PLHLAADFGHLKIVEVLLKHGADVNAEDMIGITPL HLAAYNGHLEIVEVLLKNGADVNASDVHGFTPLHL AAFIGHLGIVEVLLKYDADVNAQDKFGKTAFDISI DNRNEDLAEILQKLN	
15	C06 28D4	DLGKELLEAASAGQDDEVHILMANGADVNAVDHDG FTPLHLAAADGHLEIVEVLLKHGADVNADDNFGWT PLHLAAFFGHLEIVEVLLKHGADVNAKDQTGLTPL HLAAVDGHLEIVEVLLKNGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
16	C06 28E5	DLGKKLLEAARAGQDDEVRILMANGADVNAADDSG ITPLHLAAEDGHLEIVEVLLKYGADVNAQDNLGDT PLHLAAWTGHLEIVEVLLKNGADVNAYDISGITPL HLAAFYGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
17	C06 42A11	DLGKKLLEAASVGQDDEVHILMANGADVNATDAWG LTPLHLAALLGHLEIVEVLLKHGADVNAHDETGFT PLHLAAVEGHLEIVEVLLKYGADVNASDILGRTPL HLAANFGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
18	C06 42C7	DLGKKLLEAASAGQDDEVRILMANGADVNAVDHDG FTPLHLAAADGHLEIVEVLLKHGADVNADDNFGWT PLHLAAFFGHLEIVEVLLKHGADVNAKDQTGLTPL HLAAVDGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
19	C06 43G2	DLGKKLLEAARAGQDDEVRILMANGADVNAKDVTG ETPLHLASWEGHLEIVEVLLKHGADVNAQDLFGIT PLHLAAATDGHLEIVEVLLKNGADVNATDSNGFTP LHLAASYGHLEIVDVLLKNGADVNAHDFDGFTPLH LAASWGHLEIVEVLLKYGADVNAQDKFGKTAFDIS	

		IDNGNEDLAEILQKLN	
20	C06_44C12	DLGKKLLEAASAGQDDEVHILMANGADVNALDDSG YTPLHLAAEDGHLEIVEVLLKHGADVNAADRMGDT PLHLAAFVGHLEIVEVLLKYGADVNAVDLAGVTPL HVAAFYGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
21	C06 44F6	DLGKELLEAARAGQDDEVHILMANGADVNALDDSG YTPLHLAAEDGHLEIVEVLLKHGADVNAMDNIGNT SLHLAAFDGHLEIVEVLLKYGADVNAVDLAGVTPL HVAAFYGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
22	C06 48F3	DLGKKLLEAARAGQDDEVRILMANGADVNAFDDSG LTPLHLAADDGHLEIVEVLLKHGADVNAADRMGDT PLHLAAFVGHLEIVEVLLKYGADVNAVDLAGVTPL HVAAFYGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
23	C06 50E5	DLGKKLLEAARAGQDDEVRILMANGADANATDDWG DTLLHLAATDGHLEIVEVLLKNGADVNAIDAMGMT PLHLAAVYGYLEIVEVLLKNGADVNAMDFSGFTPL HLAAFSGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
24	C06 53E9	DLGKELLEAASAGQDDEVHILMANGADVNASDKDG STPLHLAAVYGHLEIVEVLLKYGADVNAEDMNGYT PLHLAAADGHLEIVEVLLKYGADVDAKDRTGWTPL HLAGEFGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
25	C06 53G6	NLGKKLLEAARAGQDDEVRILMANGADVNATDDWG DTLLHLAATDGHLEIVEVLLKYGADVNANDAIGDT PLHLAALYGHLEIVEVLLKYGADVNATDLHGFTPL HLAAFWGHLEIVEVLLKHGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
26	C06 54C2	DLGKKLLEAARAGQDDEVRILMANGADVNATDDWG DTLLHLAATDGHLEIVEVLLKNGADVNAIDAMGMT PLHLAAVYGYLEIVEVLLKNGADVNAMDFSGFTPL HLTAFSGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
27	C06 6E9	DLGKKLLEAARAGQDDEVRILMANGADVNAIDSDG TTPLHLAAMDGHLEIVEVLLKYGADVNAVDWNGDT PLHLAAVDGHLEIVEVLLKYGADVNAQDNLGDTPL HLAAYYGHLEIVEVLLKHGADVNASDFHGITPLHL AAFSGHLEIVEVLLKYGADVNAQDKFGKTAFDISI	

		DNGNEDLAEILQKLN	
28	C06_24H1	DLGKKLLEAARAGQDDEVRILMANGADVNAHDNSG FTPLHLAAEIGHLEIVEVLLKYGADVNAADRMGDT PLHLAAFVGHLEIVEVLLKYGADVNAVDLAGVTPL HVAAFYGHLEIVEVLLKNGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
29	C06 4A7	DLGKKLLEAARAGQDDEVRILMANGADVNAEDDWG LTPLHLAAMLGHLEIVEVLLKYGADVNAKDDTGFT PLHLAAVEGHLEIVEVLLKYGADVNASDILGRTPL HLAANFGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
30	C06 14A4	DLGKKLLEAARAGQDDEVRILMANGADVNATDAWG LTPLHLAALLGHLEIVEVLLKHGADVNAHDETGFT PLHLAAVEGHLEIVEVLLKYGADVNASDILGRTPL HLAANFGHLEIVEVLLKYGADVNAQDKFGKTAFDI SIDNGNEDLAEILQKLN	
31	AR1 C06 13A10	SDRIGNTPLH LAAVYVHLEI VEVLLKNGAD VNA	
32	AR1 C06 17A11	IDEWGDTPLH LAAIEGHLEI VEVLLKYGAD VNA	
33	AR1 C06 19C3 & AR1 C06_19F8	TDDWGDTLLH LAATDGHLEI VEVLLKNGAD VNA	
34	AR1 C06 20B8, AR1 C06_28D4, & AR1 C06_42C7	VDHDGFTPLH LAAADGHLEI VEVLLKHGAD VNA	
35	AR1 C06 26H2	YDSSGDTPLH VAAIDGHLEI VEVLLKHGAD VNA	
36	AR1 C06 28E5	ADDSGITPLH LAAEDGHLEI VEVLLKYGAD VNA	
37	AR1 C06_42A11, AR1 C06_14A4, & AR1 C06_14A4	TDAWGLTPLH LAALLGHLEI VEVLLKHGAD VNA	
38	AR1 C06 43G2	KDVTGETPLH LASWEGHLEI VEVLLKHGAD VNA	

39	AR1 C06 44C12 & AR1 C06_44F6	LDDSGYTPLH LAAEDGHLEI VEVLLKHGAD VNA	
40	AR1 C06 48F3	FDDSGLTPLH LAADDGHLEI VEVLLKHGAD VNA	
33	AR1 C06 50E5 & AR1 C06_54C2	TDDWGDTLLH LAATDGHLEI VEVLLKNGAD VNA	
42	AR1 C06 53E9	SDKDGSTPLH LAAVYGHLEI VEVLLKYGAD VNA	
43	AR1 C06 53G6	TDDWGDTLLH LAATDGHLEI VEVLLKYGAD VNA	
44	AR1 C06 6E9	IDSDGTTPLH LAAMDGHLEI VEVLLKYGAD VNA	
45	AR1 C06 24H1	HDNSGFTPLH LAAEIGHLEI VEVLLKYGAD VNA	
46	AR1 C06_4A7	EDDWGLTPLH LAAMLGHLEI VEVLLKYGAD VNA	
47	AR2 C06 13A10	LDDDGLTPLH LAAADGHLEI VEVLLKHGAD VNA	
48	AR2 C06 17A11	SDAMGMTPLH LAAVYGYLEI VEVLLKNGAD VNA	
49	AR2 C06 19C3, AR2 C06_50E5, & AR2 C06_54C2	IDAMGMTPLH LAAVYGYLEI VEVLLKNGAD VNA	
50	AR2 C06 19F8	SDSQGLTPLH LAAYYGHLEI VEVLLKYGAD VNA	
51	AR2 C06 20B8, AR2 C06_28D4, & AR2 C06_42C7	DDNFGWTPLH LAAFFGHLEI VEVLLKHGAD VNA	
52	AR2 C06_26H2	SDASGDTPLH LAADFGHLKI VEVLLKHGAD VNA	

53	AR2 C06 28E5	QDNLGDTPLH LAAWTGHLEI VEVLLKNGAD VNA	
54	AR2 C06 42A11 & AR2 C06_14A4	HDETGFTPLH LAAVEGHLEI VEVLLKYGAD VNA	
55	AR2 C06 43G2	QDLFGITPLH LAAATDGHLEI VEVLLKNGAD VNA	
56	AR2 C06_44C12, AR2 C06 48F3, & AR2 C06_24H1	ADRMGDTPLH LAAFVGHLEI VEVLLKYGAD VNA	
57	AR2 C06 44F6	MDNIGNTSLH LAAFDGHLEI VEVLLKYGAD VNA	
58	AR2 C06 53E9	EDMNGYTPLH LAAADGHLEI VEVLLKYGAD VDA	
59	AR2 C06 53G6	NDAIGDTPLH LAALYGHLEI VEVLLKYGAD VNA	
60	AR2 C06 6E9	VDWNGDTPLH LAAVDGHLEI VEVLLKYGAD VNA	
61	AR2 C06 4A7	KDDTGFTPLH LAAVEGHLEI VEVLLKYGAD VNA	
62	AR3 C06 13A10	TDSHVWTPLH LAAFFGHLEI VEVLLKYGAD VNA	
63	AR3 C06_17A11, AR3 C06_19C3, & AR3 C06_50E5	MDFSGFTPLH LAAFSGHLEI VEVLLKYGAD VNA	
64	AR3 C06 19F8	NDHHGITPLH LAAFAGHLEI VEVLLKYGAD VNA	
65	AR3 C06 20B8	KDQTGLTPLH LAAVDGHLEI VEVLLKHGAD VNA	
66	AR3 C06 26H2	EDMIGITPLH LAAYNGHLEI VEVLLKNGAD VNA	

67	AR3 C06 28D4 & AR3 C06_42C7	KDQTGLTPLH LAAVDGHLEI VEVLLKNGAD VNA	
68	AR3 C06 28E5	YDISGITPLH LAAFYGHLEI VEVLLKHGAD VNA	
69	AR3 C06 42A11	SDILGRTPLH LAANFGHLEI VEVLLKHGAD VNA	
70	AR3 C06 43G2	TDSNGFTPLH LAASYGHLEI VDVLLKNGAD VNA	
71	AR3 C06 44C12 & AR3 C06_48F3	VDLAGVTPLH VAAFYGHLEI VEVLLKYGAD VNA	
72	AR3 C06 44F6	VDLAGVTPLH VAAFYGHLEI VEVLLKHGAD VNA	
73	AR3 C06 53E9	KDRTGWTPLH LAGEFGHLEI VEVLLKYGAD VNA	
74	AR3 C06_53G6	TDLHGFTPLH LAAFWGHLEI VEVLLKHGAD VNA	
75	AR3 C06 54C2	MDFSGFTPLH LTAFSGHLEI VEVLLKYGAD VNA	
76	AR3 C06 6E9	QDNLGDTPLH LAAYYGHLEI VEVLLKHGAD VNA	
77	AR3 C06 24H1	VDLAGVTPLH VAAFYGHLEI VEVLLKNGAD VNA	
78	AR3 C06 4A7 & AR3 C06_14A4	SDILGRTPLH LAANFGHLEI VEVLLKYGAD VNA	
79	AR4 C06 6E9	SDFHGITPLH LAAFSGHLEI VEVLLKYGAD VNA	
80	AR4 C06 26H2	SDVHGFTPLH LAAFIGHLGI VEVLLKYDAD VNA	
81	AR4 C06 43G2	HDFDGFTPLH LAASWGHLEI VEVLLKYGAD VNA	
82	AR1-C	X_1DDWGX_6TPLHLAATDGHLEIVEVLLKX_{27}GADVNA	X_1 is selected from T, V, I,

			S, A, V, and H X_6 is selected from D, F, L, I, N, E, Y, and T X_{27} is selected from H, N, and Y
83	AR2-C	$X_1DX_3X_4GX_6TPLHLAAX_{14}X_{15}GHLEIVEVLLKX_{27}GADVNA$	X_1 is selected from S, I, D, Q, A, E, H, K, N, and V X_3 is selected from A, D, E, L, M, N, S, and W X_4 is selected from D, F, I, L, M, N, Q, S, and T X_6 is selected from D, M, L, F, I, and Y; X_{14} is selected from A, D, F, L, V, and Y X_{15} is selected from D, E, F, T, V, W, and Y X_{27} is selected from H, N, and Y
84	AR3-C	$X_1DX_3X_4GFTPLHLAAX_{14}X_{15}GHLEIVEVLLKX_{27}GADVNA$	X_1 is selected from M, K, V, E, N, T, S and Y X_3 is selected

			from F, H, I, L, M, N, Q, R, and S X_4 is selected from A, H, I, L, N, S, and T X_{14} is selected from E, F, N, S, V and Y X_{15} is selected from A, D, F, I, N, S, and Y X_{27} is selected from H, N, and Y
85	AR1-F	TDDWGX_6TPLHLAATDGHLEIVEVLLKX_{27}GADVNA	X_6 is selected from D, F, L, I, N, E Y, and T X_{27} is selected from H, N, and Y
86	AR2-F	X_1DAMGX_6TPLHLAAVYGHLEIVEVLLKX_{27}GADVNA	X_1 is selected from A, D, E, H, I, K, N, Q, and V X_6 is selected from D, F, I, L, M and, Y X_{27} is selected from H, N, and Y
87	AR3-F	X_1D$X_3$$X_4$GFTPLHLAAFYGHLEIVEVLLK$X_{27}$GADVNA	X_1 is selected from E, K, M, N, T, S, V and Y X_3 is selected from F, H, I,

			L, M, N, Q, R, S, and V X_4 is selected from A, H, I, L, N, S, and T X_{27} is selected from H, N, and Y
88	AR1-O	X_1DDX_4GX_6TPLHLAAX_{14}DGHLEIVEVLLKX_{27}GADVNA	X_1 is selected from A, L, and T X_4 is selected from S and W X_6 is selected from D, I, and Y X_{14} is selected from E and T X_{27} is selected from A and H
89	AR2-O	X_1DX_3X_4GDTPLHLAA$X_{14}X_{15}$GHLEIVEVLLKX_{27}GADVNA	X_1 is selected from A, N, and Q X_3 is selected from A, N, and R X_4 is selected from I and L X_{14} is selected from F, L and W X_{15} is selected from T, V, and Y X_{27} is selected from A and Y
90	AR3-O	X_1DX_3X_4GX_6TPLHLAAFX_{15}GHLEIVEVLLKX_{27}GADV	X_1 is selected

		NA	from T, V, and Y X_3 is selected from I and L X_4 is selected from A, H, and S X_6 is selected from F, I, and V X_{15} is selected from W and Y X_{27} is selected from A and H
91	C06_44C12v2	DLGKKLLEAASAGQDDEVHILMANGADVNALDDS GYTPLHLAAEDGHLEIVEVLLKHGADVNAADRLG DTPLHLAAFVGHLEIVEVLLKAGADVNAVDLAGV TPLHVAAFYGHLEIVEVLLKAGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
92	C06_28E5v1	DLGKKLLEAARAGQDDEVRILMANGADVNAADDS GITPLHLAAEDGHLEIVEVLLKAGADVNAQDNLG DTPLHLAAWTGHLEIVEVLLKAGADVNAYDISGI TPLHLAAFYGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
93	C06_53G6v1	DLGKKLLEAARAGQDDEVRILMANGADVNATDDW GDTLLHLAATDGHLEIVEVLLKAGADVNANDAIG DTPLHLAALYGHLEIVEVLLKYGADVNATDLHGF TPLHLAAFWGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
94	C01_6G9_V1	DLDKKLLEAARAGQDDEVRILMANGADVNARDSY GSTPLHLAAREGHLEIVEVLLKYGADVNAADFIG DTPLHLAAYRGHLEIVEVLLKYGADVNASDITGE TPLHLAAQIGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
95	C01_6G9_V1_C06_28E5_V1	DLDKKLLEAARAGQDDEVRILMANGADVNARDSY GSTPLHLAAREGHLEIVEVLLKYGADVNAADFIG DTPLHLAAYRGHLEIVEVLLKYGADVNASDITGE TPLHLAAQIGHLEIVEVLLKHGADVNAQDKFGKT	

		AFDISIDNGNEDLAEILQKLGGGGSGGGGSGGGG SGGGGSRSDLGKKLLEAARAGQDDEVRILMANGA DVNAADDSGITPLHLAAEDGHLEIVEVLLKAGAD VNAQDNLGDTPLHLAAWTGHLEIVEVLLKAGADV NAYDISGITPLHLAAFYGHLEIVEVLLKHGADVN AQDKFGKTAFDISIDNGNEDLAEILQKLN	
96	C06_28E5_V1_C 01_6G9_V1	DLGKKLLEAARAGQDDEVRILMANGADVNAADDS GITPLHLAAEDGHLEIVEVLLKAGADVNAQDNLG DTPLHLAAWTGHLEIVEVLLKAGADVNAYDISGI TPLHLAAFYGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLNGGGGSGGGGSGGG GSGGGGSDLDKKLLEAARAGQDDEVRILMANGAD VNARDSYGSTPLHLAAREGHLEIVEVLLKYGADV NAADFIGDTPLHLAAYRGHLEIVEVLLKYGADVN ASDITGETPLHLAAQIGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
97	C01_6G9_V1_C0 6_44C12_V2	DLDKKLLEAARAGQDDEVRILMANGADVNARDSY GSTPLHLAAREGHLEIVEVLLKYGADVNAADFIG DTPLHLAAYRGHLEIVEVLLKYGADVNASDITGE TPLHLAAQIGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLNGGGGSGGGGSGGG GSGGGGSDLGKKLLEAASAGQDDEVHILMANGAD VNALDDSGYTPLHLAAEDGHLEIVEVLLKHGADV NAADRLGDTPLHLAAFVGHLEIVEVLLKAGADVN AVDLAGVTPLHVAAFYGHLEIVEVLLKAGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
98	C06_44C12_V2_ C01_6G9_V1	DLGKKLLEAASAGQDDEVHILMANGADVNALDDS GYTPLHLAAEDGHLEIVEVLLKHGADVNAADRLG DTPLHLAAFVGHLEIVEVLLKAGADVNAVDLAGV TPLHVAAFYGHLEIVEVLLKAGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLNGGGGSGGGGSGGG GSGGGGSDLDKKLLEAARAGQDDEVRILMANGAD VNARDSYGSTPLHLAAREGHLEIVEVLLKYGADV NAADFIGDTPLHLAAYRGHLEIVEVLLKYGADVN ASDITGETPLHLAAQIGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
99	C01_6G9_V1_C0 6_53G6_V1	DLDKKLLEAARAGQDDEVRILMANGADVNARDSY GSTPLHLAAREGHLEIVEVLLKYGADVNAADFIG DTPLHLAAYRGHLEIVEVLLKYGADVNASDITGE	

		TPLHLAAQIGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLNGGGGSGGGGSGGG GSGGGGSDLGKKLLEAARAGQDDEVRILMANGAD VNATDDWGDTLLHLAATDGHLEIVEVLLKAGADV NANDAIGDTPLHLAALYGHLEIVEVLLKYGADVN ATDLHGFTPLHLAAFWGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
100	C06_53G6_V1_C 01_6G9_V1	DLGKKLLEAARAGQDDEVRILMANGADVNATDDW GDTLLHLAATDGHLEIVEVLLKAGADVNANDAIG DTPLHLAALYGHLEIVEVLLKYGADVNATDLHGF TPLHLAAFWGHLEIVEVLLKHGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLNGGGGSGGGGSGGG GSGGGGSDLDKKLLEAARAGQDDEVRILMANGAD VNARDSYGSTPLHLAAREGHLEIVEVLLKYGADV NAADFIGDTPLHLAAYRGHLEIVEVLLKYGADVN ASDITGETPLHLAAQIGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
101	Human IL13 and variant, where X is R or Q	GPVPPSTALRELIEELVNITQNQKAPLCNGSMVW SINLTAGMYCAALESLINVSGCSAIEKTQRMLSG FCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHL KKLFREGXFN	
102	Interleukin-13 receptor subunit alpha-2, Homo sapiens (>Q14627, 27-380)	DTEIKVNPPQDFEIVDPGYLGYLYLQWQPPLSLD HFKECTVEYELKYRNIGSETWKTIIT KNLHYKDGFDLNKGIEAKIHTLLPWQCTNGSEVQ SSWAETTYWISPQGIPETKVQDMDCV YYNWQYLLCSWKPGIGVLLDTNYNLFYWYEGLDH ALQCVDYIKADGQNIGCRFPYLEASD YKDFYICVNGSSENKPIRSSYFTFQLQNIVKPLP PVYLTFTRESSCEIKLKWSIPLGPIP ARCFDYEIEIREDDTTLVTATVENETYTLKTTNE TRQLCFVVRSKVNIYCSDDGIWSEWS DKQCWEGEDLSKKTLLRFWLPFGFILILVIFVTG LLLRKPNTYPKMIPEFFCDT	
103	Modified C-cap	QDKFGKTPAD IAADNGHEDI AEVLQKAA	
104	Human	DLGKKLLEAARAGQDDEVRILMANGADVNALDDS	

	bispecific (Re-engineered 44C12-linker-6G9 w modified C-cap)	GYTPLHLAAEDGHLEIVEVLLKHGADVNAADRLG DTPLHLAAFVGHLEIVEVLLKAGADVNAVDLAGV TPLHVAAFYGHLEIVEVLLKAGADVNAQDKFGKT PADIAADNGHEDIAEVLQKAAGGGGSGGGGSGGG GSGGGGSGSDLDKKLLEAARAGQDDEVRILMANG ADVNARDSYGSTPLHLAAREGHLEIVEVLLKYGA DVNAADFIGDTPLHLAAYRGHLEIVEVLLKYGAD VNASDITGETPLHLAAQIGHLEIVEVLLKHGADV NAQDKFGKTPADIAADNGHEDIAEVLQKAA	
177	Human bispecific (Re-engineered 44C12-linker-6G9 w modified C-cap)	GSDLGKKLLEAARAGQDDEVRILMANGADVNALD DSGYTPLHLAAEDGHLEIVEVLLKHGADVNAADR LGDTPLHLAAFVGHLEIVEVLLKAGADVNAVDLA GVTPLHVAAFYGHLEIVEVLLKAGADVNAQDKFG KTPADIAADNGHEDIAEVLQKAAGGGGSGGGGSG GGGSGGGGSGSDLDKKLLEAARAGQDDEVRILMA NGADVNARDSYGSTPLHLAAREGHLEIVEVLLKY GADVNAADFIGDTPLHLAAYRGHLEIVEVLLKYG ADVNASDITGETPLHLAAQIGHLEIVEVLLKHGA DVNAQDKFGKTPADIAADNGHEDIAEVLQKAA	
41	Human bispecific (44C12-linker-6G9)	DLGKKLLEAASAGQDDEVRILMANGADVNALDDS GYTPLHLAAEDGHLEIVEVLLKHGADVNAADRLG DTPLHLAAFVGHLEIVEVLLKAGADVNAVDLAGV TPLHVAAFYGHLEIVEVLLKAGADVNAQDKFGKT AFDISIDNGNEDLAEILQKAAGGGGSGGGGSGGG GSGGGGSGSDLDKKLLEAARAGQDDEVRILMANG ADVNARDSYGSTPLHLAAREGHLEIVEVLLKYGA DVNAADFIGDTPLHLAAYRGHLEIVEVLLKYGAD VNASDITGETPLHLAAQIGHLEIVEVLLKHGADV NAQDKFGKTAFDISIDNGNEDLAEILQKAA	
178	Human bispecific (44C12-linker-6G9)	GSDLGKKLLEAASAGQDDEVRILMANGADVNALD DSGYTPLHLAAEDGHLEIVEVLLKHGADVNAADR LGDTPLHLAAFVGHLEIVEVLLKAGADVNAVDLA GVTPLHVAAFYGHLEIVEVLLKAGADVNAQDKFG KTAFDISIDNGNEDLAEILQKAAGGGGSGGGGSG GGGSGGGGSGSDLDKKLLEAARAGQDDEVRILMA NGADVNARDSYGSTPLHLAAREGHLEIVEVLLKY GADVNAADFIGDTPLHLAAYRGHLEIVEVLLKYG ADVNASDITGETPLHLAAQIGHLEIVEVLLKHGA DVNAQDKFGKTAFDISIDNGNEDLAEILQKAA	
105	C06_21H2 mu	DLGEKLLEAARAGQDDEVRILMANGADVNAYDDD	

	IL4 binding protein surrogate	GMTPLHLAAKSGHLEIVEVLLKHGADVNAMDITG SAPLHLAADLGHLEIVEVLLKHGADVNAIDYLGA TPLHLAATYGHPEIVEVLLKYGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
106	21H2 mu IL13 binding protein surrogate	DLGEKLLEAARAGQDDEVRILMANGADVNAYDDD GMTPLHLAAKSGHLEIVEVLLKHGADVNAMDITG SAPLHLAADLGHLEIVEVLLKHGADVNAIDYLGA TPLHLAATYGHPEIVEVLLKYGADVNAQDKFGKT AFDISIDNGNEDLAEILQKLN	
107	11G11-21H2 Bispecific surrogate	MRGSHHHHHHGSDLGKKLMEAARAGQDDEVRILM ANGADVNAKDLFGITPLHLAAVYGHLEIVEVLLK HGADVNATDNWGSTPLHLAAQFGHLEIVEVLLKY GADVNAQDKFGKTAFDISIDNGNEDLAEILQKLG GGGSGGGGSGGGGSGGGGSRSDLGEKLLEAARAG QDDEVRILMANGADVNAYDDDGMTPLHLAAKSGH LEIVEVLLKHGADVNAMDITGSAPLHLAADLGHL EIVEVLLKHGADVNAIDYLGATPLHLAATYGHPE IVEVLLKYGADVNAQDKFGKTAFDISIDNGNEDL AEILQKLN	

Note: SEQ ID NOS: 41, 104, 177, and 178 optionally have a Met residue at the N-terminus

IL13 Binding Protein AR Sequences

SEQ ID NO:	Name	Sequence	Clones
108	AR	XDXXGXTPLH LAAXXGHLEI VEVLLKXGAD VNA	
109	AR1	R•SY•S•••• •••RE••••• ••••••Y••• ••• RDSYGSTPLH LAAREGHLEI VEVLLKYGAD VNA	6G9
110	AR1	T•EFDS•••• •••RH••••• ••••••Y••• ••• TDEFDSTPLH LAARHGHLEI VEVLLKYGAD VNA	7G11
111	AR1	S•IF•S•••• •••RH••••• ••••••Y••• ••• SDIFGSTPLH LAARHGHLEI VEVLLKYGAD VNA	9F8
112	AR1	I•HFDS•••• •••RH••••• ••••••N••• ••• IDHFDSTPLH LAARHGHLEI VEVLLKNGAD VNA	10A6

SEQ ID NO:	Name	Sequence	Clones
113	AR1	T•VF•S•••• •••RH••••• ••••••H••• ••• TDVFGSTPLH LAARHGHLEI VEVLLKHGAD VNA	5B9
114	AR1	F•DF•S•••• •••RS••••• ••••••H••• ••• FDDFGSTPLH LAARSGHLEI VEVLLKHGAD VNA	7D2
115	AR1	E•IL•I•••• •••HH••••• ••••••H••• ••• EDILGITPLH LAAHHGHLEI VEVLLKHGAD VNA	6G11
116	AR1	T•TY•S•••• •••RHC•Q•• ••••••H••• ••• TDTYGSTPLH LAARHCHQEI VEVLLKHGAD VNA	7D7
117	AR1	V•DY•S•••• •••RQ••••• ••••••N••• ••• VDDYGSTPLH LAARQGHLEI VEVLLKNGAD VNA	5D12
118	AR1	K•LF•S•••• •••RH••••• ••••••N••• ••• KDLFGSTPLH LAARHGHLEI VEVLLKNGAD VNA	5D2
119	AR1	T•MY•S•••• •••RH••••• ••••••Y••• ••• TDMYGSTPLH LAARHGHLEI VEVLLKYGAD VNA	7H3
120	AR1	W•SY•S•••• •••RE••••• ••••••Y••• ••• WDSYGSTPLH LAAREGHLEI VEVLLKYGAD VNA	5D3
121	AR1	A•MY•T••Q• •••RT••••• ••••••Y••• ••• ADMYGTTPQH LAARTGHLEI VEVLLKYGAD VNA	5H7
122	AR1	S•IY•S•••• •••RH••••• ••••••N••• ••• SDIYGSTPLH LAARHGHLEI VEVLLKNGAD VNA	9E11
123	AR1	A•DY•S•••• •••RS••••• ••••••H••• ••• ADDYGSTPLH LAARSGHLEI VEVLLKHGAD VNA	6D4
124	AR1	M•KY•S•••• •••RS••••• ••••••H••• ••• MDKYGSTPLH LAARSGHLEI VEVLLKHGAD VNA	7C6
125	AR1	F•DF•D•••• •••RE••••• ••••••K••• ••• FDDFGDTPLH LAAREGHLEI VEVLLKKGAD VNA	2F1
126		XDXXGXTPLH LAAXXGHLEI VEVLLKXGAX VNA	

SEQ ID NO:	Name	Sequence	Clones
127	AR2	K•MI•D•••• •••YR••••• ••••••N••• ••• KDMIGDTPLH LAAYRGHLEI VEVLLKNGAD VNA	6G9
128	AR2	A•FI•D•••• •••YR••••• ••••••C•V• ••• ADFIGDTPLH LAAYRGHLEI VEVLLKCGVD VNA	7G11
129	AR2	M•FI•D•••• •••YR••••• ••••••Y•V• ••• MDFIGDTPLH LAAYRGHLEI VEVLLKYGVD VNA	9F8
130	AR2	D•FL•D•••• •••YH•••D• ••••••H••• ••• DDFLGDTPLH LAAYHGHLDI VEVLLKHGAD VNA	10A6
131	AR2	T•FI•D•••• •••YH••••• ••••••H••• ••• TDFIGDTPLH LAAYHGHLEI VEVLLKHGAD VNA	5B9
132	AR2	H•MI•D•••• •••YH••••• ••••••Y••• ••• HDMIGDTPLH LAYHEGHLEI VEVLLKYGAD VNA	7D2
133	AR2	N•FA•Y•••• •••VY••••• ••••••Y••• ••• NDFAGYTPLH LAAVYGHLEI VEVLLKYGAD VNA	6G11
134	AR2	N•FI•D•••• •••WH••••• ••••••N••• ••• NDFIGDTPLH LAAWHGHLEI VEVLLKNGAD VNA	7D7
135	AR2	D•FI•D•••• •••FK••••• ••••••N••• ••• DDFIGDTPLH LAAFKGHLEI VEVLLKNGAD VNA	5D12
136	AR2	E•FI•D•••• •••YR••••• ••••••Y•V• ••• EDFIGDTPLH LAAYRGHLEI VEVLLKYGVD VNA	5D2
137	AR2	F•FI•D•••• •••YR••••• ••••••Y•V• ••• FDFIGDTPLH LAAYRGHLEI VEVLLKYGVD VNA	7H3
138	AR2	K•MI•D•••• •••YR••••• ••••••Y•V• ••• KDMIGDTPLH LAAYRGHLEI VEVLLKYGVD VNA	5D3
139	AR2	A•FL•D•••• •••YH••••• ••••••H••• ••• ADFLGDTPLH LAAYHGHLEI VEVLLKHGAD VNA	5H7
140	AR2	N•MI•D•••• •••YH••••• ••••••H••• •••	9E11

SEQ ID NO:	Name	Sequence	Clones
		NDMIGDTPLH LAAYHGHLEI VEVLLKHGAD VNA	
141	AR2	N•FI•D•••• •••YN••••• ••••••N••• ••• NDFIGDTPLH LAAYNGHLEI VEVLLKNGAD VNA	6D4
142	AR2	T•FI•D•••• •••YH••••• ••••••H••• ••• TDFIGDTPLH LAAYHGHLEI VEVLLKHGAD VNA	7C6
143	AR2	T•II•N•••• •••FR••••• ••••••H••• ••• TDIIGNTPLH LAAFRGHLEI VEVLLKHGAD VNA	2F1
144	AR3	S•IT•E•••• •••QI••••• ••••••Y••• ••• SDITGETPLH LAAQIGHLEI VEVLLKYGAD VNA	6G9
145	AR3	D•H-•D•••• •••SM••••• ••••••N••• ••• DDHYGDTPLH LAASMGHLEI VEVLLKNGAD VNA	7G11,9F8, 7H3,5D3,5D2
146	AR3	K--N•E•••• •••YH••PD• ••••••H••• ••• K--NGETPLH LAAYHGHPDI VEVLLKHGAD VNA	5B9
147	AR3	K•TS•T•••• •••DS••••• ••••••H••• ••• KDTSGTTPLH LAADSGHLEI VEVLLKHGAD VNA	7D2
148	AR3	T•AW•E•••• •••YT••••• ••••••HD•• ••• TDAWGETPLH LAAYTGHLEI VEVLLKHDAD VNA	6G11
149	AR3	M•VT•E•••• •••YH•••D• ••••••N••• ••• MDVTGETPLH LAAYHGHLDI VEVLLKNGAD VNA	7D7
150	AR3	S•IT•E•••• •••TA••••• ••••••H••• ••• SDITGETPLH LAATAGHLEI VEVLLKHGAD VNA	5D12
151	AR3	S•IT•E•••• •••HL••••• ••••••Y••• ••• SDITGETPLH LAAHLGHLEI VEVLLKYGAD VNA	5H7
152	AR3	S•IT•E•••• •••HN••••• ••••••Y••• ••• SDITGETPLH LAAHNGHLEI VEVLLKYGAD VNA	9E11
153	AR3	T•IT•E•••• •••ER••••• ••••••N••• ••T TDITGETPLH LAAERGHLEI VEVLLKNGAD VNT	6D4

SEQ ID NO:	Name	Sequence	Clones
154	AR3	E•IT•E•••• •••ES••••• ••••••H••• ••• EDITGETPLH LAAESGHLEI VEVLLKHGAD VNA	7C6
155	AR3	S•IT•E•••• •••HL••••• ••••••Y••• ••• SDITGETPLH LAAHLGHLEI VEVLLKYGAD VNA	2F1

SEQ ID NO	NAME	Sequence	Features
156	AR1-C	$X_1DX_3X_4$GSTPLHLAARHGHLEIVEVLLKX_{27}GADVNA	X_1 is selected from T, A, F, E, I, K, M, S, R, V, and W X_3 is selected from D, E, H, I, K, M, S, T, and V X_4 is F or Y X_{27} is H, N, or Y
157	AR1-F	TDYGSTPLHLAARHGHLEIVEVLLKX_{27}GADVNA	X_{27} H, N, or Y
158	AR2-C	X_1DFIGDTPLHLAAYX_{15}GHLEIVEVLLKX_{27}GADVNA	X_1 is selected from N, T, A, D, K, E, H, M, and F X_{15} is selected from H and R X_{27} is selected from H, N, and Y
159	AR2-F	X_1DFIGDTPLHLAAYX_{15}GHLEIVEVLLKX_{27}GADVNA	X_1 is selected from A, D, N, T, and K X_{15} is selected from H and

			R X_{27} is selected from H, N, and Y
160	AR3-C	$X_1DX_3TGETPLHLAAX_{14}X_{15}GHLEIVEVLLKX_{27}GADVNA$	X_1 is selected from D, E, K, M, S, and T X_3 is selected from I, A, T and V or is absent (SEQ ID NO: 179) X_{14} is selected from D, E, H, Q, S, T, and Y X_{15} is selected from M, L, H, S, A, I, N, R, and T X_{27} is selected from H, N, and Y
161	AR3-F	X_1DITGETPLHLAASMGHLEIVEVLLKX$_{27}$GADVNA	X_1 is selected from D and S X_{27} is selected from H, N, and Y
162	6G9	DLGKKLLEAARAGQDDEVRILMANGADVNA RDSYGSTPLHLAAREGHLEIVEVLLKYGADVNA KDMIGDTPLHLAAYRGHLEIVEVLLKNGADVNA SDITGETPLHLAAQIGHLEIVEVLLKYGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
163	6G9r13	ALDKKLLEAARAGQDDEVRILMANGADVNA RDSYGSTPLHLAAREGHLEIVEVLLKYGADVNA ADFIGDTPLHLAAYRGHLEIVEVLLKYGADVNA SDITGETPLHLAAQIGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKAA	

164	9F8	DLGKKLLEAARAGQDDEVRILMANGADVNA SDIFGSTPLHLAARHGHLEIVEVLLKYGADVNA MDFIGDTPLHLAAYRGHLEIVEVLLKYGVDVNA DDHGDTPLHLAASMGHLEIVEVLLKNGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
165	9F8r3	GLDKKLLEAARAGQDDEVRILMANGADVNA SDIFGSTPLHLAARHGHLEIVEVLLKYGADVNA FDFIGDTPLHLAAYRGHLEIVEVLLKYGVDVNA DDHGDTPLHLAAQIGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKAA	
166	7G11	DLGKKLLEAARAGQDDEVRILMANGADVNA TDEFDSTPLHLAARHGHLEIVEVLLKYGADVNA ADFIGDTPLHLAAYRGHLEIVEVLLKCGVDVNA DDHGDTPLHLAASMGHLEIVEVLLKNGADVNA QDKFGKTAFDISIDNGNEDLAEILQKLN	
167	7G11r7	GLDKKLLEAARAGQDDEVRILMANGADVNA TDEFDSTPLHLAARHGHLEIVEVLLKYGADVNA ADFIGDTPLHLAAYRGHLEIVEVLLKYGVDVNA DDHGDTPLHLAASTGHLEIVEVLLKHGADVNA QDKFGKTAFDISIDNGNEDLAEILQKAA	
168	AR1-O	$X_1DX_3X_4X_5$STPLHLAARX_{15}GHLEIVEVLLKYGADVNA	X_1 is R, S, or T; X_3 is S or W; X_4 is F or Y; X_5 is D or G; and X_{15} is E or H
169	AR2-O	X_1DFIGDTPLHLAAYRGHLEIVEVLLKYGADVNA	X_1 is A or F;
170	AR3-O	$X_1DX_3X_4GX_6$TPLHLAA$X_{14}X_{15}$GHLEIVEVLLKHGADVNA	X_1 is D or S; X_3 is H or S; X_4 is G or T; X_6 is D or S; X_{14} is Q or S; X_{15} is I or T
171	N-cap Variants	X_1LX_3KKLLEAA RAGQDDEVRI LMANGADVNA	X_1 is A, D, or G; X_3 is D or G;
172	C-cap variant	QDKFGKTPAD IAADNGHEDI AEVLQKAA	
173	11G11	GSDLGKKLMEAARAGQDDEVRILMANGADVNAKDLFGI TPLHLAAVYGHLEIVEVLLKHGADVNATDNWGSTPLHL AAQFGHLEIVEVLLKYGADVNAQDKFGKTAFDISIDNG NEDLAEILQKLN	
174	N-cap	X_1KKLLEAARAGQDDEVRILMANGADVNA	X_1 is D or G;
175	C-cap	QDKFGKTX_8X_9DI$X_{12}X_{13}$DNGX_{17}EDX$_{20}$AEX$_{23}$LQKX$_{27}X_{28}$	X_8 is A or P; X_9 is A or F; X_{12} is A or S; X_{13} is A or I; X_{17} is H or N; X_{20} is I or L;

			X_{23} is I or V; X_{27} is A or L; X_{28} is A or N
176	Coding sequence for human bi-specific (re-engineered 44C12-linker-6G9 with modified C-cap)	atgggatccgacctgggtaagaaactgctggaagctgc tcgtgctggtcaggacgacgaagttcgtatcctgatgg ctaacggtgctgacgttaacgctctggatgatagcggt tatacaccgctgcatctggcagcggaagatggtcatct ggaaattgttgaagttctgctgaaacacggtgccgatg tgaatgccgcagatcgtctgggtgatactccgctgcat ctggctgcctttgttggccatctggaaatcgtagaggt gctgctgaaagcaggcgcagatgtaaacgcagttgatc tggcaggcgttacccctctgcacgttgcagcattttat ggacacttagaaattgtggaggtactgctgaaggcagg tgcagacgttaacgcacaggataaatttggtaaaaccc cggcggatattgcggcggataatggccatgaggatatt gcagaagtgctgcaaaaggcggcgggcggcggtggctc tggcggtggtggctctggcggtggcggttctggcggtg gtggctctggatccgacctggataagaaactgctggaa gcagcacgtgcaggtcaggatgatgaagttcgtattct gatggcaaatggcgccgatgttaatgcacgtgatagct atggtagcacaccgctgcatctggctgcacgtgagggt catctggaaattgtggaagtgctgctgaaatacggtgc cgatgtgaatgccgcagattttattggtgataccccgt tacatctggctgcgtatcgtggccatttagaaatcgtg gaggttctgttaaaatacggcgcagacgttaatgcaag cgatattaccggtgaaacccctctgcatttagcagcgc agattggccacctggaaatcgtcgaagttttactgaaa catggcgcagatgttaacgcacaggataaatttggtaa aaccccggcggatattgcggcggataatggccatgagg atattgcagaagtgctgcagaaggcggcg	

CLAIMS

1. A binding protein comprising a binding domain, wherein the binding domain is an ankyrin repeat domain, binds to human IL4, and inhibits human IL4 binding to IL4Ralpha in vitro or in vivo and wherein the ankyrin repeat domain comprises at least one ankyrin repeat module.

2. The binding protein of claim 1, wherein the ankyrin repeat domain comprises two or more ankyrin repeat modules.

3. The binding protein of claim 2, wherein the ankyrin repeat domain comprises an amino terminal cap (N-cap) module positioned N-terminal to the ankyrin repeat domain comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 171, and 174 and a carboxy terminal cap (C-cap) module positioned C-terminal to the ankyrin repeat domain having an amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 103, 172, and 175.

4. The binding protein of claim 2, wherein the ankyrin repeat modules comprise at least one of the amino acid sequences selected from the group consisting of SEQ ID NOS: 1, 31-40 and 42-90.

5. The binding protein of claim 4, wherein the ankyrin repeat modules comprise an AR1 module, an AR2 module and an AR3 module in tandem, the AR1 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 31-40, 42-46, 82, 85, and 88; the AR2 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 47-61, 83, 86, and 89; and the AR3 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 62-78, 84, 87, and 90.

6. The binding protein of claim 5, further comprising an AR4 module in tandem, the AR4 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1 and 79-81.

7. An IL4 binding protein comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 91-93.

8. A binding protein comprising an ankyrin repeat domain, wherein the binding protein competes for binding to human IL4 with a binding protein according to any of claims 1-7.

9. A binding protein comprising a binding domain, wherein the binding domain is an ankyrin repeat domain and binds to human IL13 in vitro or in vivo, inhibiting human IL13 binding to

at least one of human IL13Ralpha1 and human IL13Ralpha2 and wherein the ankyrin repeat domain comprises at least one ankyrin repeat module.

10. The binding protein of claim 9, wherein the ankyrin repeat domain comprises two or more ankyrin repeat modules.

11. The binding protein of claim 9, wherein the binding domain binds to an IL13 R130Q variant and inhibits binding of the IL13 R130Q variant to at least one of human IL13Ralpha1 and human IL13Ralpha2.

12. The binding protein of claim 10, wherein said ankyrin repeat domain comprises ankyrin repeat modules AR1, AR2, and AR3 arranged in tandem

13. The binding protein of claim 10, wherein the ankyrin repeat domain comprises an N-cap module positioned N-terminal to the ankyrin repeat domain having an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 171, and 174 and a C-cap module positioned C-terminal to the ankyrin repeat domain having an amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 103, 172, and 175.

14. The binding protein of claim 10, wherein the ankyrin repeat modules comprise at least one of the amino acid sequences of SEQ ID NOS: 1, 108-161, and 168-170.

15. The binding protein of claim 14, wherein the ankyrin repeat modules comprise an AR1 module, an AR2 module and an AR3 module in tandem, the AR1 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 108-125, 156, 157, and 168; the AR2 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 126-143, 158, 159, and 169; and the AR3 module comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 144-155, 160, 161, and 170.

16. An IL13 binding protein comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 94, 162-167, and 173.

17. A binding protein comprising a binding domain, wherein the binding domain is an ankyrin repeat domain and binds to human IL13 in vitro or in vivo, inhibiting human IL13 binding to at least one of human IL13Ralpha1 and human IL13Ralpha2, the ankyrin repeat domain comprises two or more ankyrin repeat modules, and the ankyrin repeat domain binds to human IL13 at one or more of residues 6, 9, 10, 13, 17, 20, 96, 99, 103, 104, 106-109, 111, and 112 of SEQ ID NO:101.

18. The binding protein of claim 17, wherein the ankyrin repeat domain binds to human IL13 at one or more of residues 106, 107, and 112 of SEQ ID NO:101.

19. A binding protein comprising an ankyrin repeat domain, wherein the binding protein competes for binding to human IL13 with a binding protein according to any of claims 9-18.

20. A binding protein comprising a first binding domain and a second binding domain, wherein said first binding domain is an ankyrin repeat domain, binds to human IL4, and inhibits human IL4 binding to IL4Ralpha in vitro or in vivo and the second binding domain is an ankyrin repeat domain and binds to human IL13 in vitro or in vivo, inhibiting human IL13 binding to at least one of human IL13Ralpha1 and human IL13Ralpha2, and wherein said ankyrin repeat domains comprise at least one ankyrin repeat module.

21. A binding protein comprising a first binding domain and a second binding domain, wherein said first binding domain is an ankyrin repeat domain, binds to human IL4, and inhibits human IL4 binding to IL4Ralpha in vitro or in vivo and said second binding domain is an ankyrin repeat domain and binds to an IL13 R130Q variant and inhibits binding of the IL13 R130Q variant to at least one of human IL13Ralpha1 and human IL13Ralpha2, and wherein said ankyrin repeat domains comprise at least one ankyrin repeat module.

22. The binding protein of any of claims 1-21, wherein the binding protein binds to IL4 or IL13 with a K_D value of less than 2 nM as determined by surface plasmon resonance.

23. A binding protein comprising an IL4-binding ankyrin repeat domain and an IL13-binding ankyrin repeat domain.

24. The binding protein of claim 23, wherein the ankyrin repeat domain comprises an ankyrin repeat module binding to IL4, an N-cap module positioned N-terminal to the IL4-binding ankyrin repeat module, a C-cap module positioned C-terminal to the IL4-binding ankyrin repeat module, an ankyrin repeat module binding to IL4, an N-cap module positioned N-terminal to the IL13-binding ankyrin repeat module, a C-cap module positioned C-terminal to the IL13-binding ankyrin repeat module, and a linker, wherein the linker connects the C-cap module of the IL4-binding ankyrin repeat module and the N-cap module of the IL13-binding ankyrin repeat module or connects the N-cap module of the IL4-binding ankyrin repeat module and the C-cap module of the IL13-binding ankyrin repeat module.

25. The binding protein of claim 24, wherein the linker is a GS linker.

26. A binding protein comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 41, 104, 177, and 178.

27. A binding protein comprising an IL4-binding ankyrin repeat domain having the amino acid sequence of SEQ ID NO: 91, an IL13-binding ankyrin repeat domain having the amino acid sequence of SEQ ID NO: 94, and a linker.

28. A pharmaceutical composition comprising the binding protein of any one of claims 1-7, 9-18, and 20-27 and a pharmaceutical acceptable carrier and/or diluent.

29. The pharmaceutical composition according to claim 28, wherein the composition is suitable for the treatment of conditions of the respiratory system

30. The pharmaceutical composition according to claim 29, wherein the respiratory system condition is at least one of asthma and pulmonary fibrosis.

31. The pharmaceutical composition according to claim 28, wherein the composition is suitable for the treatment of cardiovascular conditions, cancer, dermatological, and fibrotic conditions.

32. The pharmaceutical composition according to claim 28, wherein the composition is formulated for delivery using a nebulizer.

33. The pharmaceutical composition according to claim 32, wherein the composition comprises a dry powder formulation for delivery using a dry powder device.

34. A method of administering the pharmaceutical composition of claim 28, wherein the route of administration is selected from the group consisting of intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intracardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravascular, intravesical, intralesional, vaginal, rectal, buccal, sublingual, intranasal, or transdermal.

35. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a binding protein according to any one of claims 1-7, 9-18, and 20-27.

36. An isolated nucleic acid vector comprising the nucleotide acid molecule according to claim 35.

37. An isolated host cell comprising the isolated nucleic acid molecule according to claim 35, wherein the host cell is prokaryotic or eukaryotic.

38. The host cell according to claim 37, wherein said host cell is at least one selected from E. Coli, yeast, baculovirus, COS-1, COS-7, HEK293, BHK21, CHO, BSC-1, Hep G2, 653, SP2/0, 293, HeLa, P3X63Ag8.653, and SP2/0-Ag14, myeloma, and lymphoma cells.

39. A method for producing a binding protein, comprising culturing the isolated host cell of claim 37 under conditions such that the binding protein is expressed and recovered.

40. A binding protein comprising a binding domain, wherein the binding domain is an ankyrin repeat domain, wherein said binding protein competes for binding to the IL4 protein having the amino acid sequence of SEQ ID NO: 4 and/or the IL13 protein having the amino acid sequence of SEQ ID NO: 101, with the binding protein of any of claims 1-7, 9-18, and 20-27.

41. A binding protein comprising an amino acid sequence encoded by the nucleotide sequence of SEQ ID NO:176.

42. An isolated nucleic acid molecule comprising a nucleotide sequence of SEQ ID NO: 176.

43. An isolated nucleic acid vector comprising the nucleotide acid molecule according to claim 42.

44. An isolated host cell comprising the isolated nucleic acid molecule according to claim 42, wherein the host cell is prokaryotic or eukaryotic.

45. The host cell according to claim 44, wherein said host cell is at least one selected from E. Coli, yeast, baculovirus, COS-1, COS-7, HEK293, BHK21, CHO, BSC-1, Hep G2, 653, SP2/0, 293, HeLa, P3X63Ag8.653, and SP2/0-Ag14, myeloma, and lymphoma cells.

46. A method for producing a binding protein, comprising culturing the isolated host cell of claim 44 under conditions such that the binding protein is expressed and recovered.

47. The use of a binding domain according to any of claims 1-7, 9-18, 20-27, and 41 for manufacture of a medicament for treating cardiovascular conditions, respiratory conditions, cancer, dermatological conditions, and fibrotic conditions.

48. The use of claim 47 for treating at least one of asthma and pulmonary fibrosis.

49. Any invention described herein.

Fig. 1A

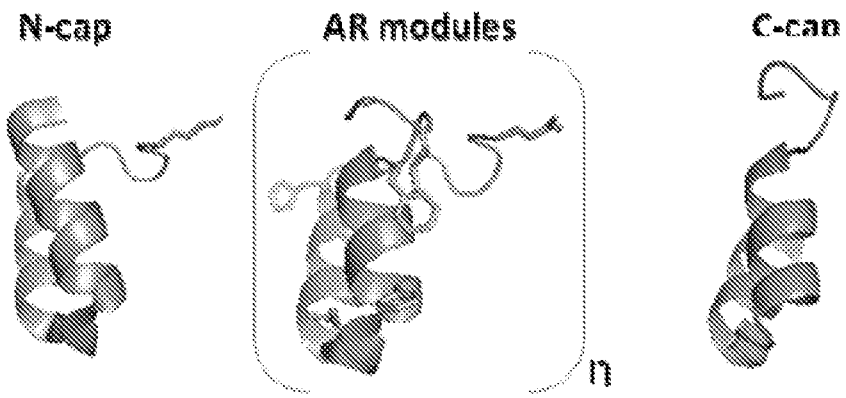

Fig. 1B

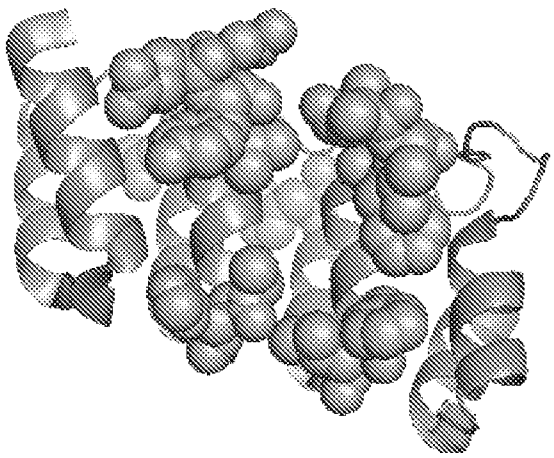

Fig. 2A

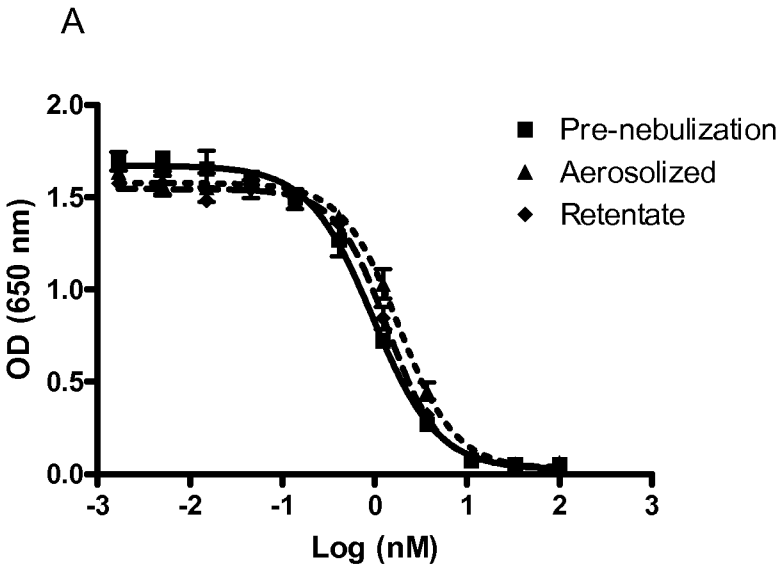
A
2.0
1.5
1.0
0.5
0.0
OD (650 nm)
-3
-2
-1
0
1
2
3
Log (nM)
Pre-nebulization
Aerosolized
Retentate

FIG. 2B

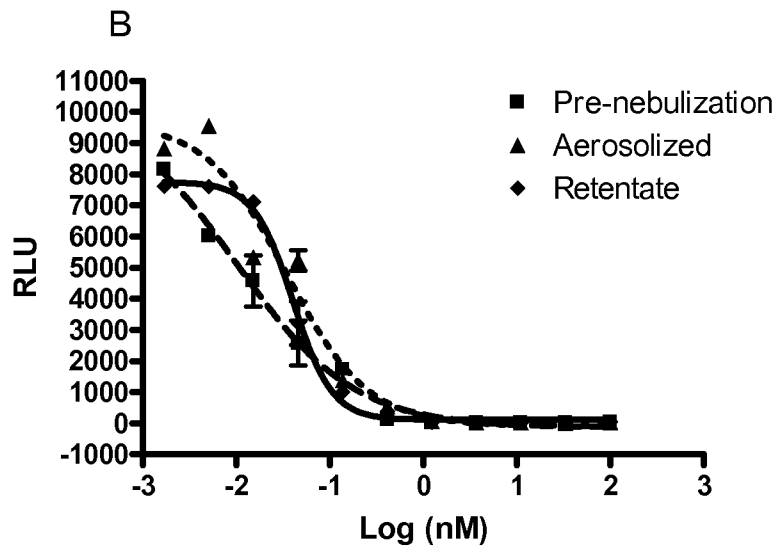
B
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
RLU
-3
-2
-1
0
1
2
3
Log (nM)
Pre-nebulization
Aerosolized
Retentate

Fig. 3

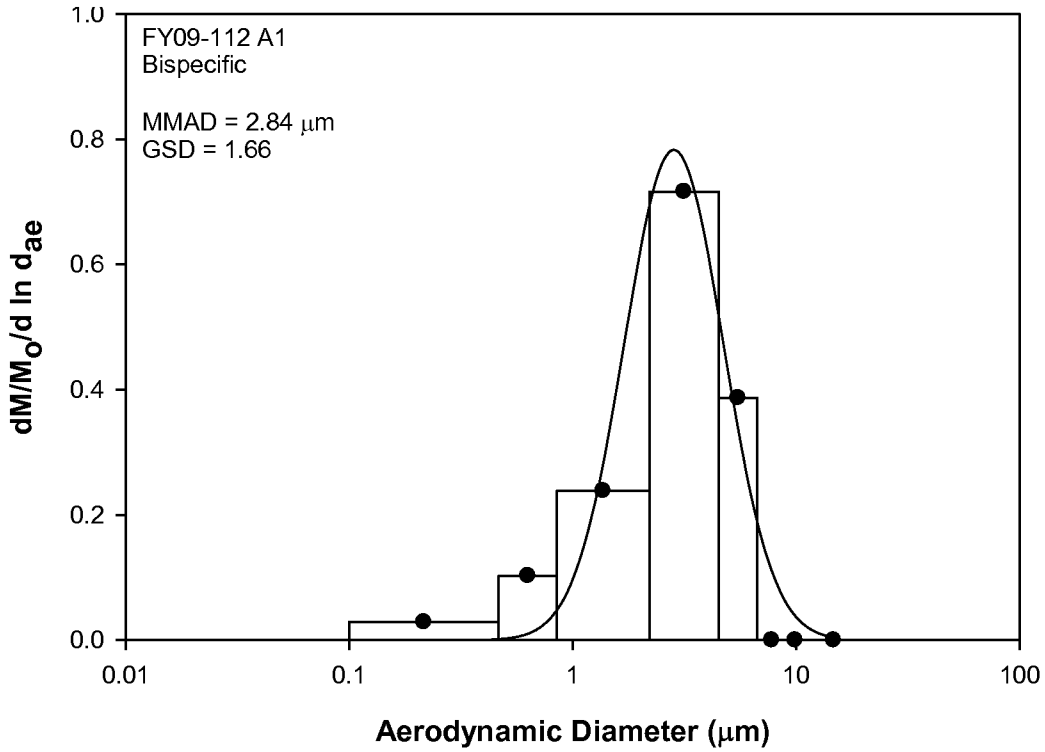
FY09-112 A1
Bispecific
MMAD = 2.84 μm
GSD = 1.66
dM/M$_O$/d ln d$_{ae}$
1.0
0.8
0.6
0.4
0.2
0.0
0.01
0.1
1
10
100
Aerodynamic Diameter (μm)

Fig. 4

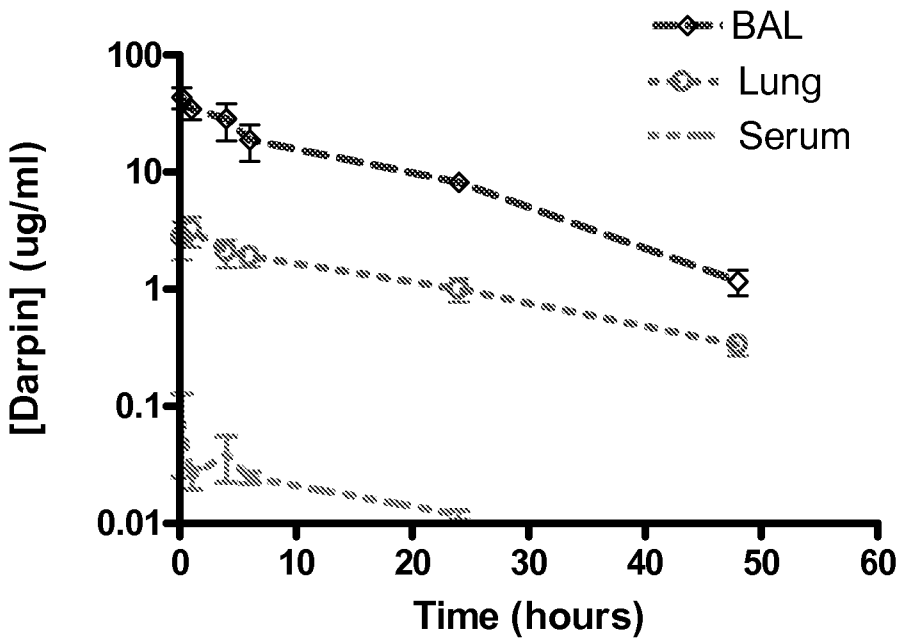
BAL
Lung
Serum
100
10
1
0.1
0.01
[Darpin] (ug/ml)
0
10
20
30
40
50
60
Time (hours)

Fig. 5

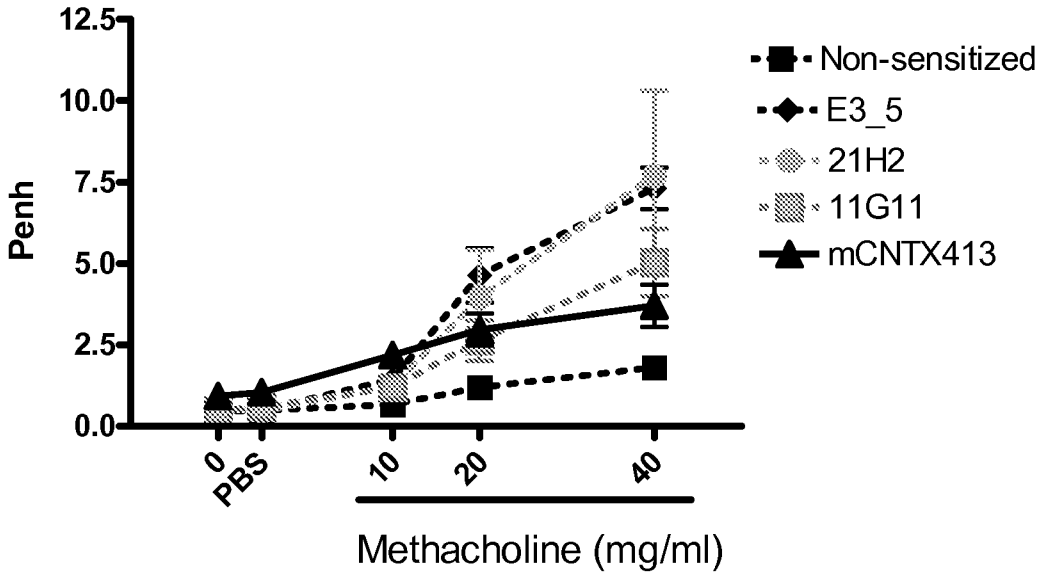
12.5
10.0
7.5
5.0
2.5
0.0
Penh
0
PBS
10
20
40
Methacholine (mg/ml)
Non-sensitized
E3_5
21H2
11G11
mCNTX413

Fig. 6

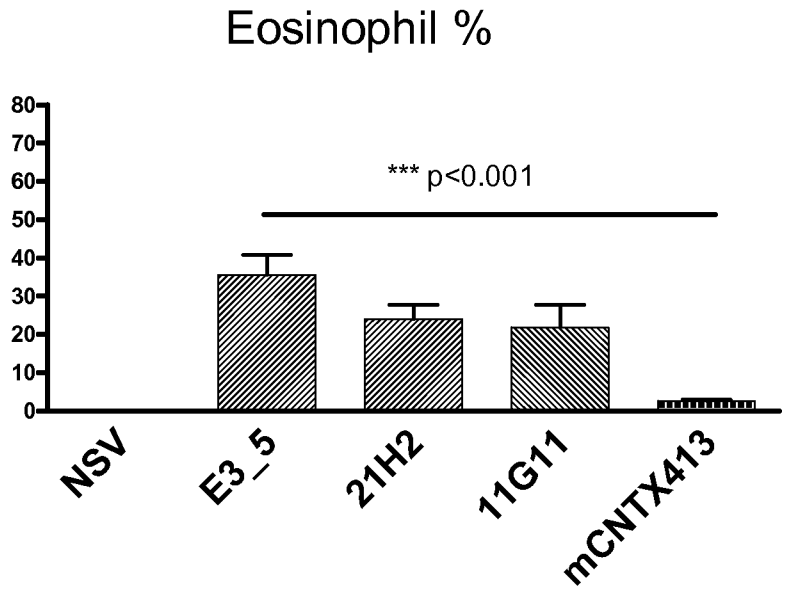
Eosinophil %
80
70
60
50
40
30
20
10
0
*** p<0.001
NSV
E3_5
21H2
11G11
mCNTX413

Fig. 7

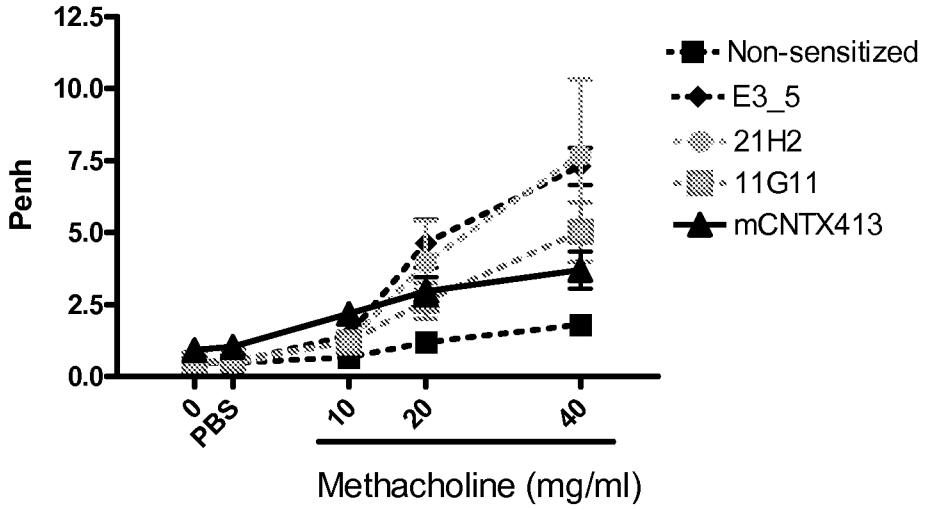
12.5
10.0
7.5
5.0
2.5
0.0
Penh
0
PBS
10
20
40
Methacholine (mg/ml)
Non-sensitized
E3_5
21H2
11G11
mCNTX413

FIG. 8

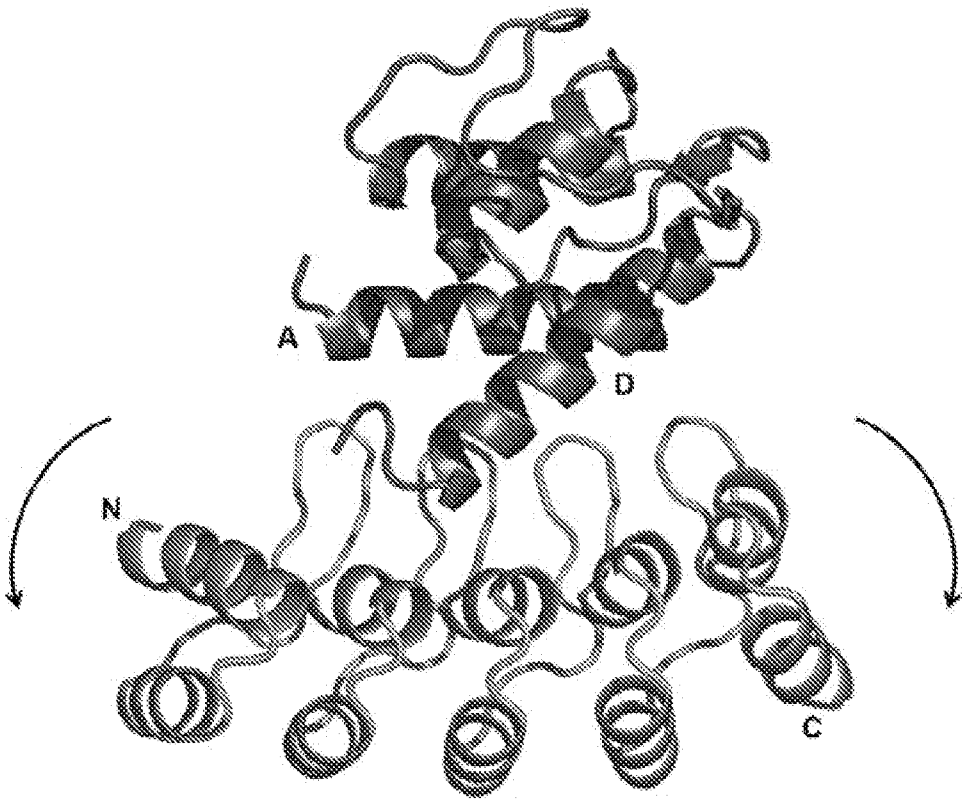

FIG. 9

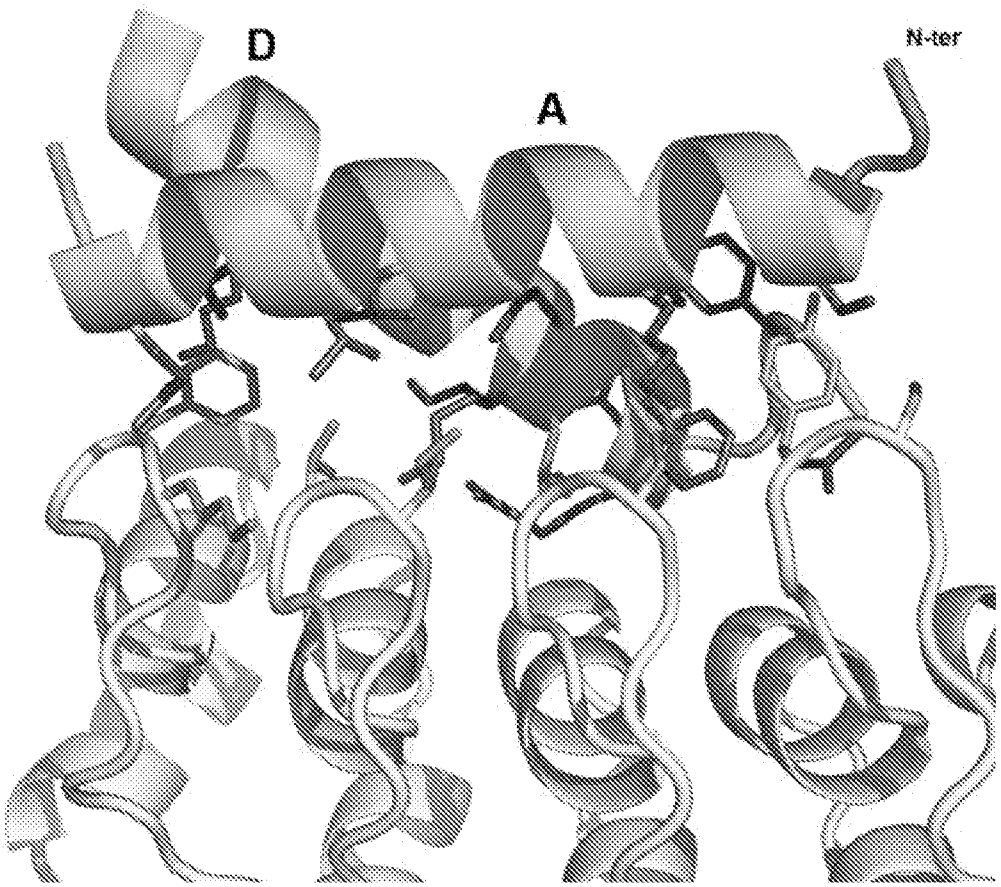

FIG. 10

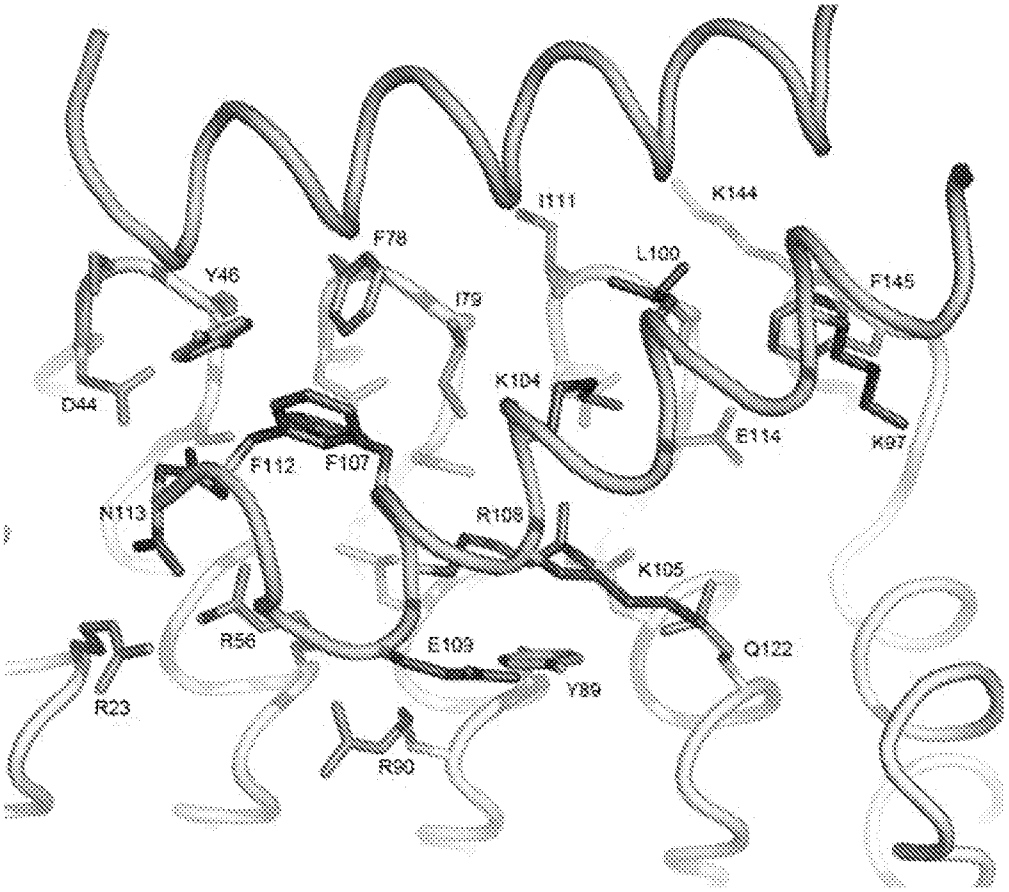
I111
K144
F78
L100
Y46
I79
F145
K104
D44
E114
K97
F112
F107
N113
R108
K105
R56
E109
Q122
Y89
R23
R90

FIG. 11

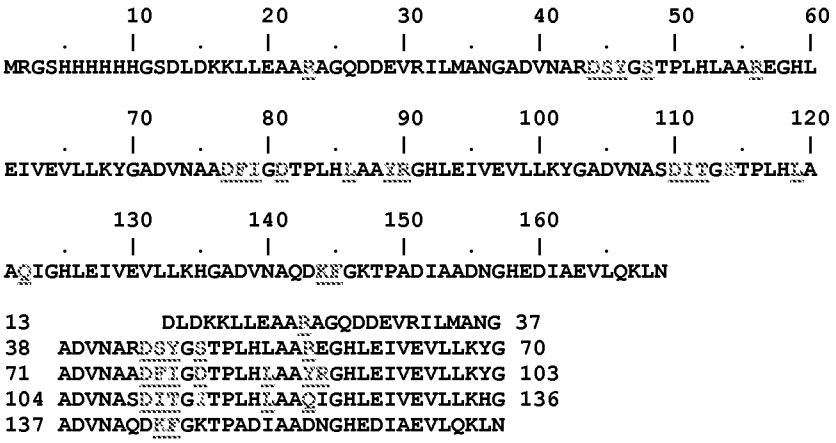
10 20 30 40 50 60
MRGSHHHHHHGSDLDKKLLEAARAGQDDEVRILMANGADVNARDSYGSTPLHLAARNEGHL
70 80 90 100 110 120
EIVEVLLKYGADVNAADFIGDTPLHLAAYRGHLEIVEVLLKYGADVNASDITGRTPLHLA
130 140 150 160
AQIGHLEIVEVLLKHGADVNAQDKFGKTPADIAADNGHEDIAEVLQKLN
13 DLDKKLLEAARAGQDDEVRILMANG 37
38 ADVNARDSYGSTPLHLAARNEGHLEIVEVLLKYG 70
71 ADVNAADFIGDTPLHLAAYRGHLEIVEVLLKYG 103
104 ADVNASDITGRTPLHLAAQIGHLEIVEVLLKHG 136
137 ADVNAQDKFGKTPADIAADNGHEDIAEVLQKLN

FIG. 12

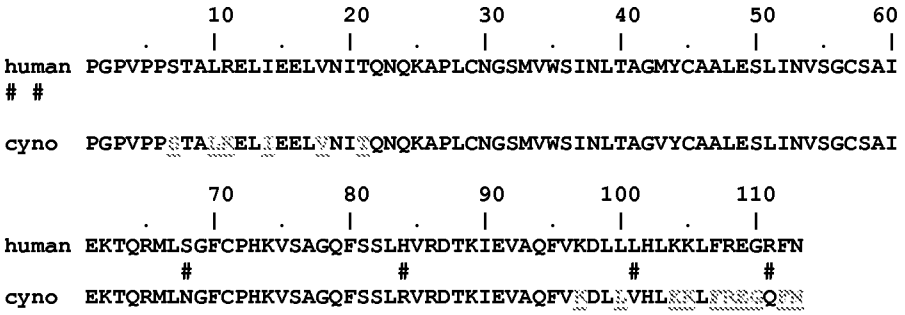
10 20 30 40 50 60
human PGPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAI
cyno PGPVPP TA EL EEL NI QNQKAPLCNGSMVWSINLTAGVYCAALESLINVSGCSAI
70 80 90 100 110
human EKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLKKLFREGRFN
cyno EKTQRMLNGFCPHKVSAGQFSSLRVRDTKIEVAQFV DL VHL KL REGQ N

FIG. 13

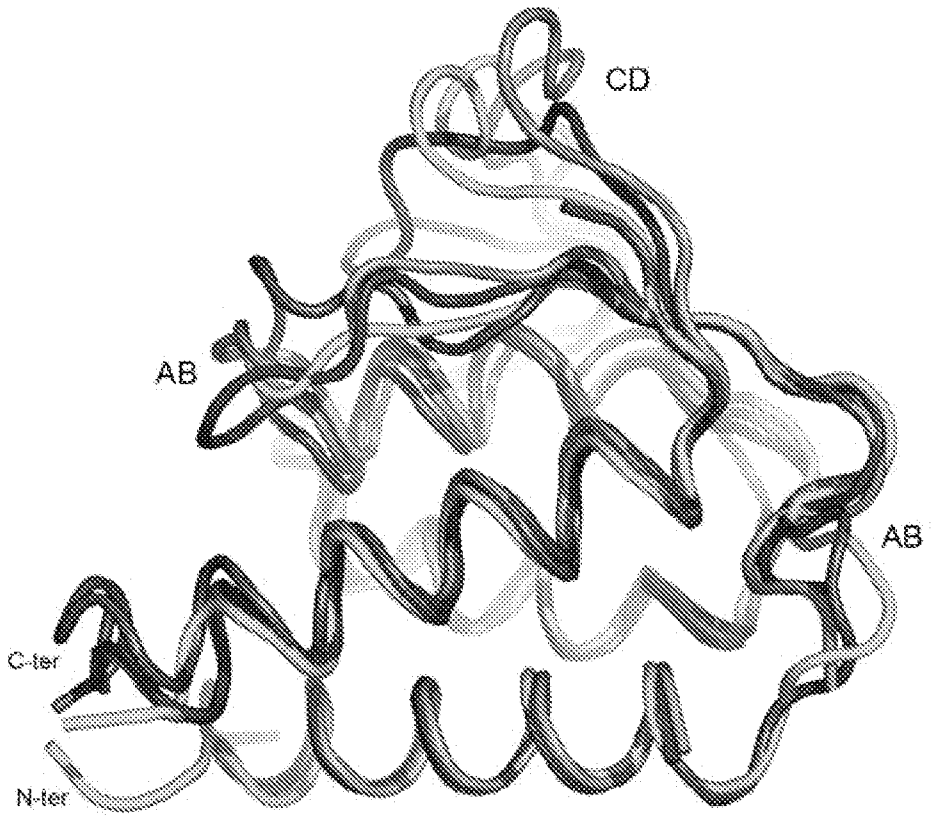

Fig. 1A

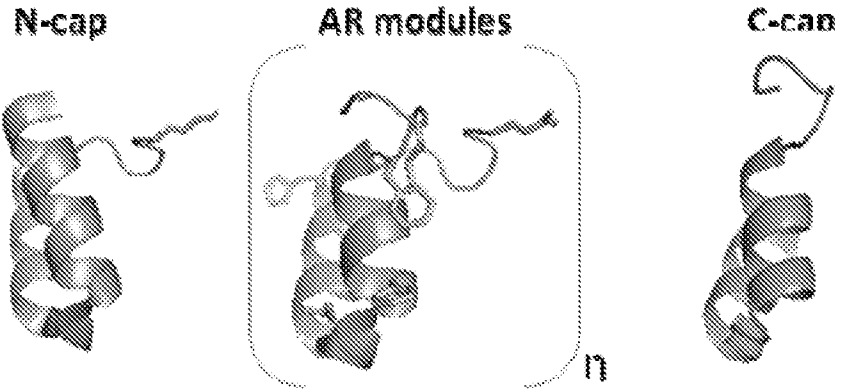

Fig. 1B